ESTIMATING THE SIZE AND STRUCTURE OF PINE MARTEN POPULATIONS USING NON-INVASIVE GENETIC SAMPLING

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Declaration

This thesis is a presentation of my original research work, of which no element has been previously submitted for a degree at this or any other research institute. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was carried out under the guidance of Dr. Catherine O' Reilly and Dr. Peter Turner in the Department of Chemical & Life Sciences, Waterford Institute of Technology, Ireland.

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-Jay-

Abstract

The pine marten is one of six mustelid species currently established in Ireland along with the badger, otter, stoat, ferret and the American mink. The ancient origin of the Irish pine marten is unknown but it was possibly introduced into Ireland in the Bronze Age as a commodity species for its luxurious pelt. The species was legally protected in Ireland in 1976 after centuries of persecution with the extensive deforestation of the island and hunting for fur or predator control. The current conservation status is considered to be favourable as the population seems to be expanding its range from core populations in the mid-west, midlands and the south-east of the country. However, the status of these core populations has not been assessed and is very difficult to evaluate based on traditional survey methods. This study aimed to develop the methods required to census pine marten populations based on non-invasive genetic sampling of hair and faeces.

Real-time PCR methods which enabled rapid species and sex identification of non-invasively collected samples were developed based on either the hybridisation of species or sex specific probes, or on differences in melting-temperature between amplified DNA sequences of less than 150 nucleotides in length. Genetic variability of the Irish population was assessed at 20 microsatellite loci. The number of alleles per locus (2.29) and expected heterozygosity (0.35) were low and a significant bottleneck signature was detected using a set of 41 road-kill individuals. The historical decline in pine marten distribution and abundance in Ireland has therefore had an impact on genetic diversity. Despite this no significant structuring was identified between regions, suggesting the dispersal ability of the pine marten is sufficient to maintain a panmictic population.

A census of two populations in south-east Ireland was then conducted by non-invasive genetic sampling of hair and scats using the methods developed in the project. Species and sex identification success rates were high for both sample types (88-100%), but individual identification was more reliable with plucked hair (94%) than scats (38%). Nine individual pine marten were identified in total in between the two sites by genotyping hair samples with eight microsatellite loci. Independent live trapping surveys carried out after the non-invasive census validated the genetic approach as the same individuals were captured with both survey methods.

Remotely plucked hair genotyping is a powerful and non-invasive survey method which can be used to inventory the magnitude and distribution of genetic diversity in pine marten, and other species with similar life history such as the stone marten, to identify any potentially important populations towards which conservation resources should be targeted in the future.

Keywords: *Martes martes*; non-invasive genetic sampling; population census; molecular sexing; microsatellite DNA; real-time PCR; Ireland.

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Table of contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1. Background to the project

There is an increasing awareness of the need to catalogue and regularly monitor our biological resources since the Convention on Biological Diversity which was adopted at the Earth Summit in Rio de Janiero, Brazil in 1992. International conservation policies such as the Conservation of European Wildlife and Natural Habitats (Bern Convention, 1979) require regular feedback from member states on the conservation status of listed wildlife species. This can be very difficult to achieve for species which are rare, nocturnal, intelligent and difficult to locate in the field. The pine marten (*Martes martes* L., 1758) is an example of one such species.

The pine marten has varying conservation priorities across Europe, being exploited for fur in some countries and virtually extinct in others due to pressures from hunting and habitat loss. There is a need for a survey method which can yield reliable information on distribution, demography and genetics across a range of habitats and population densities. Presence can be determined by the collection of field signs, road casualties or hunting statistics, but it is practically impossible to estimate population size, leading to a lack of information on population dynamics (Broekhuizen 2006).

It is vitally important to conserve biological diversity below the species level as the pressures on local populations as a result of unregulated hunting and habitat fragmentation can impede genetic exchange between populations and reduce population size to a level where chance events may threaten viability in the long term. The pine marten has full statutory protection in Ireland under the Wildlife Acts 1976 & 2000 and has expanded its range in recent decades, leading to the suggestion that the Irish population is of international importance (Whilde 1993).

The most recent evaluation of the conservation status of the pine marten in Ireland places it in the IUCN category of Least Concern (Marnell *et al.* 2009). However, the Irish population is genetically distinct from other European populations and is characterised by a low level of genetic diversity. This genetic similarity of all individuals in Ireland makes individual identification difficult with the set of genetic

markers previously used to assess genetic diversity and structure (Kyle *et al.* 2003; Statham 2005; Lynch 2006). The main focus of this study was therefore to develop and evaluate genetic techniques for estimating the size and structure of pine marten populations through non-invasive genetic sampling of hair and faeces.

1.2. The European pine marten, *Martes martes*

The pine marten belongs to the most diverse carnivore family, the mustelids (Mustelidae), which contains 59 species grouped into 22 genera (Koepfli *et al.* 2008). Martens occupy forests in tropical, temperate and boreal zones of both the Old and New World (Proulx *et al.* 2004). There are eight species in the genus *Martes*: the stone marten *M. foina*, Nilgiri marten *M. gwatkinsii*, yellow-throated marten *M. flavigula*, fisher *M. pennanti*, European pine marten *M. martes*, Japanese marten *M. melampus*, sable *M. zibellina* and the American pine marten *M. americana*. The pine marten can hybridize with the sable where the two species meet in Russia, though backcrossing of the 'kidas' offspring with the parent forms is generally unsuccessful, maintaining reproductive isolation (Grakov 1994). Hybridization with the American marten has also been documented in England, presumably with fur farm escapees (Davison *et al.* 2001). Although sympatric over much of continental Europe, the pine marten and stone marten do not interbreed, or at least this has not been published to date (Davison *et al.* 2001). The pine marten is one of five mustelid species currently in the Irish mammalian fauna. The other representatives are the Eurasian badger *Meles meles*, Irish stoat *Mustela erminea hibernica*, Eurasian otter *Lutra lutra* and the recently introduced American mink *Neovison vison*. The polecat-ferret *Mustela furo* has also established feral populations, particularly in Co. Monaghan (Buckley *et al.* 2007).

The pine marten is a little smaller than a domestic cat (the pine marten is known as the 'cat crainn', meaning tree cat in Gaelic), with a dark brown sleek coat in the summer months, filling out to a luxurious, sometimes reddish fur in the winter time. Sexual

dimorphism is marked with males 1.60-2.15 kg, about a third larger than females 1.1-1.45 kg (Birks 2002; Balharry *et al.* 2008). The tail, which the marten uses for balance, also becomes bushier and along with the limbs, is of a darker colour than the main pelt. The head is wedge-shaped with round, pale rimmed ears. A throat patch of cream to yellow/orange is present in most animals, the markings of which can be used to distinguish individuals. In continental Europe, where the pine marten is sympatric with the stone marten in places, the throat patch of the latter is usually white and extends farther ventrally. Large paws with haired soles, semi-retractile claws and flexible ankles afford the marten great arboreal agility and although it mainly hunts on the ground, it can prey on squirrels in the trees.

Figure 1: The European pine marten, *Martes martes* in summer coat. The coat lightens and the tail becomes bushier in winter. The extensive cream throat patch and inside ear, along with relatively long legs are distinctive features. ©Terry Whitakker, www.terrywhitakker.com.

The name pine marten is misleading as pine marten utilise a variety of habitats, including limestone pavement, scrub, fields, hedgerows and human constructions (O'Sullivan 1983; Pereboom *et al.* 2008; Balestrieri *et al.* 2009). A core area of about 2 km^2 of woodland may be required to support adult breeding martens (Balharry 1993), but home range sizes vary depending on the habitat and prey availability and where these conditions are favourable individual home ranges can be very small, for example in the order of 13-15 ha for a study of a marten population in Dromore Wood, Co. Clare (Fairley 2001). In the primeval forest of Bialowieza National Park in Poland, radio-tracking over a five year period showed females travelled further in spring and summer and males increased their range size in summer to almost three times the autumn-winter range to access females for mating (Zalewski *et al.* 2004). Larger home ranges are covered in coniferous plantations. In Scotland, Bright & Smithson (1997) recorded an average distance travelled of 7.3 km per night, with a maximum of 15.8 km. The data available for Irish populations suggest population densities are average to high compared to the rest of Europe (Warner & O'Sullivan 1982; Lynch *et al.* 2006).

Most of the foraging time is spent on the ground, patrolling a system of regularly used pathways, which are marked with scats in conspicuous places (Warner & O'Sullivan 1982). These scats are thought to be important in the maintenance of home ranges, along with urine and anal secretions (DeMonte & Roeder 1990; Hutchings & White 2000), but may also be used as a form of autocommunication to familiarise the individual with its home range (DeMonte & Roeder 1990). Pine marten are primarily a solitary, nocturnal species though they may be active during the day, especially during the summer months (Zalewski 2000). Intrasexual territories are maintained where same sex individuals are excluded but some overlap is tolerated with opposite sex and non-breeding individuals (Schröpfer *et al.* 1997). Females are sexually mature in their third year with mating, which is thought to be promiscuous, occurring in late summer (Balharry *et al.* 2008). The delayed implantation of the blastocyst results in the young kits being born in early March-April, blind, deaf and sparsely furred. Rocky crevices may be chosen as a natal den (Ussher 1898) in the absence of suitable tree cavities which are scarce in the

relatively young coniferous forests which dominate the Irish landscape (Birks *et al.* 2005). Kits are weaned by their second month and are able to kill by their third. They remain with their mother until they are about 6 months old when it is thought the lack of resources or aggression between the family group members causes the juveniles to disperse to establish their own territory (Sleeman 1989).

Food preferences vary seasonally and geographically (Zalewski *et al.* 2004). Small rodents such as voles are an important food item in most European countries, whereas larger rodents such as the red squirrel *Scuirus vulgaris* are more common in the diet of martens inhabiting boreal forests at higher latitudes (de Marinis & Masseti 1995; Zalewski *et al.* 2004). A wider variety of food items have been recorded from island populations, most likely due to the depauperate mammalian prey base and reduced intraguild competition (Warner & O'Sullivan 1982; Clevenger 1993). In Ireland, two major dietary studies have been conducted. Warner & O'Sullivan (1982) analysed over 600 scats from Dromore Wood, Co. Clare over a four-year period. Martens were found to avail of a wide range of food items, including birds (mainly Passerines) and their eggs, berries (*Sorbus, Rubus, Fragaria*), fruits (*Malus* and *Prunus*), nuts (*Corylus* and *Fagus*), invertebrates (Coleoptera), small mammals (*Apodemus sylvaticus*), and carrion. Lynch & McCann (2007) described the diet of a population of translocated pine marten in broadleaf woodlands in Killarney National Park, Co. Kerry. Fruit was the main component of the diet, but for the small mammal group the recently introduced bank vole *Myodes glareolus* was as important as the wood mouse *Apodemus sylvaticus*. The latest addition to the small mammal inventory in Ireland, the greater white-toothed shrew *Crocidura russula* (Tosh *et al.* 2008) may also add to the prey base for the pine marten, though Clevenger (1993) found it rarely featured in the diet of Spanish pine martens in the Cantabrian Mountains.

1.3. Distribution and conservation status

The pine marten has a Palaearctic distribution, occurring across most of Europe, extending into the Caucasus and Asia Minor (Figure 2). Insular populations also occur on Majorca, Minorca, Sicily, Sardinia, Elba and Corsica. Hunting or trapping is permitted in 13 European countries (Austria, Croatia, Czech Republic, France, Germany, Latvia, Lithuania, Poland, Romania, Switzerland, Turkey, Sweden and Yugoslavia) and the species is protected by wildlife legislation in 10 countries (Albania, Britain, Bulgaria, Denmark, Hungary, Ireland, Italy, Portugal, Spain and The Netherlands) (Proulx *et al.* 2004). The global conservation status of pine marten under IUCN guidelines is Least Concern as it maintains a wide distributional range, a large population, it occurs in a number of protected areas and in a variety of habitats and because it is unlikely to decline at the rate required to qualify for listing in a threatened category (Kranz *et al.* 2008).

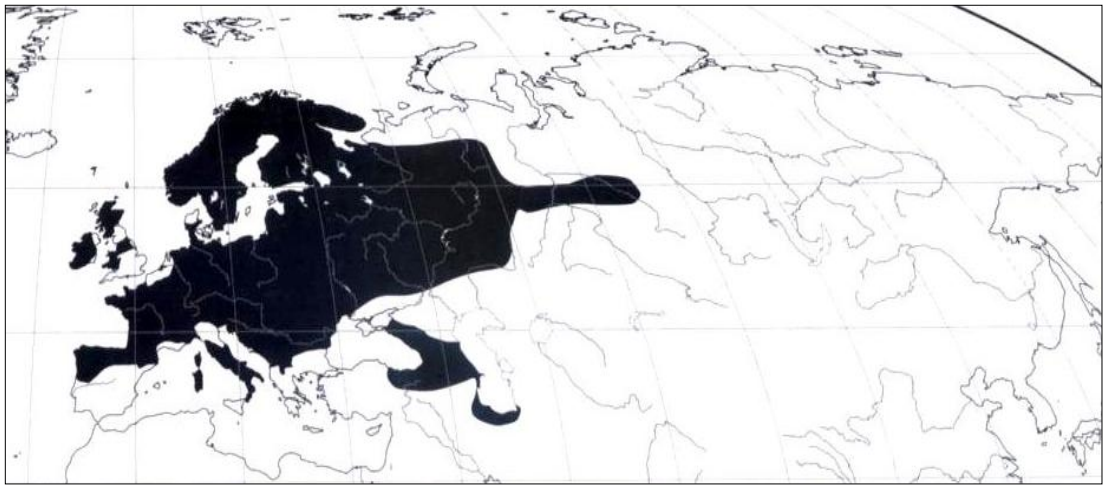


Figure 2: Global pine marten distribution. From Balharry (2008) in *Mammals of the British Isles: Handbook, 4th Edition.*

The pine marten is considered native to Ireland but its origin is currently unknown. Archaeological evidence shows the pine marten was present on the island at least as early as the Bronze Age, with bones from Kilgreaney Cave, Co. Waterford radiocarbon dated to 2780±55 years before present (Woodman *et al.* 1997). The early human immigrants arrived in Ireland approximately 9,000 years ago (Lynch 1996) and may have introduced the pine marten into Ireland as a commodity species for their luxurious fur or as pets (Searle 2008). Molecular evidence suggests the founder individuals came from the Iberian peninsula, with a single unique mitochondrial DNA haplotype found in Irish pine marten (hap *p*) which is most similar to the Iberian haplotype (hap *i*, Davison *et al.* 2001). Searle (2008) suggests this is quite possible given the trading links between south-west Europe and Ireland since Mesolithic times, or alternatively the introduction of Iberian genes into Ireland may have occurred through Britain as an intermediate. The discovery of hap *i* in Welsh museum specimens recently provided some support for this hypothesis (C. O'Reilly, unpublished data).

Pine martens have been hunted for their pelts for thousands of years in Europe and in Ireland since at least medieval times (Charles 1997; Fairley 2001). The value placed on their skins in the fur trade and the widespread destruction of Irish forests in the 17th century has had major impacts on the population with written accounts of their rarity emerging from the late 1600s onwards. In the 19th century the improvement of firearm technology and the development of the sporting estate placed further pressures on the species as it was considered vermin by gamekeepers (Langley & Yalden 1977; Fairley 2001). Despite this pest status the pine marten was still considered to be 'found all over the island in suitable localities' (Thompson 1856). Fairley (2001) compiled a list of all the records of pine marten presence in Ireland between 1870 and 1975 which confirmed the widespread distribution of the species, but it also highlighted a population decline over the period.

The first comprehensive assessment of pine marten distribution in Ireland was carried out in 1978-1980 by searching 428 10 km squares of the national grid for pine marten scats (O'Sullivan 1983). All suitable habitats were searched within each square based on

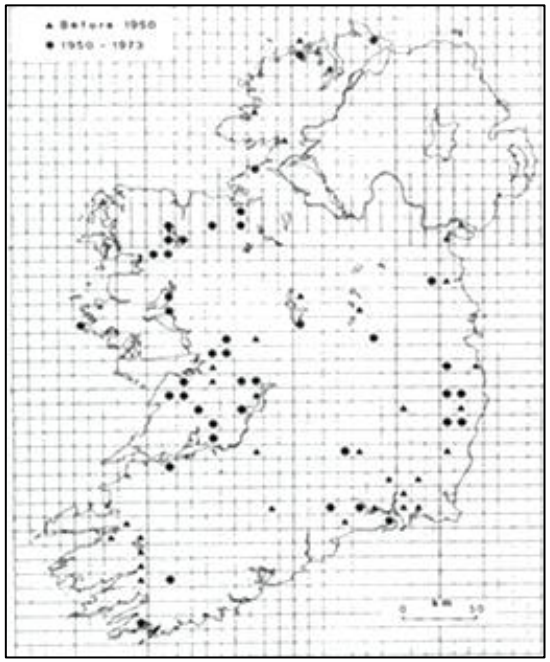
historical records or anecdotal information collated prior to the survey. Presence was only recorded in a survey unit by direct observation or the collection of characteristic scats, which led to the detection of pine marten in 97 of 428 squares surveyed (23%). The population was found to be mainly concentrated in the mid-west with other important populations in the midlands, south-east and north-east (Figure 3b) (O'Sullivan 1983). The range of the pine marten in Ireland had diminished significantly and it was more abundant as recently as the 1930s. The population decline was attributed in part to the increased demand for meat and wool in the 1950s and 1960s which led to government incentives to develop upland areas for farming, leading to the increased use of strychnine-laced fresh meat as bait for vermin control (O'Sullivan 1983; Fairley 2001). The main target for the poison was foxes, but pine marten were also vulnerable as they readily took carrion and hunted along the edges of woodland where the poison was placed (Fairley 2001). The species was finally protected under the Wildlife Act in 1976 making it illegal to hunt or injure an individual or to interfere with its breeding or resting place (Balharry *et al.* 2008). A decade later numbers had increased sufficiently to include the pine marten in Ireland's IUCN Red Data List for terrestrial mammal species under the category 'Internationally Important' as the species was in a more precarious state in continental Europe (Whilde 1993).

The national distribution of pine marten was reassessed in the National Pine Marten Survey (O'Mahony *et al.* 2006), which began in 2005 with a repeat survey of a random sample of 55 10 km squares originally surveyed by O'Sullivan (1983), and is ongoing (O'Mahony *et al.* 2007). Comparing both surveys, a statistically significant increase (↑ 18.1%) in the number of squares with pine marten presence was recorded. This range expansion was attributed to increased afforestation rates as the forest cover almost doubled in the years between the surveys from 420,000 ha to 700,000 ha, and pine marten are known to utilise thicket stage conifer plantations with an abundance of prey (Forbes 1937; O'Sullivan 1983; Fairley 2001). Legal protection, the ban on the use of strychnine poison in 1991 and deliberate translocations to areas which recently lost local populations were other possible factors which possibly worked in synergy to reverse the

decline in the Irish population (O'Mahony *et al.* 2006). The available data for the current distribution of the pine marten in Ireland from scat surveys is shown in Figure 3c. The favourable conservation status of the pine marten in Ireland places the species in the category of Least Concern using the new IUCN guidelines, along with the majority of the other terrestrial mammal species in Ireland at present (Marnell *et al.* 2009). However, estimates of population densities from a variety of habitats need to be obtained for conservation assessments in the future (O'Mahony *et al.* 2007).

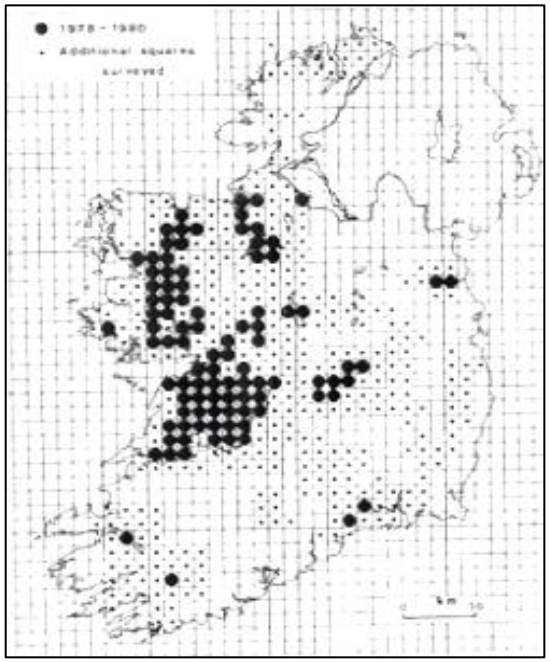
Figure 3: Distributional range of pine marten in Ireland from 1850 to present. The decline and expansion of pine marten across Ireland can be seen from (a) to (c). In Figure 3b 428 10km squares were surveyed and detection (large filled circles) and non-detection (small dots) are represented. Figure 3c is the survey data from the NPMS 2005-2006 (O'Mahony *et al.* 2007). A selection of squares surveyed in 1978-1980 were resurveyed and the change in status was assessed. It shows the current range expansion from core refuge areas identified in the previous survey (Figure 3b).

(a) pre-1973



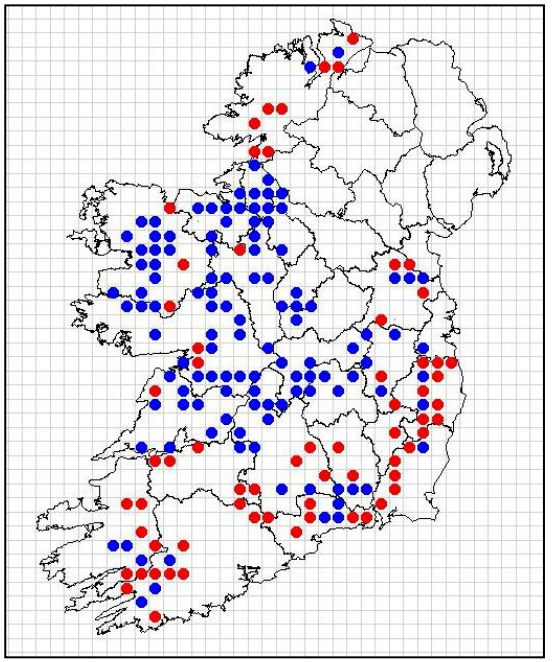
Source: O'Sullivan (1983)

(b) 1978-1980



Source: O'Sullivan (1983)

(c) 2005-2006



Source: O'Mahony (2007)

1.4. Survey techniques

As forest-dwellers and furbearers, martens are heavily influenced by habitat change and hunting pressures therefore it is important to regularly monitor their distribution and abundance (Proulx *et al.* 2004). Such information is essential for wildlife conservation and research, but is difficult to collect for carnivores which are often nocturnal, secretive and range over large areas (Wilson & Delahay 2001). Various methods for surveying for mustelid presence can be used separately or in combination.

1.4.1. Direct handling or observation

Direct observation of pine marten in the wild is rare, but sightings can be used effectively to identify possible core populations if the reliability of questionnaire records can be assessed and verified (Messenger & Birks 2000). However, a considerable increase in density would probably be necessary before the frequency of casual sightings would increase (Lockie 1964) therefore sightings are of limited use for indices of relative abundance. Live trapping can yield important information on the number, age, sex, reproductive status and parasite load of individuals but it is time consuming and expensive as a survey method with generally low capture rates in the order of 0.6-0.8 captures/100 trap nights (Ruette *et al.* 2003) or fewer in low density populations (Messenger & Birks 2000). As the pine marten is a protected species in many European countries, the risks to animal welfare from live capture warrant the investigation of alternative non-invasive survey methods based on the detection of field signs.

1.4.2. Road casualties

Animals found dead on roads as a result of traffic collisions provide useful records for determining broad scale distribution and material for post mortem analysis (Baker *et al.* 2004). The small number of records in low density populations may limit the potential use of this method for quantifying population status. In such cases, a major survey effort may be required to improve power. Road mortality rates can also change between survey years due to the upgrading of roads, leading to fluctuations in traffic volume and therefore road deaths independently of local population dynamics. Above a certain level of traffic roads may be avoided altogether, resulting in a non-linear relationship between abundance and road-kill density (Baker *et al.* 2004). Nevertheless, the collation of road-kill records over space and time can be used to identify core populations and monitor their relative abundance in order to target conservation efforts towards the most vulnerable populations. Broekhuizen (2006) demonstrated the use of road-kill data to monitor pine marten populations in The Netherlands. The number of pine marten found dead on Dutch roads in the north, south and east of the country has declined gradually since the 1940s, signalling the loss of regional populations.

1.4.3. Scat collection

The collection of pine marten faeces (scats) from the field is relatively straightforward and is the most commonly employed method for monitoring pine marten populations across Europe (O'Sullivan 1983; McDonald *et al.* 1994; Strachan *et al.* 1996; O'Mahony *et al.* 2007; Pilot *et al.* 2007; Ruiz-Gonzalez *et al.* 2008). Scats are often the only sign of occupancy in an area. They are collected non-invasively without disturbing the natural behaviour of the study population and contain a large amount of information per survey effort (Kohn & Wayne 1997). Pine marten scats are sweet-smelling and cylindrical in shape with pointed ends, approximately 8 to 10 cm long and 1 cm in diameter, and are deposited on forest vehicular trails and paths, large rocks, or other features such as old

stone walls and fallen trees (Figure 4) (Fairley 2001). However, morphology can change depending on diet and environmental exposure, and scats may be interfered with by other mammals or invertebrates (Birks *et al.* 2004). Scent marking behaviour can also break down at low population densities, making scats as elusive as the species itself.

The accuracy of species identification is reliant on expert knowledge to identify scats to species level. This has proven to be difficult as scats from other species such as red fox *Vulpes vulpes*, polecat *Mustela putorius*, stoat *Mustela erminea* or American mink *Mustela vison* may also be present in the study area and have been mistaken for pine marten even by experienced field workers leading to inaccurate inferences about species status (Davison *et al.* 2002; Harrington *et al.* 2009). Such survey methods are also largely confined to habitats with a network of trails to collect scats such as plantation forestry. Scat deposition behaviour may vary within the species' range and recent studies suggest that even in established populations only a small percentage of the total number of scats (< 2%) may be deposited on forest trails (Roche 2008).

Figure 4: Characteristic pine marten scat. Scats are often placed on grass in the middle of forest paths. The same spot may be marked repeatedly, as can be seen from the remains of an older scat in the right of the picture.

1.4.4. Hair collection

Mammal fur may also be collected from resting sites (Bradley *et al.* 2008), birds' nests (Toth 2008), from faeces due to autogrooming (Zhang *et al.* 2009), or more commonly plucked from individuals using a specially designed hair snare. Hair-traps have several advantages over the cage traps used in live capture. They are cheaper to construct, generally more lightweight and therefore less cumbersome to carry to remote field sites, and they can also be left in the field for longer periods thus facilitating their use over larger areas (Mills *et al.* 2002). The use of hair-traps was first described over thirty years ago for a survey of arboreal small mammals in Australia (Suckling 1978). Baited PVC (polyvinylchloride) tubes were nailed to trees about 2-3 m above the ground. Hair-tube visitors left hair on adhesive tape lining the tube, which was later verified to species level by examination under a dissecting microscope and comparison to reference hair samples from potential species.

Several modifications of this original design have been used successfully to retrieve hair from other species by varying trap morphology, the bait type and the material used to capture hair. One of the first hair-traps to be used for martens was an open-ended wooden triangular or square squeeze tube with glue patches tacked inside for plucking hair (Foran *et al.* 1997). A possible disadvantage of using this type of trap design is the opportunity for multiple trap visits from different individuals within a single sampling session causing samples to be of mixed origin. Foran *et al.* (1997) used a single-serving bait and examined tracks in the snow at each hair-tube site to ensure the hair sample was left by one individual Snow tracking is not possible for countries with milder climates such as Ireland. Williams *et al.* (2009) used the same hair snare design to monitor the distribution and abundance of *Martes americana* and *Martes pennanti* in areas of sympatry, but with meat bait which could be removed more easily to deter repeat visits.

Another solution is to design single-sampling hair snares. Messenger & Birks (2000) designed a hair-trap for pine marten with a single entrance across which a spring is stretched. Bait is placed at the end of the trap, forcing the visitor to brush past the spring,

which snaps shut, snagging a sample of dorsal guard hairs from a single individual. This trap design was successfully field tested in Britain and Ireland and routinely removed over 10 hairs per individual for species identification (Messenger & Birks 2000; Lynch *et al.* 2006). Other mechanical removal methods include currycombs attached to the door of a cage trap (Belant 2003) and steel brushes secured in a PVC tube, which is a lighter design, less expensive and easier to construct (Pauli *et al.* 2008). The most suitable type of hair snare may depend on the budget and the goal of the study as all designs can be used to physically remove hair for morphological examination after casting in gelatin, but glue-based traps may be more suitable for studies intending to carry out downstream genetic analyses (Zielinski *et al.* 2006). The hair-trap used in this project combines the portability of the PVC material with the suitability of glue-based capture for genetic analysis, the efficacy of which has previously been confirmed in field trials (Figure 5) (Statham 2005; Roche 2008).

Figure 5: Pine marten using a hair-tube. Picture obtained using a remote camera. © Terry Whittaker, www.terrywhittaker.com.

A limitation of the use of biological remains is the inability to distinguish between transient martens passing through a study area and individuals resident and contributing to a viable breeding population (Messenger & Birks 2000). Wildlife surveys making use of non-invasively collected samples such as faeces or plucked hair can carry out additional genetic analyses to confirm the species, sex and individual identity of the sample.

1.4.5. Remote camera surveillance

Remote cameras can be useful for assessing the presence and sometimes individual identification of the target species and other members of the mammal community in a survey area. Bait or lure is placed in front of a camera which takes a picture or video footage when triggered by infra-red motion detection. Both remote camera placement and scat transect surveys were found to be reliable methods for assessing the presence and absence of pine marten and stone marten in a 67 km^2 area of north-west Spain (Rosellini *et al.* 2008).

1.5. Genetic analysis of non-invasively collected samples

The analysis of DNA from biological samples collected in the field allows researchers to address key questions in ecology which are difficult to answer in any other way (Beebee & Rowe 2008). Non-invasive genetic sampling allows researchers to study many individuals and populations in the wild without capturing or even observing the study animals, which is beneficial for rare, elusive or potentially dangerous species. Faeces and hair are commonly used but a variety of DNA sources have been used for mammals including museum specimens, saliva, blood and urine (reviewed in Beja-Pereira *et al.* 2009).

1.5.1. Polymerase chain reaction

Non-invasively obtained samples generally contain nanogram or picogram quantities of DNA. The polymerase chain reaction (PCR) is a standard laboratory technique used to generate enough genetic material for analysis by enzymatic synthesis of DNA to produce thousands or millions of copies. A heat-resistant DNA polymerase copies a region of DNA defined by two oligonucleotide primers complementary to the target DNA, with the addition of deoxynucleotides, magnesium, template DNA, buffer, and in some cases extra adjuvants to enhance amplification. The replication process is generally repeated 30-40 times resulting in exponential amplification of the desired target sequence. For some more difficult samples the number of PCR cycles may be increased to improve sensitivity, though this may increase the risk of amplifying contaminant molecules. Negative controls containing all the reagents with the same volume of water as DNA template in the other samples are used to monitor for PCR contamination (Butler 2005). Similarly, positive controls using a DNA template which is known to be reliable are used to ensure the reaction is working efficiently.

In contrast to standard PCR where DNA is detected post-amplification, in real-time PCR reactions are monitored throughout the cycling using either fluorescent DNA binding dyes or fluorogenic probes, a sophisticated detection system and computer software to display amplification plots and carry out analysis. No gels are required to analyse the products of a real-time PCR, which considerably reduces sample analysis time. There are three phases to any PCR; the exponential phase where PCR product doubles after every cycle (if the reaction is at 100% efficiency), the linear phase where one or more components are limiting and the plateau phase where amplification ends as some reaction components are used up. End-point PCR methods detect PCR products in the plateau phase which is inefficient as a quantitation method as the reaction is not at 100% efficiency and the relationship between cycle number and amount of PCR product is not linear. Real-time PCR detects product accumulation in the exponential phase after each PCR cycle. For calculating DNA quantity, an arbitrary fluorescence threshold is used.

This threshold is usually set by the software to be above background fluorescence but in the exponential amplification phase. As the amplification proceeds, fluorescence increases with PCR product formation. The fractional cycle number at which the sample fluorescence reaches this threshold value is called the cycle threshold (C_T) value (Figure 6).

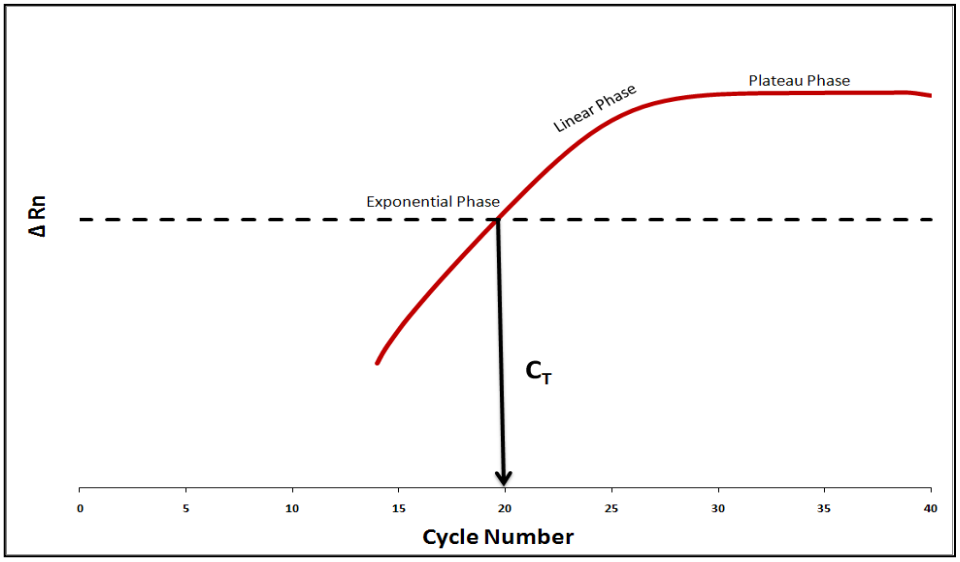


Figure 6: Real-time PCR amplification plot. Schematic amplification plot showing threshold fluorescence (dashed line) and C_T value. ΔR_N is the fluorescence at each cycle normalized against an internal reference dye, minus the baseline fluorescence.

The C_T value provides a measure of the initial DNA concentration in the sample, as a sample with many copies of the target DNA sequence will reach this value sooner and have a lower C_T value than a sample with low target DNA copy number (high C_T). In this project real-time PCR was used as a means to confirm the amplification of the intended target DNA, rather than the absolute DNA concentration, but samples were compared by C_T value as a relative measure of the initial target DNA concentration. There are many different reaction chemistries available for real-time PCR analysis

(Andersen *et al.* 2006). Two main chemistries were used in this project for the detection of amplified target DNA: intercalating dyes (SYBR® Green) and fluorogenic probes (TaqMan® MGB). The major difference between TaqMan® MGB probe assays and SYBR® Green assays is sequence specificity. Intercalating dyes will bind to any double-stranded DNA whereas the TaqMan® MGB probes are specific to the target DNA sequence.

SYBR® Green

SYBR® Green is an intercalating dye commonly used in real-time PCR applications as it binds specifically to double-stranded DNA (dsDNA). When the dye is added to the DNA sample, it binds to all dsDNA present. As the PCR proceeds, more dsDNA is produced, binding more SYBR®. The net increase in fluorescence is therefore proportional to the increase in amplicon and related to the starting copy number of target DNA sequences. Although this method is applicable to any target sequence amplifiable in a real-time PCR reaction, the lack of dye specificity can generate false signals especially if the reaction is not optimised as primer artefacts will also bind SYBR® and fluoresce. Confirmation of specific amplification of the target DNA is therefore required after the PCR cycling. A melt-curve analysis (MCA) is carried out which calculates the melting temperature (T_M) of the amplified DNA based on the loss of fluorescence when the two DNA strands separate. Primer-dimer artefacts are differentiated from target DNA amplification by a characteristically lower T_M value (Ririe *et al.* 1997). The T_M of an amplicon can also be used for species identification based on differences between species in the amplified region (Moran *et al.* 2008; O'Reilly *et al.* 2008).

TaqMan® MGB fluorogenic probes

TaqMan® MGB probes are synthesized oligonucleotides which are dual-labelled with a reporter and quencher dye at opposite ends (Livak *et al.* 1995). When intact, the

fluorescence emitted from the 5' reporter dye is transferred to the 3' quencher dye and no fluorescent signal is detected by the real-time PCR instrument. The probes are designed to have high annealing temperatures (65 - 67 °C), to hybridize between the forward and reverse primer sites before the PCR primers, which usually have annealing temperatures of 58 – 60 °C. The annealing and extension of the primers then displaces the hybridized probe through the 5' to 3' exonuclease activity of *Taq* DNA polymerase (Figure 7).

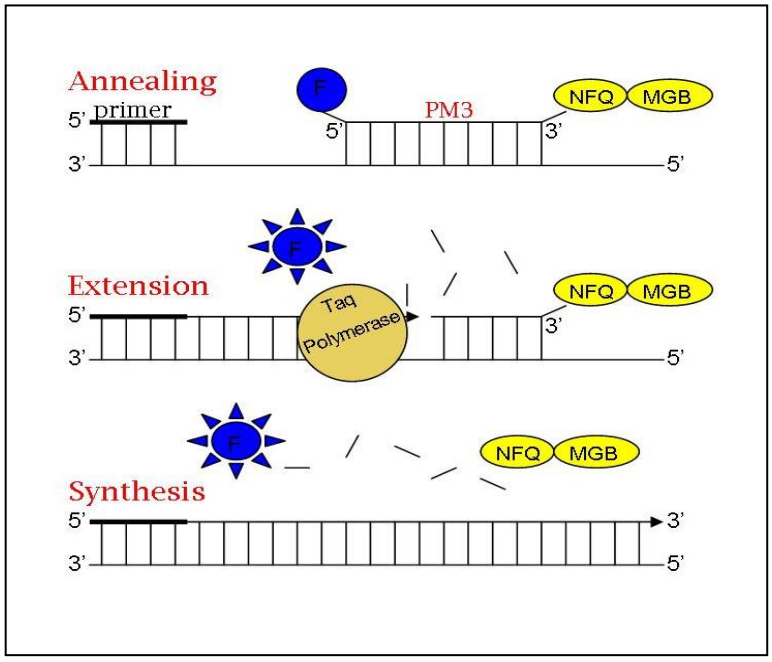


Figure 7: Schematic of a TaqMan® MGB real-time PCR assay. The 6-FAM (F) labelled TaqMan® MGB probe anneals at a higher temperature than the PCR primers. Following the annealing and extension of the PCR primers by *Taq* polymerase, the probe is disintegrated, releasing F from the quencher dye (NFQ) which is detected by the instrument as an increase in fluorescence. After synthesis of the target region the reaction is repeated, leading to an increase in fluorescence after every PCR cycle.

Cleavage of the probe releases the reporter dye from the quencher dye, leading to an increase in fluorescent signal which is plotted in real-time after each PCR cycle, as for SYBR® Green assays (Figure 6). The sequence specificity of TaqMan® MGB probes is enhanced through the use of a 3' Minor Groove Binder (MGB) which stabilises the probe-template hybrid allowing shorter probes (usually less than 20 nucleotides) with high sequence specificity to be used (Kutyavin *et al.* 2000).

1.5.2. Species identification

The mustelids are difficult to survey as they are often nocturnal, shy and present at low population densities. Surveying for their presence can also be hampered by the presence of scats from sympatric carnivores which can confuse identification of the target species. These species can have differing conservation priorities to the pine marten such as the red fox and stone marten (Davison *et al.* 2002; Pilot *et al.* 2007) and the invasive American mink (Harrington *et al.* 2009). Genetic species identification is recommended in situations where misidentification with other species is possible (Birks *et al.* 2004) and accuracy is important as correct species identification of field signs is important to properly inform conservation management plans (Vercillo *et al.* 2004).

Species discrimination is typically carried out using mitochondrial DNA analysis. Mitochondria are cellular organelles involved in respiration with their own haploid, circular, maternally inherited genome of approximately 15-20 kb long in animals encoding 13 protein subunits, 2 rRNAs and 22 tRNAs with a general gene arrangement that is conserved among many species (Boore 1999). Replication of the mitochondrial genome is initiated in the non-coding control region, termed the D-loop. The mutation rate of mitochondrial DNA (mtDNA) is 5-10 times higher than that of single copy nuclear DNA (scnDNA) (Butler 2005). As a result the control region has high intra- and inter-specific variability, making it a useful genetic marker for inferring evolutionary relationships and for species discrimination. The mtDNA cytochrome b gene is also used

in molecular ecology as it is a coding sequence with more conserved regions, making it better suited to the design of universal primers to amplify homologous mtDNA sequences from many species (Kocher *et al.* 1989). No mtDNA sequence polymorphism has been detected so far in Irish pine marten, with only one haplotype for either D-loop or cytochrome b (Davison *et al.* 2001; Statham *et al.* 2005; Lynch 2006).

A major advantage of mtDNA for genetic analysis of non-invasive samples is its high copy number, with hundreds to thousands of mitochondria per cell compared with only two copies of scnDNA sequences, yielding sufficient genetic material from biological samples with sub-optimal DNA quality. All methods begin with the PCR amplification of a target DNA region. Direct sequencing of approximately 500-bp of mtDNA was used to identify the presence of the mustelids *Martes melampus*, *Mustela sibrica* and *Martes zibellina* in Japan (Murakami 2002), however the success rate of species identification for scats was very low (2.6%), possibly due to the large size of the target region. DNA sequence information is commonly used to identify species-specific sites for restriction enzymes. PCR products are then digested to create restriction fragment length polymorphisms (PCR-RFLP). This method has been used extensively (Hansen & Jacobsen 1999; Vercillo *et al.* 2004; Colli *et al.* 2005; Statham *et al.* 2005; Ruiz-Gonzalez *et al.* 2008) but PCR-RFLP requires relatively large product sizes (>250-bp) and an extra handling step which may increase the contamination risk. PCR inhibitors may also interfere with the restriction digest, giving false results for species identification. Shorter DNA regions can be targeted if one or both PCR primers are species-specific resulting in species discrimination in a single step which both time and cost effective (Namba *et al.* 2007; Fernandes *et al.* 2008).

The potential of real-time PCR for the amplification and analysis of DNA from non-invasively collected samples has been recognised for its high sensitivity, small product size (typically 70-150-bp) and high throughput, but very few studies have employed the technology to date for free-ranging species (Beja-Pereira *et al.* 2009). Protocols have been developed for species identification to detect mammal and poultry DNA for quality

control of animal and human food using real-time PCR fluorescent detection methods (Brodmann & Moor 2003; Dooley *et al.* 2004). For wild populations, real-time PCR has mainly been used as a means to rapidly quantify DNA extracts in order to select samples with the highest quality DNA for downstream microsatellite genotyping (Morin *et al.* 2001).

Some species identification methods have been based on nuclear DNA amplification. Microsatellite loci with length polymorphisms between species have enabled discrimination between pine marten and stone marten giving insights into their habitat and dietary preferences (Domingo-Roura 2002; Pilot *et al.* 2007; Posłuszny *et al.* 2007). Furthermore, the use of microsatellite genotypes had the additional advantage of giving an estimate of the minimum number of individuals alive in the study area and the potential for the analysed samples to yield full multilocus genotypes (Posłuszny *et al.* 2007). The *Interphotoreceptor Retinoid-Binding Protein* (IRBP) nuclear gene also has potential for species identification and its use has been demonstrated for a wide range of carnivore species in Iberia, including the pine marten (Oliveira *et al.* 2009). Again, the use of a short nuclear DNA region is useful for screening samples which may be useful for sex or individual identification and the success rate for non-invasive samples was high at 81.13% (Oliveira *et al.* 2009).

1.5.3. Sex identification

The ability to recognise males and females is important for understanding the biology of sexually reproducing species and developing management plans (Lynch & Brown 2006). Sexing of animals may be carried out by direct observation of genitalia, sexual dimorphism or sex-biased behaviour. Molecular sexing of biological tissues is required where it is not possible to directly observe and distinguish the sex of animals in the field, or for juveniles and species which lack sexual dimorphism, masking the underlying genetic sex of the individual.

Molecular sexing uses variation in DNA between the sexes to determine gender (Vidya *et al.* 2003). Mammals have a chromosomal system of sex determination where females possess two X chromosomes and males carry an X chromosome copy and a smaller Y chromosome. Genetic techniques used for sex determination of mammals include the amplification of Y-specific sequences or the co-amplification of homologous genes from both sex chromosomes which is more reliable as the X chromosome fragment serves as an internal control for the PCR in the event of low or degraded DNA in the sample.

The SRY gene is located in the Non-Recombining Region of the Y chromosome and is critical for normal male development. The SRY gene is male specific and conserved in most mammal species (Sinclair *et al.* 1990). PCR primers can be designed to amplify a region of the SRY with gender identification based on the presence (male) or absence (female) of a PCR product. A lack of SRY amplification cannot be taken as confirmation that a sample originated from a female donor (Taberlet *et al.* 1993). Many factors can contribute to amplification failure such as insufficient or degraded target DNA and the presence of inhibitors. It is therefore necessary to include an internal positive control in the PCR. Researchers have used primers for the zinc finger (ZF) gene on the sex chromosomes (Bryja & Konecny 2003), amelogenin, mitochondrial DNA (Palsboll *et al.* 1992; Taberlet *et al.* 1993) and a microsatellite marker (Dallas *et al.* 2000) for this purpose. The ideal control should be single locus, of equal copy number in the genome, approximately the same size as the SRY product and amplifiable under the same reaction conditions. Although sex typing has been carried out on mustelids by SRY amplification (Lynch & Brown 2006), it is more desirable to use a single primer pair in a PCR to amplify sequences from both the X and Y chromosomes, with amplification of X-linked sequences acting as an internal control for the reaction.

Amelogenins are a family of extracellular matrix enamel proteins with homologs on the X (AMGX) and Y (AMGY) chromosomes (Salido *et al.* 1992). Differentiation between the sexes is based on a deletion of six nucleotides in the first intron of the AMGX, resulting in a 106-bp PCR product for females and products of 106-bp and 112-bp for

males (Sullivan *et al.* 1993). The amelogenin marker has been successfully used to sex sheep and deer (Pfeiffer & Brenig 2005), cattle (Ennis & Gallagher 1994) and prosimians (Fredsted & Villesen 2004). The short and similar product sizes for AMGX and AMGY are suitable for amplification from degraded samples, however this assay may not be diagnostic for mustelids as Hattori *et al.* (2003) found no size polymorphism between the sexes for sea otter *Enhydra lutris* tissue.

Sex determination can also be achieved by targeting the zinc finger gene present on both the X and Y chromosomes. Eutherian mammals generally have one copy of the gene on each chromosome, termed ZFX and ZFY. Similar to species identification methods, PCR primers have been designed based on conserved regions to amplify and directly sequence (Fernando & Melnick 2001) or digest the PCR product with PCR-RFLP (Mucci & Randi 2007; Statham *et al.* 2007) to discriminate between the sexes. Researchers have designed assays based on different regions of the zinc finger gene, targeting conserved exon or more variable intron sequences. Non-coding regions can accumulate mutations at a higher rate than coding regions of a gene; the ZFY intron evolves at about twice the rate of the ZFX intron in felids (Slattery & O'Brien 1998). The size of the ZFY intron also varies with respect to ZFX. In a study of eight mammalian species the ZFX intron product size ranged from 912-1013-bp whereas the ZFY intron size variation was 868-1278-bp (Shaw *et al.* 2003). Thus gender can be determined from biological samples in a single PCR based on differences in size between ZFX and ZFY on agarose gels with no further restriction digestion or sequencing. Recent studies have extended this method to enable the molecular sexing of non-invasively collected samples by targeting short PCR products less than 200-bp which still differ in size between the sexes (Durnin *et al.* 2007; Peppin *et al.* 2009). Real-time PCR has also been used for sexing cetacean species on the basis of single nucleotide polymorphisms between ZFX and ZFY genes (Morin *et al.* 2005). The assay was highly sensitive and the small product of 105-bp enabled sex identification from samples which had failed with other sexing methods based on larger SRY or ZFY sequences.

Non-invasive samples can yield small amounts of DNA, which may also be degraded. Allele dropout may occur where only one allele of the homologs amplifies e.g. X only in a male sample therefore gender can be incorrectly assigned. This type of error can lead to a positive bias in the number of females in the population, thus affecting conservation management decisions. One of the earliest studies to employ an extensive non-invasive genetic sampling protocol was a population census of brown bears *Ursus arctos* in the Pyrenees, France. The population was shown to be in immediate danger of extinction with only five individuals remaining on the basis of genetic analysis, track counts and remote camera surveillance. Only one remaining adult female was discovered. This gave the project a sense of urgency and conservation managers were advised to augment the population with females only (Taberlet *et al.* 1997). The accuracy of sex determination was crucial for proper conservation management at such low numbers. It is suggested that more replicates will be required for samples which amplify an X fragment only (in ZFX/Y) or internal control only (in SRY) to be sure PCR results are due to lack of the gene amplification rather than stochastic effects in the PCR. Taberlet *et al.* (1997) used a multiple tubes approach with nine replicates whereby the SRY gene needed to be observed at least twice for accurate sexing as male and female samples were only confirmed after all reactions amplified a microsatellite locus internal control but failed to amplify the SRY fragment. Lynch & Brown (2006) used the SRY sexing assay without an internal control for pine marten, but demonstrated the reliability of the sexing assay with hair and tissue samples using two replicates. Another possible source of error when using conserved mammalian sexing primers is the amplification of Y-linked sequences from prey which is only detectable when known females are genetically identified as males (Murphy *et al.* 2003). A potential solution to this problem is to design species-specific sex assays, where primers anchored in conserved exon regions are used to obtain sequence data for more variable intron regions. Hedmark *et al.* (2004) successfully used this approach to develop wolverine-specific and possibly mustelid-specific primers to amplify the Y-linked DBY gene, with amplification from wolverine faeces and urine and no amplification from possible prey species.

1.5.4. Individual Identification

The ability to recognise individuals is vitally important in wildlife management for estimating population size. Genetic techniques can be used to identify individuals of rare and elusive species without observation, through the amplification and genotyping of several polymorphic microsatellite loci from non-invasively collected samples such as scats and hair (Frantz *et al.* 2003; Frantz *et al.* 2004). Microsatellites are repetitive DNA sequences whose repeat unit is typically 2-6 base pairs tandemly repeated 5-40 times (Selkoe & Toonen 2006). They are mostly found in non-coding DNA regions and are assumed to be selectively neutral, though some microsatellites are located in coding DNA and are responsible for genetic disorders such as Fragile X Syndrome (Schlotterer 2000). Microsatellites are often highly polymorphic as a result of high mutation rates in the order of 1×10^{-4} mutations per generation (Beebee & Rowe 2008). DNA slippage during replication is proposed to be the main cause for repeat unit variation in microsatellites (Schlotterer 2000). During polymerisation, the nascent or template strand may dissociate in the repeat unit region and realign out of register. If the error occurs on the new strand, the repeat unit number will have increased by one unit whereas if the template strand re-aligns incorrectly then the new strand will be one repeat shorter than the original DNA template. There are three major theoretical models of microsatellite mutation (Beebee & Rowe 2008). The Step-wise Mutation Model (SMM) assumes microsatellites length is altered by replication slippage, with alleles derived by increases or decreases in repeat units. Under the SMM model, the mutational history of alleles in a sample is potentially recoverable as, for example, a microsatellite locus with n repeats is assumed to share a more recent common ancestor with alleles of n-1 repeats than with alleles of n-5 repeats. The Infinite Alleles Model (IAM) is the generally accepted model for markers defined by point mutations such as for allozymes. Alleles are not derived from one another by single repeat mutations under this model but arise spontaneously and therefore a large amount of allelic diversity is expected (Beebee & Rowe 2008). An intermediate model, the Two-Phased Model (TPM) was developed to account for the

possibility of more complex mutation at microsatellite loci. Under the TPM, the majority of allelic diversity is explained by IAM, with a percentage of step-wise changes.

Microsatellites are highly informative molecular markers due to their single locus, codominant nature and high allelic diversity. They are also PCR-based markers ranging in size from approximately 75 to 300 base pairs, making them particularly useful for the analysis of degraded or low copy number DNA (Allendorf & Luikart 2007). The isolation and characterisation of microsatellite loci involves screening a genomic DNA library for positive clones containing a particular microsatellite sequence, such as $(GT)_n$, followed by sequence analysis and PCR primer design (Zane *et al.* 2002). PCR primers are designed to hybridise to conserved sequences flanking the repeat region, which varies in length if polymorphic, giving a variable length PCR product which is resolved on a polyacrylamide gel. The process of microsatellite development can be time consuming and is often bypassed by examining the literature for previously characterised microsatellites from congeneric species or other related taxa. However, estimates of genetic variability can be significantly lower using microsatellites transferred from other species and amplification success may decrease with increasing phylogenetic distance (Barbara *et al.* 2007). Nevertheless, several studies have developed mustelid microsatellite loci and reported their polymorphism across a range of related species (Davis & Strobeck 1998; Fleming *et al.* 1999; Beheler *et al.* 2005; Cabria *et al.* 2007).

To create a genetic profile a DNA sample must be genotyped at several loci, often 10 or more, but this number depends on the polymorphism of the microsatellite markers used and the research question. When two multilocus DNA profiles are different (provided there are no errors in analysis) the two samples can be excluded as being derived from the same individual. A positive match between two multilocus profiles does not necessarily mean both samples came from the same individual however, as this depends on the ability of the microsatellite set to identify genotypes as being unique. The most commonly used statistic to quantify the power of microsatellite loci to identify

individuals is the probability of identity (PI), which is the probability that two different individuals sampled from the population will have identical locus or multilocus genotypes by chance. PI can be calculated based on the assumption that the two sampled genotypes are from unrelated individuals (PI*ave*) or from siblings (PI*sibs*) (Taberlet & Luikart 1999). It is important to calculate PI statistics before carrying out a population census as using too few loci can underestimate population size due to individuals becoming genetic shadows of each other (Mills *et al.* 2000), whereas scoring more loci than are necessary can inflate population size estimates from an increased risk of genotyping errors and it also depletes precious template DNA (Creel *et al.* 2003). The microsatellite genotype of a sample equals individual identity only when the PI is zero (Mills *et al.* 2000). Researchers attempt to bring the PI as close to zero as possible so that the chance of obtaining the same genetic profile for multiple individuals is negligible. The level of heterozygosity of each locus is inversely correlated with PI so it is desirable to type polymorphic microsatellites with high heterozygosities. For wildlife studies, PI values range from 0.061 to 0.51 per locus (Mills *et al.* 2000). The actual PI required for a study is relative to the actual number of animals which may be sampled in the field and the level of certainty required for individual identification. The Northern hairy-nosed wombat *Lasiorhinus krefftii* has a population of about 100 individuals, therefore the eight variable microsatellite loci used were sufficient for reliable individual identification, with a PI*sibs* of 0.005 (Sloane *et al.* 2000).

The simplest application of microsatellites for estimating population size is to count the number of unique genotypes to give the minimum number alive in the survey area during the period of sample collection. A large number of samples are required (approximately three times the number of expected individuals in the population) when using non-invasive sampling to improve the precision of the estimate (Solberg *et al.* 2006; Puechmaille & Petit 2007). The wolverine is a mustelid with a history of persecution similar to the pine marten in Britain and Ireland. The population in Norway and Sweden has been heavily persecuted since the 19th century, with legal protection given in 1969 in Sweden and in 1973-1983 in Norway (Hedmark & Ellegren 2007).

Subsequent recovery and expansion in recent years has led to the establishment of new populations outside the main range of the wolverine along the Norwegian-Swedish border. Hedmark & Ellegren (2007) estimated the minimum population size, the number of founders, territoriality, genetic isolation and the relatedness of individuals in two newly founded wolverine populations using genetic analysis of faeces and glandular excretions over five field seasons. The number and sex of individuals was identified by genotyping 227 (of 416 collected) samples with a multilocus tag of 10 microsatellites isolated from wolverine and American mink. An analysis of a further set of microsatellites was used to estimate relatedness between individuals, parentage, and territory size by repeat captures of individual genotypes at different locations and inbreeding between individuals in the population, all without ever capturing a single animal. Microsatellite DNA analysis has also been applied to pine marten and stone marten scats to estimate the distribution and minimum population size for both species simultaneously in a 105 km^2 area of central Poland, which would have been impossible without genetic analysis due to the morphological similarity between the scats of both species (Posłuszny *et al.* 2007).

Plucked hair is also a popular source of DNA for molecular tracking using microsatellite analysis. Mowat & Paetkau (2002) estimated the size of an American marten population in a 797 km^2 area of the Central Selkirk Mountains in Canada by collecting 180 hair samples using a modification of the hair-trap designed by Foran *et al.* (1997). Multilocus microsatellite genotypes were obtained for 139 (77.2%) samples, which gave a minimum population size estimate of 88 martens for the area as some samples represented recaptures of the same individual. Further exploration of the data enabled the researchers to estimate the total population size with capture-recapture models (213, 95% CI: 148-348), the average movement distance between captures (2.6 km, range 0.5-8.9 km) and the population density (0.33 martens/km^2, 95% CI: 0.23-0.55). Williams *et al.* (2009) used the same hair-trap design to record the distribution and abundance of fishers *Martes pennanti* and martens *Martes americana* over a 672 km^2 area of the

Ottawa National Forest, Michigan, USA. There have been no published studies to date on the use of plucked hair genotyping to census *Martes martes* populations.

1.5.5. Technical issues associated with non-invasive genotyping

Microsatellite genotypes are unique and permanent tags for individual identification as long as enough loci are scored to assign individual identity based on the PI of the markers and the multilocus fingerprint is not misread by genotyping error. Non-invasively collected samples typically have low quality and/or quantity DNA and are therefore particularly susceptible to errors in genotype determination (Pompanon *et al.* 2005). Genotyping errors can have a significant impact on population size estimation by increasing the number of unique genotypes in a sample set, possibly biasing estimates upwards by several times the actual population size (Creel *et al.* 2003). The cause of genotyping error may not be known, but usually arise due to issues with DNA quantity, quality and sequence, biochemical artefacts or human errors (Pompanon *et al.* 2005). For microsatellite analysis, a common problem is 'allele dropout' which is the failure of one allele of a heterozygous genotype to amplify. This is the most common type of genotyping error in non-invasive genetic studies and can lead to an apparent excess of homozygotes at problematic loci. Alleles can also fail to amplify due to primer binding site mismatches ('null alleles') which is a particular problem for cross-species applications where sequence information may not be available for the primer binding site. Another form of genotyping error is 'false alleles' where an allele-like artefact is generated by PCR. This type of error is less common and may be a result of amplification of non-specific DNA, particularly in scat samples (Bradley & Vigilant 2002). Finally, human error in genotype determination is a major cause of error in microsatellite datasets as a result of difficulties in allele calling or data input (Pompanon *et al.* 2005).

It has become standard practice to systematically replicate genotypes for each sample in order to define a consensus genotype, particularly in the case of homozygote genotypes which may be a result of allele dropout. The widely used multiple-tubes protocol requires at least seven replicates of a homozygote genotype before it is accepted and two replicates of a heterozygote to obtain reliable genetic typing with a confidence level of 99% (Taberlet *et al.* 1996). The extensive number of amplifications required for each sample is both time and sample consuming. Frantz *et al.* (2003) developed the comparative multiple tubes method which involves genotyping each locus in triplicate and comparing provisional multilocus genotypes on a pairwise basis to identify samples which need to be repeated in order to be confident each genotype represents a different individual. Hansen *et al.* (2008) compared both multiple-tubes protocols and found that reliable genotypes were obtained with fewer PCRs and reduced error rates with the comparative tubes protocol than the original strategy of Taberlet *et al.* (1996) for otter faeces, which are notoriously difficult to genotype with low amplification success rates.

Allele dropout occurs widely in non-invasive genotyping as the quantity of DNA in samples such as faeces and hair is often in the picogram range, with further amplification problems when the DNA is degraded due to environmental exposure to UV radiation, humidity and heat (McKelvey & Schwartz 2004). The collection and preservation of such samples is therefore an important first step towards obtaining reliable multilocus genotypes. In general, samples should be collected as fresh as possible from the field in dry field conditions (Murphy *et al.* 2007; Santini *et al.* 2007). Preservation methods aim to slow the action of nucleases and include methods based on drying, freezing and storage in specially formulated buffers (Beja-Pereira *et al.* 2009). The next step, DNA extraction, is also crucial for genotyping success as it determines the quantity and quality of DNA available for all downstream applications. Commercial extraction kits are generally used for scats, either based on DNA adsorption to silica or magnetic beads (Flagstad *et al.* 1999), while hair samples are often digested in the presence of Chelex resin (Walsh *et al.* 1991). PCR inhibitors such as heme,

polysaccharides, bile salts, humic compounds and urea should be removed at the DNA extraction stage as they can interfere with amplification procedures (Butler 2005).

Prior to PCR amplification, DNA extracts may be screened to discard samples with low quantities of amplifiable nuclear DNA using either one or all of the microsatellite loci to be typed (Mowat & Paetkau 2002; Banks *et al.* 2003) or by quantification of target DNA using fluorescent dyes (Morin *et al.* 2001; Ball *et al.* 2007). The use of a two-step PCR protocol and redesigned primers amplifying shorter microsatellite PCR products has also shown promising application to improve amplification success (Bellemain & Taberlet 2004). The interpretation of electropherograms for capillary based microsatellite analysis has been made easier by the use of modified primers and specialised PCR protocols to reduce stuttering and split peaks (Brownstein *et al.* 1996). Considering all the factors which can influence the amplification and analysis of DNA from non-invasively collected samples, it is recommended to conduct a pilot study to determine whether it is possible to achieve the high amplification success rates and low genotyping error rates desirable to warrant the adoption of genetic techniques into population monitoring (Taberlet & Luikart 1999).

1.6. Genetic diversity and structure of natural populations

1.6.1. Importance of genetic variability

Genetic diversity is recognised as a major component of biological diversity which requires conservation as it is responsible for the biochemical, morphological and behavioural variability in a species (Frankham *et al.* 2004). Genetic variability is therefore seen as fundamental for adaptation to environmental changes and ultimately for speciation (Amos & Harwood 1998). The variety of domestic dog breeds is an example of the stunning array of phenotypic variation which can be expressed from a

single genome, the ancestral grey wolf *Canis lupus* (Frankham *et al.* 2004). Evaluation of the levels of genetic variability in a species is therefore important to determine whether conservation actions should be taken to preserve evolutionary potential. Microsatellite analysis was used in this project to measure genetic variability in the pine marten population in Ireland.

1.6.2. Estimation of genetic variation

A locus is polymorphic when it has at least two different alleles in a population, one of which has a frequency of at least 95% in small populations (<50 individuals) or 99% in large populations (Allendorf & Luikart 2007). Allelic diversity is the average number of alleles per locus across several microsatellite loci, called allelic richness when rarefaction analysis is used to account for differences in the size of sampled populations. Eutherian mammals are diploid and thus possess two copies of each locus in a genome. When both copies are the same the individual is homozygous and when both copies differ the individual is heterozygous. Observed heterozygosity (H_O) refers to the observed proportion of heterozygotes in a sample of individuals. Expected heterozygosity (H_E) is the expected proportion of heterozygotes in a randomly mating population and is the best general measure of genetic variation (Allendorf & Luikart 2007). H_E is calculated from the allele frequencies in the population as one minus the expected homozygosity for each locus. If a population is mating randomly and is experiencing no factors which change genetic variation from generation to generation such as mutation, selection, migration or fluctuations in population size then the allele and genotype frequencies will remain constant from generation to generation, which is known as Hardy-Weinberg equilibrium (Allendorf & Luikart 2007). Testing for significant differences between H_O and H_E in a population is important to identify if the above processes may be affecting the distribution of allelic variation in a population and is generally carried out using a chi-square or exact test in computer programs such as

GENEPOP (Raymond & Rousset 1995) as the calculations can be computationally intensive for polymorphic loci such as microsatellites.

1.6.3. Loss of genetic variability

A severe reduction in population size, known as a population bottleneck, can have a drastic effect on allelic diversity. When census population size decreases, the number of breeding individuals contributing to the next generation (the effective population size, Ne) may be reduced to a level where some alleles are not transmitted to the next generation due to sampling effects. This leads to the fixation of certain alleles over others by chance, termed random genetic drift (Frankham *et al.* 2004). Rare alleles which may be important for adaptation to future environmental conditions are easily lost in a population with low Ne due to the process of genetic drift (deYoung & Honeycutt 2003). The total amount of heterozygosity lost in a bottleneck depends on how severe the reduction in population size was and for how long it persisted. Species with slow growth rates are particularly vulnerable as the effective population size may remain at low numbers for several generations (Allendorf & Luikart 2007).

The founder effect is a special type of population bottleneck where a new population is founded by a small number of individuals which may have allele frequencies which are not representative of the parent population. Founder effects can occur naturally as a result of processes such as post-glacial colonisation or from deliberate translocations by humans (Beebee & Rowe 2008). Such a pattern has been detected for pine marten in Ireland. Genetic diversity at both nuclear and mitochondrial DNA markers was found to be lower in Ireland than continental Europe which may be a result of a small founding population post-glaciation (Davison *et al.* 2001; Kyle *et al.* 2003; Lynch 2006). In addition, Lynch (2006) detected a further reduction in genetic variation within Ireland due to the foundation of the population in Killarney National Park with individuals

translocated from Counties Mayo and Clare in the west, with lower H_E and allelic diversity values in the founded population compared to the source population.

1.6.4. Genetic structure

Genetic diversity can be further eroded by habitat fragmentation where populations are isolated and unable to exchange genetic information with one another through the dispersal and reproductive contribution of migrant individuals. Kyle *et al.* (2000) examined the genetic variability of American marten populations across northern Canada using microsatellite analysis and detected no significant genetic structuring at vast spatial scales, despite the presence of large geographic barriers. This study was subsequently extended to include genetic samples from populations across Canada, including southern regions where habitat fragmentation was expected to lead to some structuring through isolation and genetic drift (Kyle & Strobeck 2003). Contrary to this, no evidence for structuring was found across all sampled regions except for the insular Newfoundland population. The dispersal capacity and large effective population size of martens in Canada appears to be enough to maintain the species as one panmictic unit. The authors noted that although no genetic structure could be detected at a broad scale, there may be some fine scale genetic structuring due to barriers to gene flow such as transportation corridors. Habitat connectivity is important in the long term to maintain viable marten populations and is seen as a key conservation priority (Proulx *et al.* 2000).

An additional comparison was made between genetic structure in the American marten and the European marten (Kyle *et al.* 2003). Genetic samples were obtained from nine European countries to assess *Martes martes* genetic variability and population structure, with comparisons made to two populations of *Martes americana*. Many European populations were found to have a lower level of genetic variation than their counterparts in North America. This was suggested to be the result of extended anthropogenic impacts such as habitat fragmentation and direct persecution in Europe with a longer

cultural history. The continental *Martes martes* had similar genetic variation to the contiguous populations of *Martes americana* in the Yukon, but the island populations of *M. martes* had much lower levels of variability, similar to *Martes americana atrata* on the island of Newfoundland. Scottish and Irish populations were found to be differentiated from each other and both were significantly different to continental populations. Assignment tests based on the probability of a multilocus genotype belonging to one population over another further confirmed the genetic distinction of Scotland and Ireland as 4 (7%) and zero individuals were cross assigned to other populations on the basis of genetic data at 8 microsatellites. Finland and the Netherlands were also relatively structured, with >77% of individuals assigned to the sampled population. Evidence was found for hybridization between *M. martes* and *M. americana*, supporting earlier studies based on mitochondrial DNA. Kyle *et al.* (2003) suggested that populations could be considered as separate management units, but that historical and contemporary causes of genetic structure needed to be disentangled.

The inclusion of historic samples in a genetic study provides a useful comparison to identify whether low genetic variability is a result of recent reductions in population size or ancient demographic fluctuations (Pertoldi *et al.* 2001). The spatio-temporal population dynamics of Danish pine marten populations was investigated by genotyping historical (<1970) and recent (>1970) tissue samples from the Jutland peninsula and the island of Sealand, which are geographically isolated (Pertoldi *et al.* 2008). Evidence for a recent population bottleneck was found in the Jutland population by comparison to historical genetic variation, but no such pattern was found in the Sealand population. Differentiation between populations in Denmark was primarily caused by loss of genetic variability in Jutland. Agricultural development since the 1970s impacted heavily on the effective population size in Jutland as revealed by the genetic signature of a population bottleneck. This comparison was only achievable by comparing historical and recent samples, but the loss of genetic variation was not an immediate conservation concern. No further substructuring was found in the Jutland population therefore the dispersal

capacity of the pine marten appeared to be sufficient for the maintenance of gene flow, as for the American marten.

1.7. Outline of thesis

The main objective of this project was to develop the genetic methods required to census pine marten populations accurately and non-invasively. In order to achieve this, a new DNA extraction method was developed to improve the recovery of DNA from scats, novel real-time PCR techniques were applied to tissue, scat and hair samples for species and sex identification and a panel of microsatellite markers was developed for individual identification. The full suite of molecular techniques was then tested on samples collected from two forests to evaluate the feasibility of carrying out a census using non-invasive genetic sampling. The results chapters were divided in four to reflect the main stages of the project:

1. An evaluation of methods for extracting DNA from scats, incorporating two different commercially available purification kits, a commercial preservation buffer and relative DNA quantification by real-time PCR amplification.
2. The development of novel real-time PCR techniques for species and sex identification.
3. Selection and optimisation of a microsatellite marker panel capable of discriminating between individual pine marten, with particular emphasis on the application to non-invasively collected samples.
4. The application of the optimised molecular techniques to a population census of two forests, with comparison of the results to the more invasive survey method of live capture.

Chapter 2

Extraction and amplification of DNA from pine marten scats:
An evaluation of sampling methods and commercial DNA purification kits

Related publication:

O'Reilly C, Statham M, Mullins J, Turner PD, O'Mahony D (2008) Efficient species identification of pine marten (*Martes martes*) and red fox (*Vulpes vulpes*) scats using a 5' nuclease real-time PCR assay. *Conservation Genetics*, **9**, 735-738. Doi: 10.1007/s10592-007-9371-6.

2.1. Introduction

The accurate identification of scats to species level is important for the proper interpretation of survey data. Researchers are increasingly turning to genetic methods to validate the results of species identification by survey personnel in the field. Faeces are challenging samples for genetic analysis as DNA from the defecator is present in low quantities in a complex mixture with non-target DNA and PCR inhibitors (Kohn & Wayne 1997). In addition, many external factors affect the success rate of DNA amplification from scats, including the season of collection and diet, therefore a pilot study should be carried out to determine the optimal DNA extraction protocol for the species of interest (Piggott & Taylor 2003a).

Efficient sampling methods can increase the ratio of shed epithelial cells taken through to DNA extraction and purification techniques (Flagstad *et al.* 1999; Palomares *et al.* 2002), while inhibitory compounds are usually removed by commercial DNA purification kits. Real-time polymerase chain reaction (real-time PCR) technology represents a relatively untapped resource for conservation genetics despite its ability to detect and quantify target DNA from difficult environmental samples (O'Reilly *et al.* 2008; Beja-Pereira *et al.* 2009). Rolfe *et al.* (2007) described an efficient real-time PCR method for norovirus detection from faecal samples which were pre-treated with a specialised Stool Transport and Recovery (S.T.A.R.) buffer. Three properties of S.T.A.R. buffer make it potentially useful for the genetic analysis of wild animal faeces: (i) pathogenic organisms are inactivated, (ii) nucleic acid degradation is minimized and (iii) the binding of nucleic acids to magnetic beads in commercial DNA purification kits is enhanced (Espy et al. 2006).

The aim of this study was to develop a standard protocol for the isolation and purification of DNA from pine marten scats. Target DNA isolation success was evaluated with real-time PCR amplification of a short (60-bp) region of mtDNA. Specifically, three factors affecting the recovery and amplification of DNA were considered:

1. *The sampling technique.* Three commonly used techniques (surface scrape, surface wash and homogenisation) were compared for target DNA isolation success. Surface treatments aim to increase the number of epithelial cells (target DNA) in the extract whereas a sample homogenisation is often used in cases where a surface treatment is not possible.

2. *Sampling buffer.* The recently developed Stool Transport and Recovery (S.T.A.R.) buffer and a phosphate-buffered-saline digestion buffer were compared for efficiency of epithelial cell isolation. S.T.A.R. buffer is a commercially available buffer which is specially formulated to inactivate pathogenic organisms and minimize DNA degradation in faecal samples.

3. *Purification kit technology.* Two purification kits were used to extract DNA from scat samples, based on either DNA adsorption to silica in a spin-column or to magnetic beads in a buffered solution.

2.2. Methods

2.2.1. S.T.A.R. buffer trial

The use of S.T.A.R. buffer as a preservation agent for the recovery and amplification of DNA from scats was tested in a preliminary trial using a scat sample which was divided in four parts and tested with one of the following methods: (i) the scat surface was sampled with a dry sterile swab and placed in 1 ml of S.T.A.R. buffer (ii) the same procedure as in (i) was used, except with a wet swab saturated with S.T.A.R. buffer, (iii) 1.5 g of scat material was added to 5 mls of S.T.A.R. buffer and (iv) 2.5 g of scat material was added to 10 mls of S.T.A.R. buffer. All samples were vortexed for 1 min and left overnight at room temperature. Chloroform was then added to all S.T.A.R. buffer mixtures (at a 1:10 ratio), vortexed for 15 s followed by a 1 min centrifugation step at 4,000 r.p.m. DNA was extracted from the supernatant with the QIAamp DNA Stool Mini Kit (Qiagen, cat. no. 51504) using the standard protocol for the isolation of genomic DNA from stool for human DNA analysis.

The success of DNA isolation was evaluated by the amplification of a fragment of mitochondrial DNA control region using primers LMS2 (5'-CRT CAG CAC CCA AAG CTG A-3') and HMS3 (5'-CAA GGR TTG ATG GTT TCT CG-3') (Statham 2005). These primers amplify a product of approximately 251-bp and 278-bp of pine marten and red fox mitochondrial DNA respectively (Statham 2005). The scat sample was provisionally identified as originating from fox (or dog) on the basis of morphology, but was selected as representing a fresh carnivore scat collected under field conditions. The PCR reaction mix contained 20 µl template DNA, 2.5 mM $MgCl_2$, 1X PCR buffer (Promega, Madison, WI, USA), 0.25 mM dNTP, 0.4 µM of each primer and 1.25 units of standard *Taq* polymerase (Promega, cat. no. M1661) in a total volume of 50 µl. The PCR program used was 95 °C for 1 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, with a 5 min final extension step at 72 °C.

2.2.2. Sampling methods

Five sampling methods were further evaluated using species known scats (n = 20) collected from the cages of four captive martens held at Wildwood Trust, Kent, UK. Each scat was divided in half to provide 40 samples. Eight samples were assigned to each method (two from each individual). Sampling methods were carried out as follows:

1. *Surface wash with S.T.A.R. buffer (SWSTAR)*: Each scat was placed in a 20 ml plastic universal and washed with 2 ml of S.T.A.R. buffer by pipetting over the scat surface several times. The wash supernatant (1 ml) was transferred to a sterile 2 ml microfuge tube, mixed well and left at room temperature for 30 mins, before centrifugation at 10,000 r.p.m. The supernatant was transferred to a new sterile microfuge tube containing 150 µl of chloroform. The contents were mixed well by repeated inversion and centrifuged for 3 mins at 10,000 r.p.m.

2. *Homogenisation with S.T.A.R. buffer (HOSTAR)*: Scat material (1.5 g) was homogenised in 5 ml of S.T.A.R. buffer by vortexing and left at room temperature for 30 mins. Chloroform (500 µl) was added just before DNA isolation, vortexed to mix and centrifuged for 10 mins @ 4,000 r.p.m.

3. *Surface scrape with S.T.A.R. buffer (SSCSTAR)*: Approximately 200 mg of scat material was scraped from the surface of the scat and placed into a sterile microfuge tube with 1 ml of S.T.A.R. buffer. The contents were mixed by vortexing and left to stand for 30 mins followed by centrifugation at 10,000 r.p.m. for 3 mins. The supernatant was transferred to another sterile microfuge tube containing 150 µl of chloroform and vortexed briefly. The samples were centrifuged again at 10,000 r.p.m. for 3 mins.

4. *Homogenisation with P.B.S. buffer containing SDS and Proteinase K (HOPBS)*: This method was modified from Flagstad et al (1999). P.B.S. buffer (5 ml, pH

7.4) containing 0.5% (w/v) sodium-dodecyl-sulphate (SDS) was added to a glass 20 ml screw cap sample bottle along with Proteinase K to a final concentration of 200 µg/ml. Approximately 1.5 g of scat was homogenised in the buffer by vortexing and left to stand at room temperature for 30 mins. The samples were digested overnight at 50 °C, and then centrifuged at 4,000 r.p.m. for 10 mins.

5. *Surface scrape with P.B.S. buffer containing SDS and Proteinase K (SSCPBS)*: The sampling procedure was the same as with SSCSTAR. P.B.S. buffer (1 ml) containing 0.5% SDS was added to the scat material along with Proteinase K to a final concentration of 200 µg/ml. The tube contents were mixed by repeated inversion and incubated at 50 °C overnight. After the overnight lysis, the tubes were centrifuged at 10,000 r.p.m. for 3 mins.

2.2.3. DNA extraction kits

DNA was extracted from 200 µl of the wash supernatant for each of the 40 scat samples with both a silica spin-column (GenElute Mammalian Genomic DNA Miniprep Kit, SIGMA, cat. no. G1-N70) and magnetic bead purification kit (Chargeswitch® Genomic DNA plant kit, Invitrogen, cat no. CS18000), giving 80 DNA extracts for real-time PCR analysis. Extraction negative controls were included in each batch of replicates to monitor for cross contamination.

2.2.4. Real-time PCR analysis

A short mitochondrial DNA control region sequence (60-bp) was targeted in a real-time PCR using the primers PM_FOR (5'-CAC CAG GCC TCG AGA AAC CAT-3') and PM_REV (5'-GGC CCG GAG CGA GAA G-3') (O'Reilly *et al.* 2008). Amplifications were performed in a 7300 real-time PCR system (Applied Biosystems, cat no. 4351103) in MicroAmp® Optical 96-well reaction plates (Applied Biosystems, cat no. N8010560).

Each reaction contained 12.5 µl of SYBR® Green Jumpstart™ Taq Readymix™ (SIGMA, cat. no. S4438), 0.5 µl ROX internal reference dye, 0.2 µM of each primer and 10 µl of template DNA dilution. The thermal profile used was 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Positive controls of pine marten tissue were included in each real-time PCR. DNA was extracted from approximately 20 mg of tissue with the GenElute Mammalian Genomic DNA Miniprep kit (SIGMA, cat. no. G1-N70) following the manufacturer's protocol for mammalian cells.

Melt curve analysis was carried out directly after the PCR cycling using one cycle of the following thermal profile; 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. The melting curves were acquired during the transition from 60 °C to 95 °C at a transition rate of 1.75 °C/min. The T_M of an amplicon varies with sequence length and composition and was used to differentiate between specific and non-specific amplification as primer-dimer artefacts have a characteristically lower T_M value (Ririe *et al.* 1997) than target DNA amplicons.

2.2.5. Evaluation of DNA extraction methods

The proportion of positive amplifications and the number of cycles taken for each sample fluorescence to reach a threshold value set by the detection software (cycle threshold, C_T), were used to compare the amount of DNA present in each sample, giving an indication of which extraction method was the most successful. The optimal template DNA input was determined prior to analysing all the samples by carrying out a dilution series (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) on one extract from each method. For the full sample set each extract was amplified once at the optimal dilution, reactions which failed or had an amplification plot slope lower than the tissue DNA were repeated at a higher dilution to reduce possible inhibition (Evans *et al.* 2007).

2.3. Results

2.3.1. Sampling methods

Four different faecal DNA sampling methods were initially evaluated based on either a surface swab or whole sample homogenisation in Stool Transport and Recovery (S.T.A.R.) buffer, followed by DNA purification through silica in a spin-column. Both homogenisation methods effectively isolated mitochondrial DNA from the scat samples (Figure 8).

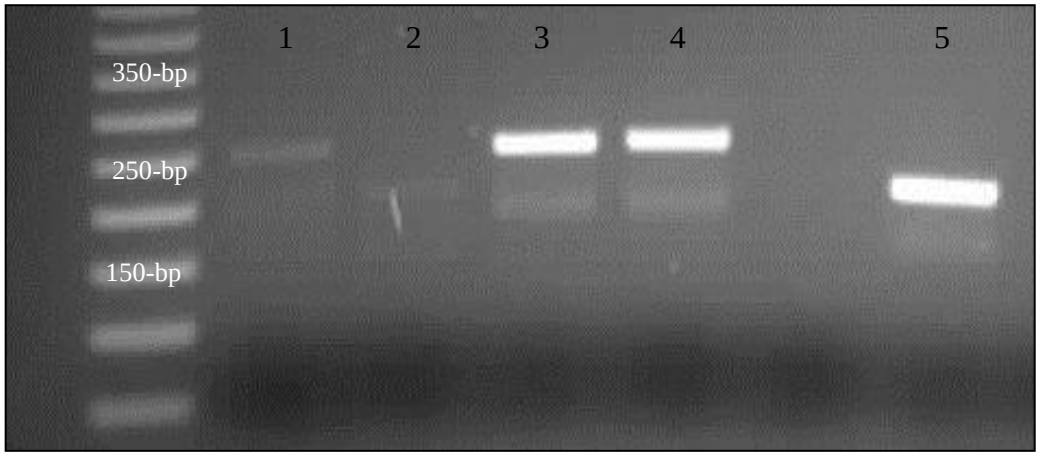


Figure 8: Mitochondrial DNA amplification from scats treated with S.T.A.R. buffer. (1) dry surface swab, (2) wet surface swab, (3) 1.5g/5ml homogenisation, (4) 2.5g/5ml homogenisation, (5) positive control of *Martes martes* DNA.

Some amplification was obtained for the surface sampling techniques with the wet and dry sterile cotton swabs but the PCR products were faint in comparison to the homogenisation methods. The DNA amplification intensity for the homogenisation methods was comparable to the positive control of tissue DNA indicating high quality

DNA free from inhibition was present in the extract (Figure 8). The larger PCR product size for the scat samples in comparison to the positive control of pine marten DNA indicated the scat may have originated from fox, as expected (section 2.2.1.). There was little difference in the PCR product intensity for the homogenisation methods using either 1.5 g or 2.5 g of scat in the DNA extraction procedure. The starting quantity of faecal material used in the DNA extraction procedure was thus reduced further to approximately 200 mg in 1 ml (20% w/v). Along with homogenisation in S.T.A.R. buffer (HOSTAR), four other sampling methods were further evaluated: a surface scrape or wash into S.T.A.R. buffer (SSCSTAR, SWSTAR) and a surface scrape or homogenisation in P.B.S. buffer (SSCPBS, HOPBS). Each method was tested on eight scat samples, with the resulting supernatant from the sampling method extracted with both a magnetic bead and silica spin-column DNA purification kit.

2.3.2. Optimal template DNA concentration

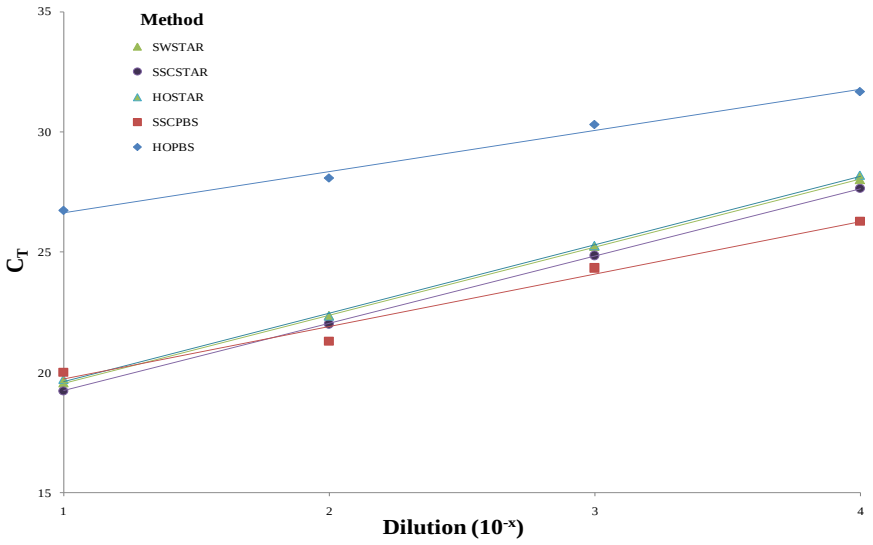
The optimal template DNA concentration suitable for real-time PCR analysis of the whole sample set of 80 DNA extracts was determined from serial dilution of DNA from one scat for each of the five sampling methods (purified using the silica spin-column kit). Real-time PCR C_T values and plots are given in Table 1. Preliminary results indicated that there was an effect of sampling method on the quantity of target DNA isolated from scat, with higher C_T values for the HOPBS method at each template DNA dilution relative to the other methods (Table 1). The sensitivity of the reaction is demonstrated by the linearity of the regression of C_T values against template DNA dilution over a range of four orders of magnitude (r^2 values, Table 1). DNA extracted with all methods showed signs of PCR inhibition as evidenced by the slope values lower than the value of 3.33 expected at 100% reaction efficiency (Smith *et al.* 2002). The S.T.A.R. buffer methods did not seem to be affected by PCR inhibitors to the same extent as the P.B.S. methods with less variability in the C_T values (higher r^2 values, Table 1).

Table 1: Real-time PCR C_T values (a) and amplification plots (b) for serially diluted scat DNA amplified using the primers PM_FOR and PM_REV, to determine the optimal DNA concentration for analysis.

(a)

	Dilution series (C_T value)					
Method	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**Amplification plot slope**	**Linearity (r^2)**
SWSTAR	19.56	22.36	25.25	28.03	2.83	0.9999
HOSTAR	19.72	22.35	25.28	28.31	2.84	0.9993
SSCSTAR	19.25	22.03	24.86	27.64	2.80	1
HOPBS	26.74	28.09	30.32	31.69	1.71	0.9897
SSCPBS	20.03	21.31	24.36	26.30	2.19	0.9786

Table 1 (b):

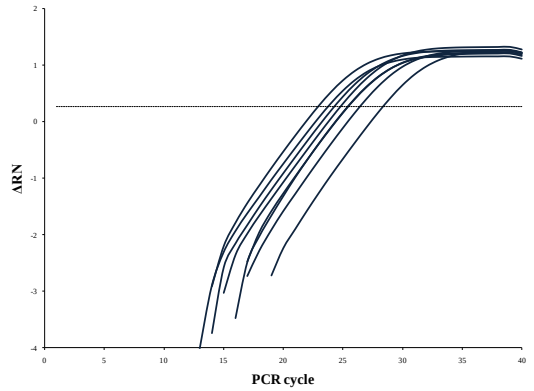


A 10^{-2} dilution of template DNA was chosen as the standard for the rest of the sample set in case some of the other samples contained high concentrations of PCR inhibitors. The real-time PCR C_T values for the set of 80 DNA extracts are given in Table 2 and the amplification plots for each method are presented in Figure 9. Samples which did not amplify at the 10^{-2} dilution were repeated at 10^{-3} to check for PCR inhibition.

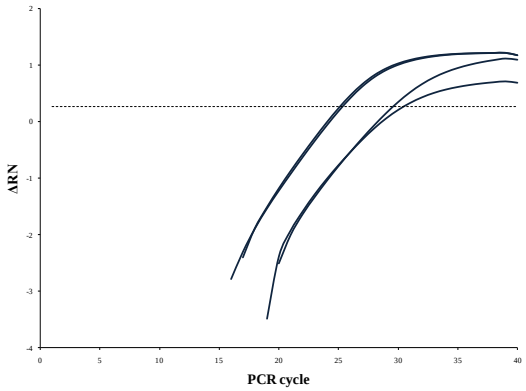
Table 2: Real-time PCR results for each sample method and DNA purification kit technology. Failed reactions are indicated (U).

		Silica-spin column Kit			**Magnetic bead Kit**		
Sampling method	**Scat**	C_T (10^{-2})	C_T (10^{-3})	T_M	C_T (10^{-2})	C_T (10^{-3})	T_M
HOSTAR	A	24.63		82.2	U	26.60	82.9
	B	24.25		82.2	U	30.15	82.9
	C	25.94		81.9	U	26.31	82.9
	D	23.70		81.9	25.10		82.9
	E	24.05		81.9	U	30.42	82.9
	F	24.73		82.4	U	25.64	83.2
	G	23.42		82.6	26.04		82.6
	H	23.13		82.6	U	29.09	82.9
Average (% amplified)		24.23 (100)			25.57 (25)		
HOPBS	A	29.97		82.2	27.77		82.6
	B	29.24		81.9	U	28.22	82.9
	C	U	31.31	81.9	U	U	72.7
	D	30.06		82.6	24.88		82.4
	E	29.29		82.9	32.75		81.9
	F	21.45		82.9	U	24.41	82.7
	G	31.20		82.9	31.61		82.2
	H	26.30		82.9	U	30.01	82.7
Average (% amplified)		28.22 (88)			30.72 (50)		
SSCSTAR	A	26.25		82.6	U	29.80	82.7
	B	21.55		82.9	U	27.98	82.9
	C	22.49		82.9	U	28.70	82.4
	D	25.14		82.9	23.22		82.9
	E	22.65		82.9	U	27.60	82.9
	F	21.65		82.6	U	26.06	82.9
	G	19.11		82.6	32.15		82.2
	H	20.28		82.6	U	23.73	82.1
Average (% amplified)		22.39 (100)			27.69 (25)		
SSCPBS	A	22.38		82.6	27.92		82.4
	B	22.44		82.6	22.70		81.9
	C	U	U	69.9	U	37.00	81.9
	D	30.67		82.4	24.50		82.4
	E	27.17		82.4	27.46		82.6
	F	U	31.03	82.7	U	32.11	82.4
	G	24.81		82.2	25.01		82.6
	H	30.37		81.9	26.51		82.6
Average (% amplified)		26.31 (75)			25.68 (75)		
SWSTAR	A	22.08		82.9	U	26.39	82.9
	B	22.59		82.6	22.94		82.6
	C	24.25		82.6	26.92		82.4
	D	23.23		82.4	23.09		82.4
	E	23.21		82.2	U	27.75	82.9
	F	26.18		82.9	U	U	72.5
	G	21.49		82.6	U	24.98	82.9
	H	20.70		82.9	27.04		81.7
Average (% amplified)		22.97 (100)			25.00 (50)		
Overall		24.65 (92.5)			26.53 (45)		

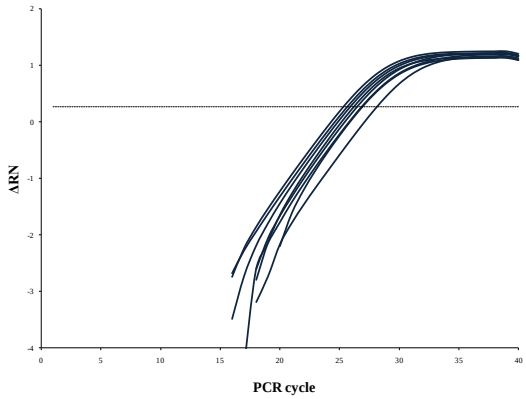
Figure 9: Real-time PCR amplification plots for each sampling method and purification kit.



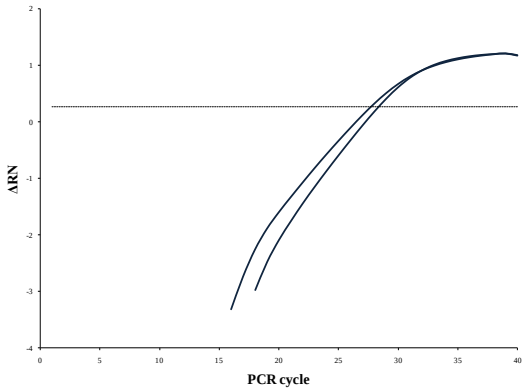
SWSTAR – silica column



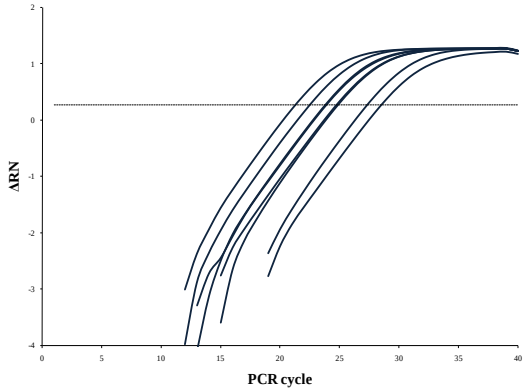
SWSTAR – magnetic bead



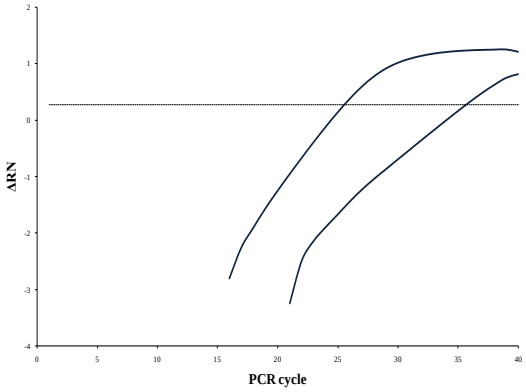
HOSTAR – silica column



HOSTAR – magnetic bead

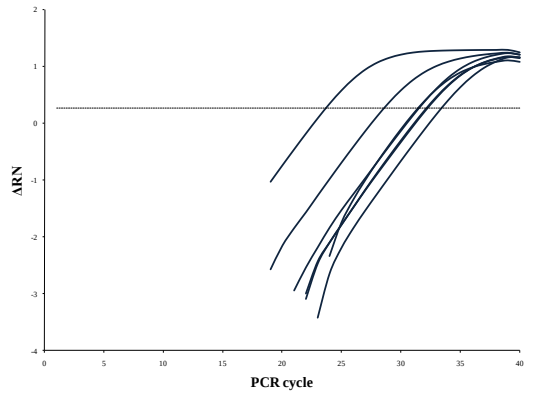


SSCSTAR – silica column

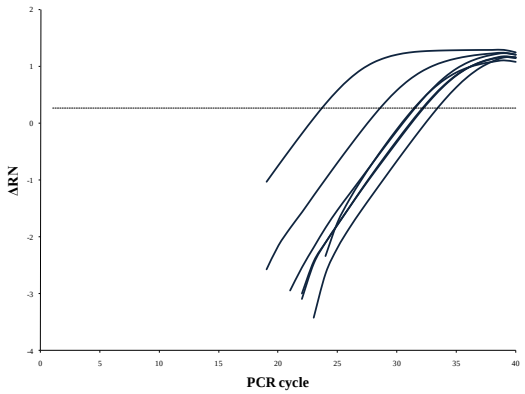


SSCSTAR – magnetic bead

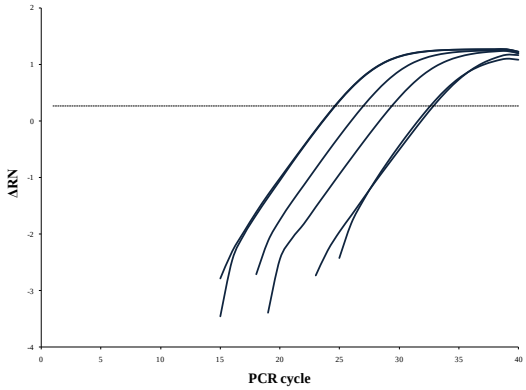
Figure 9 (Continued from previous page):



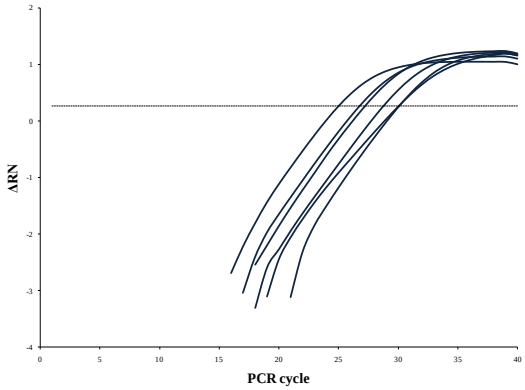
HOPBS – silica column



HOPBS – magnetic bead



SSCPBS – silica column



SSCPBS – magnetic bead

2.3.3. Real-time PCR amplification success

The overall amplification success rate for the full set of 80 DNA extracts was 68.75% at the 10^{-2} dilution (55/80 samples, Table 2). The amplification plots of these samples are shown for each method in Figure 9. More samples amplified with the silica spin-column purification kit than the magnetic bead purification kit, with amplification success rates of 92.5% and 45% respectively for each kit, despite having the same starting material for DNA extraction. The S.T.A.R. buffer sampling methods (SWSTAR, SSCSTAR and HOSTAR) were clearly more successful than the P.B.S. buffer and digestion with SDS and proteinase K methods (SSCPBS and HOPBS) in terms of the higher amplification success and lower, less variable C_T values for positive reactions (Table 2). All but three

of the 25 failed reactions were positive at a higher (10^{-3}) dilution, supporting the earlier evidence that PCR inhibitors were present in the DNA extracts from scat samples. One sample had a C_T value of 37.00, which was high but the T_M value of 81.9 °C was within the range of the other amplified samples and not the failed reactions (69.9 to 72.7 °C), and was therefore taken as specific amplification of pine marten DNA. The overall proportion of scat samples containing sufficient DNA for real-time PCR amplification was therefore 96.3% (77/80).

2.3.4. Standard protocol for DNA extraction from scats

The highest amplification success rates and lowest average C_T values were obtained with either a surface scraping, wash or whole sample homogenisation of scat material in S.T.A.R. buffer with silica spin-column DNA extraction. Of the three S.T.A.R. buffer methods, only the homogenisation method was considered to be feasible for handling pine marten scats as it was not always possible to wash the scat surface without sample degradation. Wild-collected scats may contain fur, bones, feathers, eggshells or berries, making a surface wash even more difficult to carry out as standard. A homogenisation of a small amount of scat material (0.2 g) in 1 ml of S.T.A.R. buffer (HOSTAR) followed by silica column purification was therefore chosen as the standard method for scat DNA extraction for the remainder of the project.

2.4. Discussion

The overall mtDNA amplification success (96.3%) compared favourably to other studies for faecal DNA analysis (53-100% - Hansen & Jacobsen 1999; Davison *et al.* 2002; Livia *et al.* 2007; Pilot *et al.* 2007). PCR inhibition was evident for all five sampling methods, with low amplification plot slope values less than the theoretical 3.33 at 100% amplification efficiency (Table 1a), and target DNA amplification for only 55 out of 80 samples on the first attempt (Table 2). Further dilution of the template DNA (and thus the inhibitor concentration) enabled the target DNA to be amplified for all but three of 80 samples. The DNA extracts for these three samples were dark brown in colour, an indication of the presence of a high level of PCR inhibitors as previously identified for coyote *Canis latrans* faeces (Prugh *et al.* 2005).

There are many compounds which are known PCR inhibitors and may be present in pine marten scats. These extracts may have contained high concentrations of humic acid, which is a potent inhibitor of *Taq* DNA polymerase found in soil (Graham 2007) or chemicals from the wood chippings from the floor of the enclosure of the captive animals. Some examples of PCR inhibitors which may be present in scats collected from the field include bile salts, complex polysaccharides, collagen, haemoglobin, humic acid, melanin and eumelanin, myoglobin, calcium ions and urea (Bessetti 2007). Santini *et al.* (2007) recorded a lower amplification success rate for wolf *Canis lupus* scats in direct contact with the ground, presumably due to increased DNA degradation with humidity and invading decomposer organisms. As the majority of pine marten scats are collected from the ground on forest tracks, the level of inhibitory compounds carried through to the DNA extract must be controlled. The diet or health of wild animals may also affect the DNA quality (Idaghdour *et al.* 2003; Piggott & Taylor 2003a), which necessitates the use of a robust DNA extraction method which consistently isolates and purifies high quality DNA for genetic analysis.

There is a point beyond which further dilution of the template DNA will result in failed amplifications due to a reduced number of target DNA molecules rather than a persistence of PCR inhibitors. Other options include the addition of protein adjuvants such as BSA, more polymerase or the use of a DNA extraction kit which reduces the quantity of inhibitors co-extracted with the target DNA (Bessetti 2007). In this study two kits were compared on the basis of recommended use in previous studies (Flagstad *et al.* 1999; Piggott & Taylor 2003a). The silica spin-column kit extracted more high quality DNA for all five sampling methods than the magnetic bead kit according to the higher amplification success and lower C_T values (Table 2). This is in agreement with Piggott & Taylor (2003a) for the scats of another carnivore, the red fox *Vulpes vulpes*, and in contrast with the findings of Flagstad *et al.* (1999) for sheep *Ovis aries* and reindeer *Rangifer tarandus* faeces.

The compact nature of herbivore faeces facilitates the use of surface swabbing (Ball *et al.* 2007), scraping (Wehausen *et al.* 2004) or surface washing (Flagstad *et al.* 1999) sampling methods which reduce the inclusion of solid faecal material in the extraction and maximize the collection of epithelial cells. Initial trials involving a surface swab of scat material into S.T.A.R. buffer in this study amplified a mitochondrial DNA PCR product of much lower amplification intensity than the homogenisation method (Figure 8). Furthermore, while a surface wash or scrape into S.T.A.R. buffer was carried out successfully for scat samples in this study, the scats were collected from captive pine marten and would not be representative of scats deposited by wild pine marten with an omnivorous diet, containing berries, eggshells, feathers, hair, bones and insect remains, giving scats varying morphologies and making any surface treatment difficult. A homogenisation of a small amount of scat material was therefore regarded as the most feasible sampling method for pine marten scats. In this respect, the results of Flagstad *et al.* (1999) also support the use of a silica spin-column purification kit, as the magnetic bead kit completely failed to amplify nuclear DNA from scats which were homogenized prior to amplification.

PCR inhibition can still affect samples with low DNA content (< 200 pg/µl) as not all inhibitory compounds are removed with silica purification (Idaghdour *et al.* 2003). The combination of S.T.A.R. buffer treatment with silica spin-column purification gave the best results with 100% amplification success, even for the scrape and homogenisation methods which include some inner scat material and consequently should negatively affect amplification success (Wehausen *et al.* 2004). The success of the S.T.A.R. buffer methods was presumably due to the additional inhibitor-binding and nuclease deactivation properties of the buffer, along with the amplification of a very short (60-bp) DNA sequence in the real-time PCR.

Another possible application of real-time PCR is the absolute quantification of target DNA in genomic DNA extracts (Morin *et al.* 2001). However, the reliability of DNA quantification is confounded by the presence of PCR inhibitors which delay the increase in fluorescence in affected samples, resulting in quantitation estimates lower than the actual value (Kontanis & Reed 2006). An internal positive control can be included to detect inhibition as a target which should amplify irrespective of the presence or absence of target DNA (Swango *et al.* 2006) but may require extensive optimisation. The selection of an optimal template DNA concentration for all samples followed by selective reanalysis of samples which failed to amplify was sufficient to detect inhibition for this assay while still enabling target DNA detection due to the sensitivity of the assay. A larger mitochondrial DNA target of approximately 280-bp was also amplified successfully by conventional PCR using the homogenisation in S.T.A.R. buffer method, which proves the application of this method in laboratories where real-time PCR equipment is not available.

In summary, real-time PCR was shown to be a reliable and powerful method for the genetic analysis of carnivore scats, in particular as target DNA quality and quantity can be readily evaluated and input DNA concentration adjusted, avoiding the unnecessary removal of samples from the dataset which do not amplify on the first attempt. Both real-time PCR and S.T.A.R. buffer are underutilized in wildlife studies and have huge

potential for future applications. The next chapter explored the use of real-time PCR for species and sex identification of non-invasively collected samples through the additional use of melting-curve analysis post amplification or the hybridisation of specific probes between real-time PCR primer binding sites.

Chapter 3

Application of real-time PCR for species and sex identification of non-invasively collected samples

3.1. Introduction

Pine marten are difficult to trap or observe in the wild due to their secretive nature and large home range size. Fortunately, pine marten often defecate on forest trails and other prominent features such as stone walls and banks. These field signs can then be collected and identified to species level by expert naturalists according to characteristic appearance and odours. However, independent genetic verification of species identity is recommended where scats of other species such as the red fox *Vulpes vulpes*, which are similar in appearance, are likely to be sampled (Davison *et al.* 2002).

Species identification methods based on conventional PCR generally target over 300-bp of DNA sequence, which may be difficult to amplify from rain-washed scats as DNA degrades rapidly in an environment of high humidity, temperature and sunlight. Real-time PCR technology holds great potential for non-invasive genetic sampling as the target amplicons are small (under 150-bp) and the assay is carried out in a sealed 96-well plate with no post-PCR manipulation, which reduces the contamination risk and increases throughput. Despite these advantages, relatively few studies have been published on the use of real-time PCR for the reliable identification of field signs from mammal species. Berry & Sarre (2007) used species-specific primers and post-amplification melt-curve analysis (MCA) to distinguish three marsupial species (tiger quoll *Dasyurus maculatus*, eastern quoll *Dasyurus viverrinus* and the Tasmanian devil *Sarcophilus harrisii*) and three eutherian species (feral cat *Felis catus*, feral dog *Canis familiaris* and the red fox *Vulpes vulpes*) on the basis of differences in melting temperature (T_M) of the amplified DNA, which is determined by nucleotide content and length. Moran *et al.* (2008) also developed a MCA approach to discriminate between five small mammal species (bank vole *Myodes glareoulus*, wood mouse *Apodemus sylvaticus*, pygmy shrew *Sorex minutus*, water shrew *Neomys fodiens* and common shrew *Sorex araneus*), with successful application of the methodology to a large-scale distribution survey in Great Britain (Moran 2009). The use of fluorogenic TaqMan® probe technology for mammal species identification has largely been restricted to

commercial applications relating to food safety (Dooley *et al.* 2004; Chisholm *et al.* 2005).

The sex of non-invasively collected samples cannot be determined visually and requires the analysis of sex-specific DNA sequences. Genetic techniques for sex determination in mustelids have recently been developed (Statham *et al.* 2007), but all are based on conventional PCR amplification which requires post-PCR manipulation with size fractionation in agarose gels, restriction digestion or direct sequencing. Some real-time PCR sex determination methods have been published for wildlife research. Morin *et al.* (2005) targeted a short 105-bp region of the zinc finger gene for sexing several cetacean species, while Chang *et al.* (2008) developed a TaqMan® probe assay targeting the Chromo-Helicase-DNA binding (*CHD*) gene for gender determination of eagles.

The aim of this study was to test the performance of two different assays for species identification representing two different real-time PCR chemistries (melt curve analysis with SYBR® Green I dye and TaqMan® MGB probe hybridisation), and one sex typing method based on TaqMan MGB probes, with particular emphasis on applicability to scat and hair samples. All real-time PCR assays were designed by Dr. Catherine O'Reilly, Waterford Institute of Technology.

3.2. Methods

3.2.1. Species identification

Real-time PCR methods were developed during this project for species identification of hair and scat samples, particularly for the discrimination between pine marten and fox scats. Two assays were initially developed to target species-specific single nucleotide polymorphisms (SNPs) in the mtDNA control region. Species assignment was based on either melt-curve analysis or TaqMan probe fluorescence.

Melt-curve analysis

MCA was carried out as described in Chapter 2, section 2.2.4 using the primers PM_FOR and PM_REV, targeting a 60-bp sequence of mtDNA (Figure 10). The melting temperature (T_M) was expected to differ between pine marten and fox as there were five sequence differences between the two species in the amplified region. Positive controls of tissue DNA were included in each 96-well plate to monitor the specificity of each probe. Tissue DNA was extracted from approximately 20 mg of tissue with the GenElute Mammalian Genomic DNA Miniprep kit (SIGMA, cat. no. G1-N70) following the manufacturer's protocol for mammalian cells.

```
                                                                                  Species  Genbank
333 CACCAGGCCTCGAGAAACCATCAACCCTTGCCCGATGTGTACCTCTTCTCGCTCCGGGCCC marten   AJ585357
359 CACCATGCCTCGAGAAACCATCAATCCTTGCTCGAAGTATCCTCTTCTCGCTCCGGGCCC fox      AJ585358
```

Figure 10: Mitochondrial DNA control region sequence alignment for pine marten and fox in the region amplified by the primers PM_FOR and PM_REV (underlined) for melt-curve analysis.

The MCA method was applied to a set of species unknown hair (N = 31) and scat (N = 25) samples which were collected from Curraghmore Forest, Co. Waterford (52.25° N, 7.3° W) as part of a separate research project (Roche 2008). DNA was extracted from plucked hair samples following a modified Chelex-100® protocol (Walsh *et al.* 1991; Statham 2005). The root end of approximately 10-30 hairs were cut into a sterile microfuge tube and centrifuged briefly. In cases where the hairs were encased in the glue patch, a pair of flamed tweezers was used to remove a clump of hairs for DNA extraction. All scissors and tweezers used in the extractions were flamed with alcohol between samples to prevent cross contamination. Sample digestion was carried out for at least 2 hours at 56 °C with 2 µl (40 mg) Proteinase K (Applied Biosystems, cat. no. AM2546), 7 µl (40 mM) dithiothreitol (Molekula, cat. no. 578517) and 200 µl (5% w/v) Chelex-100 resin (Bio-Rad, cat. no. 142-1253) in a Biosan TS-100 shaking incubator. After digestion, the tubes were heated to 100 °C for 8 minutes then centrifuged at 13,000 r.p.m. for 3 minutes. The supernatant was removed carefully from the Chelex pellet and stored at -20 °C for downstream applications. DNA was extracted from scats following the standard protocol described in the results section 2.3.4.

Species identification of field signs was carried out by comparing the sample T_M values to the T_Ms of the tissue DNA positive controls simultaneously amplified in each assay.

TaqMan probes

The probe-based assay was similar to the melt-curve analysis, targeting the same mtDNA region with the primers PM_FOR and PM_REV, with the addition of two fluorescently labelled oligonucleotide probes. These probes were designed to be very short (16 to 19-bp) and complementary to either pine marten or fox DNA as shown in Figure 11.

```
                                                                  Species  Genbank
333 CACCAGGCCTCGAGAAACCATCAACCCTTGCCCGATGTGTACCTCTTCTCGCTCCGGGCCC marten   AJ585357
359 CACCATGCCTCGAGAAACCATCAATCCTTGCTCGAAGTATCCTCTTCTCGCTCCGGGCCC fox      AJ585358
                          CCTTGCCCGATGTGTACCT (PM2)
                         TCCTTGCTCGAAGTAT     (FOX)
```

Figure 11: Mitochondrial DNA control region sequence alignment for the TaqMan probe species typing assay. Sequence mismatches to pine marten in the probe binding region are highlighted.

Each reaction contained 12.5 µl TaqMan® MGB Universal PCR mastermix (Applied Biosystems, cat. no. 4304437), 1 µl each of forward primer (PM_FOR), reverse primer (PM_REV), pine marten probe (PM2:5'-6FAM-CCT TGC CCG ATG TGT ACC T-MGB-3') and fox probe (FOX:5'-VIC-TCC TGG CTC GAA GTA T-MGB-3'), all at 0.2 µM final concentration, with 8.5 µl of a ten-fold dilution of template DNA. The real-time PCR amplifications were carried out in an ABI Prism 7300 sequence detection system using the standard PCR programme as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Positive controls were included in each PCR as for the melt-curve analysis. The TaqMan probe assay was also tested on the set of 56 hair and scat DNA extracts which were species typed by melt-curve analysis. Species typing in this case was based on the detection of accumulated fluorescence (C_T value) from either the FAM-labelled (pine marten) or VIC-labelled (fox) probe.

The positive controls used in the real-time PCR assays were pine marten and fox tissue as the scats from these species are most often confused with each other on the basis of morphology (Davison *et al.* 2002). Fox tissue was not a relevant positive control for the hair samples as foxes cannot reach the hair-tubes, which are fixed to trees about 1m off the ground. Stoats may enter the hair-tubes used in this study (Statham 2005) and the primers PM_FOR and PM_REV will amplify stoat DNA. Therefore the PM2 and FOX TaqMan® MGB probes were checked for species-specificity using a dilution series of stoat, pine marten and fox tissue DNA.

A second TaqMan probe assay was designed later in the project as the PM2 probe was found to cross-react with stoat DNA. PCR conditions were the same as for the PM2/FOX assay, except the following oligonucleotides were used: PM3F forward primer (5'-CTT GCC CCA TGC ATA TAA GCA-3'), PM-REV2 reverse primer (5'-GCC TGG TGA TTA AGC TCG TGA T-3') and PM3 pine marten probe (5'-6FAM-CGT GCA CCT CAC TTA G-MGB-3'). The mtDNA sequence alignments showing primer and probe binding sites for both assays is given in Figure 12. The PM3 probe was tested for specificity with tissue DNA of pine marten *Martes martes*, stoat *Mustela erminea*, red squirrel *Scuirus vulgaris*, grey squirrel *Scuirus carolensis*, American mink *Mustela vison*, brown rat *Rattus norvegicus* and wood mouse *Apodemus sylvaticus*.

```
                                                                                Species   Genbank
255 CTTGCCC---CATGCATATAAGCATGTA---CATATTATGCTTGATCTTGCA-----                   marten    AJ585357
103 .C.....---.....................---.........A..A..T..A..-----                stoat     AJ585356
249 .A..TA.ATAT.CTT......TT.CA..GGA....C....T..A.....A..ATAAT                   fox       AJ585358
    PM3F→

301 ------------------TTCGTGCACCTCACTTAGATCAC---GAGCTTAATCACC                   marten    AJ585357
149 ------------------.GTA.C..TT...GC....C...---......T......                   stoat     AJ585356
306 CTCTGTCAGGAGCATATA.GAC.....G........TC..GTAA.G.A..T......                   fox       AJ585358
                            PM3              PM_FOR→

337 AGGCCTCGAGAAACCATCAACCCTTGCCCGATGTGTACCTCTTCTCGCTCCGGGCC                    marten    AJ585357
185 .T.............................C.........................                   stoat     AJ585356
363 .T..................T......T...A..A.C...................                   fox       AJ585358

    ←PM-REV2             PM2          ←PM_REV
```

Figure 12: Positioning of real-time PCR primers and probes for species identification. Numbers refer to the base positions of the sequences for *Martes martes* (AJ585357), *Mustela erminea* (AJ585356) and *Vulpes vulpes* (AJ585358). Probe binding sites are highlighted in grey and bold underline indicates where the binding sites for the PM_FOR forward primer (PM2/FOX assay) and PM-REV2 reverse primer (PM3 assay) overlap.

3.2.2. Sex identification

A TaqMan probe assay was designed to distinguish between the sexes on the basis of a single nucleotide polymorphism in a 76-bp region of the last exon of the zinc finger gene (Figure 13). The sequence of this gene was obtained previously for pine marten (Statham *et al.* 2007). Primer and probe sequences were designed using the Applied Biosystems Custom Assay Design Tool based on submission of *M. martes* ZFX and ZFY sequences (Accession Numbers AM039478 and AM039479).

```
103 AGCCAACAAAATGCACAAGTGTAAATTCTGTGAATACGAGACAGCCGAACAAGGGTTGTTGAATCGCCACCTTTTGG ZFX
103 AGCCAACAAAATGCACAAGTGTAAATTCTGTGAATACGAGACAGCTGAACAAGGGTTGTTGAATCGCCACCTTTTGG ZFY
```

Figure 13: Alignment of the sequences for the ZF exon sex typing assay. Alignment of ZFX (AM039478) and ZFY (AM039479) sequences with the primer binding sites underlined and the probe target regions shaded in grey. The SNP which differentiates ZFX and ZFY is highlighted in black.

The standard real-time PCR contained 5 µl TaqMan® Universal PCR mastermix, 0.25 µl of the Custom Assay SNP Genotyping Mastermix (supplied at a 40X concentration, Applied Biosystems) and 4.75 µl of diluted template DNA. Universal reaction conditions were the same as for the species typing assays, with the following primer and probe sequences: forward primer PMZF_SNP1F (5'-AGC CAA CAA AAT GCA CAA GTG TAA A-3'), reverse primer PMZF_SNP1R (5'-CCA AAA GGT GGC GAT TCA ACA A-3'), ZFX probe PMZF_SNP1X (5'-6FAM-CCT TGT TCG GCT GTC T-MGB-3') and ZFY probe PMZF_SNP1Y (5'-VIC-CCC TTG TTC AGC TGT CT-MGB-3') with 45 cycles to allow for the later amplification of single copy nuclear DNA. Samples were identified as female by FAM fluorescence (C_T value) for the ZFX only, and as male by detected fluorescence from both the ZFX and ZFY sequences, with C_T values for both ZFX and ZFY.

Assay specificity

The ZF exon assay was tested for sex-specificity with tissue from martens of known sex (n = 9) and scats from captive pine marten of known sex (2 males and 2 females) held at Wildwood Animal Trust, Herne Bay, Kent, UK. Four scats were genotyped in triplicate from each captive marten giving 12 PCR replicates per individual to assess consistency between replicates and identify genotyping errors. Plucked hair samples of unknown sex (n = 26) collected remotely in Curraghmore Forest were also typed in triplicate with the assay to evaluate the success rate of nuclear DNA amplification and molecular sexing for hair samples. Cross-reactivity tests were carried out for five other mustelid species (otter *Lutra lutra*, stoat *Mustela erminea*, mink *Neovison vison*, badger *Meles meles* and stone marten *Martes foina*), fox *Vulpes vulpes*, domestic cat *Felis catus*, wood mouse *Apodemus sylvaticus* and pygmy shrew *Sorex minutus*.

3.3. Results

3.3.1. Identification of pine marten field signs by real-time PCR analysis

Mitochondrial DNA was successfully amplified from pine marten and fox tissue with the primers PM_FOR and PM_REV in both the SYBR Green MCA and the PM2/FOX TaqMan probe real-time PCR assays. Both species were easily discriminated with at least a 1.0 °C difference in T_M value between the species (Table 3a and 3b). The positive controls in the scat MCA assay (Table 3a) were 82.5 °C and 81.4 °C for pine marten and fox respectively, while the values were lower in the hair MCA assay (pine marten 81.4 °C; fox 80.4 °C) which was carried out independently on a different day. However, a 1 °C difference in T_M value was still maintained between the two species, which suggested that the MCA method could be used effectively for species assignment, if the T_M values for tissue DNA were used as positive controls for comparison with unknown samples within each assay.

Pine marten and fox tissue were also easily discriminated with the TaqMan probe assay. Fluorescence was detected for the PM2 probe in the presence of pine marten DNA and for the FOX probe in the presence of fox DNA for the tissue DNA positive controls (Tables 3a and 3b). Neither probe cross-reacted with tissue DNA of the other species. Both PCR methods were subsequently used to species type 25 scats and 31 hair samples of unknown species origin.

Scats

The PCR amplification success rate for the scats was high at 92% for both the MCA and the TaqMan assay (Table 3a). Twenty-four of 25 scats (96%) contained DNA which was amplifiable by real-time PCR. One of the scats (S17) did not amplify with either method while scat S2 did not amplify for the MCA assay and scat S8 did not amplify for the

TaqMan probe assay. Scats of both pine marten (n = 14) and fox (n = 9) were identified in the sample set on the basis of fluorescence (C_T value) for either the PM2 or the FOX TaqMan probe.

Table 3: Species identification of field signs by real-time PCR analysis.

(a) Scat

	MCA		TaqMan		
Sample	**C_T**	**T_M**	**C_T**	**Probe**	**Species**
Marten	13.20	82.5	15.51	PM2	
Fox	15.72	81.4	21.30	FOX	
S1	23.76	82.2	33.50	PM2	marten
S2	U	U	24.24	FOX	fox
S3	26.37	81.1	29.00	FOX	fox
S4	26.88	80.2	37.62	FOX	fox
S5	22.88	82.6	23.75	PM2	marten
S6	22.39	82.4	30.41	PM2	marten
S7	27.20	81.3	33.27	FOX	fox
S8	24.92	82.0	U	U	marten
S9	21.58	82.6	22.37	PM2	marten
S10	25.90	82.7	27.32	PM2	marten
S11	19.93	82.7	24.43	PM2	marten
S12	23.29	82.1	25.47	PM2	marten
S13	24.92	82.4	29.33	PM2	marten
S14	25.40	81.2	35.41	FOX	fox
S15	20.96	82.6	21.29	PM2	marten
S16	22.81	82.2	22.89	PM2	marten
S17	U	U	U	PM2	Undet
S18	18.92	82.1	27.26	PM2	marten
S19	25.19	81.9	30.21	PM2	marten
S20	21.18	82.7	24.81	PM2	marten
S21	18.26	81.6	29.19	FOX	fox
S22	21.07	80.9	34.74	FOX	fox
S23	16.75	82.4	23.45	PM2	marten
S24	18.29	81.1	23.05	FOX	fox
S25	20.37	80.6	26.03	FOX	fox

(b) Plucked hair

	MCA		TaqMan		
Sample	**C_T**	**T_M**	**C_T**	**Probe**	**Species**
Marten	20.97	81.4	27.00	PM2	
Fox	20.01	80.4	27.62	FOX	
H1	21.03	81.4	25.44	PM2	marten
H2	19.54	81.7	24.24	PM2	marten
H3	19.42	81.7	23.93	PM2	marten
H4	19.35	81.7	23.63	PM2	marten
H5	16.92	81.7	21.29	PM2	marten
H6	16.96	81.7	21.50	PM2	marten
H7	U	U	33.92	PM2	marten
H8	18.44	81.7	23.33	PM2	marten
H9	19.99	81.1	23.53	PM2	marten
H10	17.74	81.4	23.47	PM2	marten
H11	18.83	81.4	23.20	PM2	marten
H12	17.37	81.1	22.00	PM2	marten
H13	18.44	81.7	23.07	PM2	marten
H14	17.21	81.7	21.48	PM2	marten
H15	U	U	U	PM2	Undet
H16	24.00	82.0	29.66	PM2	marten
H17	U	U	24.20	PM2	marten
H18	22.13	81.4	24.43	PM2	marten
H19	U	U	25.56	PM2	marten
H20	17.55	81.4	23.27	PM2	marten
H21	19.25	81.4	24.67	PM2	marten
H22	U	U	27.07	PM2	marten
H23	18.79	82.0	24.17	PM2	marten
H24	U	U	29.52	PM2	marten
H25	23.68	82.3	27.80	PM2	marten
H26	34.04	82.0	U	PM2	marten
H27	U	U	26.58	PM2	marten
H28	U	U	U	PM2	Undet
H29	19.11	82.0	25.18	PM2	marten
H30	U	U	34.25	PM2	marten
H31	U	U	24.89	PM2	marten

The T_Ms of the scats identified as pine marten with the PM2 TaqMan probe (average 82.4 °C, range 81.9-82.7 °C; n = 13) did not overlap with the T_Ms of the fox scats identified with the FOX probe (average 81.0 °C, range 80.2-81.6 °C; n = 9). The one

remaining sample which did not give a result for the TaqMan assay (S8, Table 3a) was identified as pine marten on the basis of T_M value (82.0 °C), which was within the range of T_Ms for the other scats (81.9-82.7 °C).

Hair

Of the 31 hair samples assayed, 28 (90%) were assigned to pine marten with C_T values for the PM2 probe ranging from 21.29 to 34.25 (Table 3b). Two of the remaining samples (H15 and H28) also failed to amplify with the MCA assay, possibly due to low target DNA copy number. The success rate for the hair MCA assay was much lower at 67%. The MCA reactions may have been affected by PCR inhibitors as was found for scat DNA extracts in Chapter 2. The T_M values for the hair samples which were identified as pine marten with the PM2 TaqMan probe averaged 81.6 °C, with a range of 81.1-82.3 °C (n = 20). The hair sample H26 which failed to amplify in the TaqMan assay (Table 3b) was identified as pine marten by T_M value (82.0 °C), which was within the range of the other pine marten hair samples. Overall, 53 out of 56 (95%) hair and scat samples were assigned to species with the real-time PCR assays.

3.3.2. Species typing assay specificity

The PM2 probe was tested for specificity to pine marten with a dilution series of pine marten, fox and stoat DNA. There was an increase in fluorescence for the PM2 probe in the presence of stoat DNA for all template DNA dilutions from 10^{-1} to 10^{-5}, while the FOX probe was specific to fox DNA (Table 4). A new probe, PM3, was subsequently designed to specifically detect pine marten DNA for application to scat and hair samples.

Table 4: Specificity test for the PM2 and FOX TaqMan probes with a dilution series of pine marten, stoat and fox DNA.

TaqMan® MGB Probe	Template DNA Dilution	Marten	Fox	Stoat
PM2	10^{-1}	17.61	U	19.33
FOX		U	25.45	U
PM2	10^{-2}	20.75	U	22.41
FOX		U	26.92	U
PM2	10^{-3}	24.32	U	25.96
FOX		U	30.97	U
PM2	10^{-4}	27.72	U	29.44
FOX		U	36.95	U
PM2	10^{-5}	31.28	U	32.82
FOX		U	33.52	U

The PM3 assay was designed to target a mitochondrial DNA control region sequence with more differences between pine marten and stoat in the probe region (Figure 14). The PM3 probe was tested with tissue DNA from stoat, red squirrel, grey squirrel, mink, otter, rat and mouse. Only pine marten amplified, with a low C_T value of 16.06. The probe was tested further with a dilution series of stoat and red squirrel DNA and an increase in fluorescence for the PM3 probe was only observed for pine marten over a wide range of template DNA concentrations (Figure 15). The PM3 assay was therefore a sensitive and reliable assay for identification of DNA samples as pine marten, and was subsequently used as the standard species identification method.

```
                                                                Species  Genbank
255 CTTGCCCCATGCATATAAGCATGTACATATTATGCTTGATCTT                 marten   AJ585357
187 CCTGCCCCATGCATATAAGCATGTACATACTATGATTAATTTT                 stoat    AJ585356

298 GCATTCGTGCACCTCACTTAGATCACGAGCTTAATCACCAGGC                 marten   AJ585357
230 ACATGTATCCATTTCAGCTAGATCACGAGCTTTATCACCATGC                 stoat    AJ585356
```

Figure 14: Mitochondrial DNA control region sequence alignment for pine marten and stoat for the PM3 real-time PCR species typing assay. PM3F and PM_REV2 primer binding sites are underlined. Sequence mismatches to pine marten in the probe binding region are highlighted.

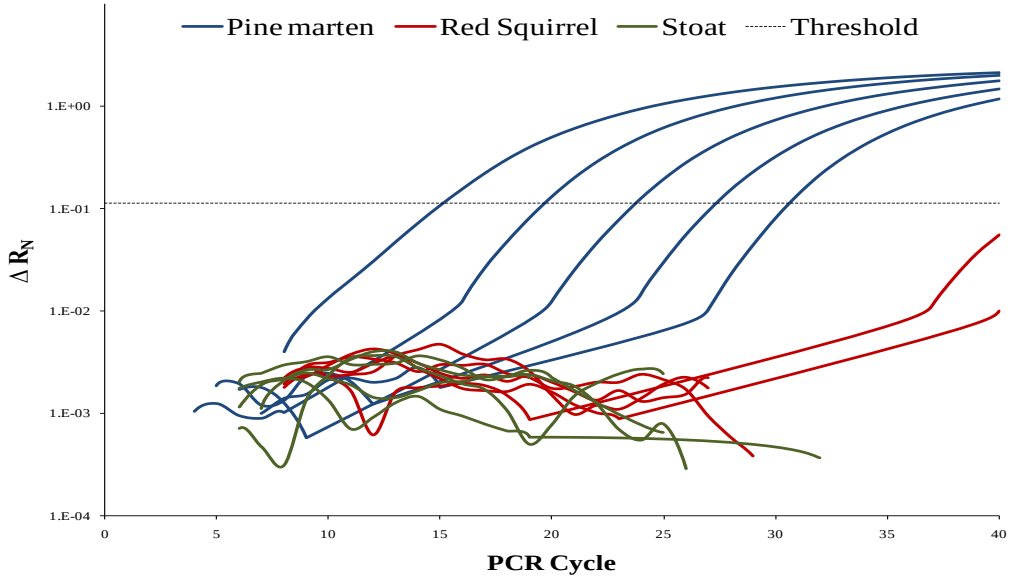


Figure 15: Specificity test for the PM3 TaqMan® MGB probe. Template DNA dilution series from left to right: 10^{-1} to 10^{-5}.

3.3.3. Sex typing using TaqMan® MGB probes

The TaqMan® MGB ZF exon assay for sex identification was tested for reliability with tissue DNA of known sex pine marten (n = 9, 5 males and 4 females), a set of scats of known sex collected from the enclosures of captive martens in Wildwood Trust (n = 4, 2 males and 2 females) and remotely plucked hair samples (n = 26) of unknown sex collected from Curraghmore Forest. The sex of all tissue samples identified by real-time PCR sexing matched the known sex of the individual with low C_T values for both the ZFX and ZFY (Table 5a). The C_T values for the hair and scat samples were several cycles higher, signifying the lower quantities of target DNA extracted from these samples. Nevertheless, amplification success rates were high for both scat (85.7%, Table 5b) and hair (92.3%, Table 5c) for nuclear DNA. All the known female scats amplified the ZFX only, as expected. Both male (21 samples) and female (3 samples) pine marten were identified from the 24 hair samples containing amplifiable DNA which were sampled non-invasively from free-ranging pine marten in Curraghmore Forest.

Table 5: ZF exon assay applicability to tissue (a), scat (b) and hair (c) samples. CT values are averages of three PCR replicates. A lack of amplification is indicated (U).

(a) Tissue

Sample Type	I.D.	Known sex	ZFX-FAM Average C_T	ZFY-VIC Average C_T	ΔC_T (ZFY-ZFX)	Molecular Sex
Tissue	IRL18	♂	18.02	21.06	3.04	♂
Tissue	IRL19	♂	19.38	22.21	2.83	♂
Tissue	FR01	♂	19.89	22.55	2.66	♂
Tissue	FR02	♀	19.66	U	-	♀
Tissue	FR03	♂	21.33	24.46	3.13	♂
Tissue	FR04	♀	19.89	U	-	♀
Tissue	FR05	♂	16.94	20.01	3.07	♂
Tissue	FR06	♀	21.54	U	-	♀
Tissue	FR07	♀	24.57	U	-	♀

(b): Scat

Individual	Scat	ZFX 1	ZFX 2	ZFX 3	Ave	ZFY 1	ZFY 2	ZFY 3	Ave	ΔC_T
Captive 1 (♂)	1	33.17	32.35	33.91	33.14	35.87	36.05	38.16	36.69	3.55
	2	35.39	35.41	39.00	36.60	39.85	40.00	36.31	38.72	2.12
	3	33.36	33.08	33.01	33.15	38.36	U	35.74	37.05	3.90
	4	31.37	32.16	31.65	31.73	37.58	35.53	36.62	36.58	4.85
Captive 2 (♂)	1	30.66	30.65	31.12	30.81	34.96	35.61	34.34	34.97	4.16
	2	33.93	U	34.57	34.25	U	U	U	U	-
	3	31.09	29.19	31.42	30.57	34.33	33.14	35.14	34.20	3.64
	4	35.54	38.71	35.5	36.58	38.07	38.39	36.24	37.57	0.98
Captive 3 (♀)	1	32.40	32.04	32.54	32.33	U	U	U	U	-
	2	33.72	33.34	32.98	33.35	U	U	U	U	-
	3	32.11	32.33	34.13	32.86	U	U	U	U	-
	4	30.03	29.88	30.25	30.05	U	U	U	U	-
Captive 4 (♀)	1	31.24	31.22	33.00	31.82	U	U	U	U	-
	2	U	U	U	U	U	U	U	U	-
	3	U	U	U	U	U	U	U	U	-
	4	31.44	32.02	32.19	31.88	U	U	U	U	-

(c): Plucked hair

Sample	Known sex	ZFX-FAM Average C_T	ZFY-VIC Average C_T	ΔC_T (ZFY-ZFX)	Molecular Sex
Hair 1	-	34.09±0.71	37.45±0.88	3.37	♂
Hair 2	-	31.32±0.11	U	-	♀
Hair 3	-	31.61±0.20	35.78±0.38	4.16	♂
Hair 4	-	31.69±0.42	35.80±0.60	4.11	♂
Hair 5	-	28.87±0.02	33.29±0.34	4.42	♂
Hair 6	-	30.05±0.11	34.43±0.04	4.37	♂
Hair 7	-	U	U	-	U
Hair 8	-	31.07±0.18	U	-	♀
Hair 9	-	28.90±0.12	33.21±0.13	4.31	♂
Hair 10	-	40.38±0.82	U	-	U
Hair 11	-	30.47±0.23	34.18±0.33	3.71	♂
Hair 12	-	28.64±0.17	32.77±0.23	4.13	♂
Hair 13	-	30.32±0.19	38.67±0.70	8.34	♂
Hair 14	-	29.51±0.16	33.58±0.12	4.08	♂
Hair 15	-	32.25±0.41	38.18±0.81	5.93	♂
Hair 16	-	29.14±0.10	33.52±0.28	4.38	♂
Hair 17	-	30.60±0.05	34.58±0.09	3.98	♂
Hair 18	-	37.12±0.14	41.59±0.63	4.46	♂
Hair 19	-	29.30±0.12	33.49±0.34	4.19	♂
Hair 20	-	28.86±0.15	32.97±0.22	4.11	♂
Hair 21	-	29.56±0.21	34.15±0.44	4.59	♂
Hair 22	-	28.67±0.18	33.08±0.28	4.41	♂
Hair 23	-	30.18±0.12	34.39±0.14	4.21	♂
Hair 24	-	35.27±0.63	40.19±3.36	4.92	♂
Hair 25	-	29.21±0.12	U	-	♀
Hair 26	-	30.05±0.09	34.28±0.03	4.23	♂

The ZFY allele was not detected in four replicates (Table 5b) of 24 reactions for the two known males, giving an allele dropout rate of 16.7%. The dropout of the ZFY allele for the scat samples was not attributed solely to low DNA quantity as the ZFX C_T values for each of replicate were relatively low (range 33.01 to 34.57). The ZFY C_T values across all male samples were almost five cycles higher than the ZFX values (ΔC_T values ranging from 0.98 to 4.85, Table 5b). The normalised fluorescence for each probe after amplification was obtained using the allelic discrimination function of the real-time PCR instrument software (Figures 16-18). This revealed a four-fold higher fluorescence for the FAM-labelled probe (ZFX) compared to the VIC-labelled probe (ZFY) for male samples, which was higher than the expected two-fold difference as a result of two copies of ZFX to every ZFY.

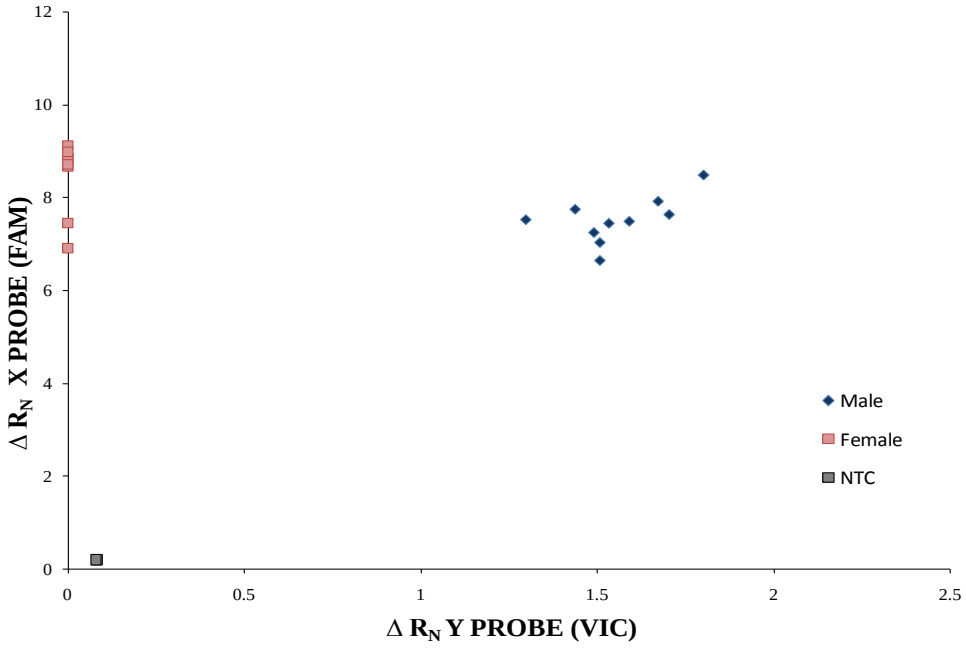


Figure 16: Sex typing of tissue samples using TaqMan® MGB probes. Each point represents a PCR reaction with fluorescence for each probe. Note the difference in the scale of the two axes due to stronger FAM fluorescence (ΔR_N).

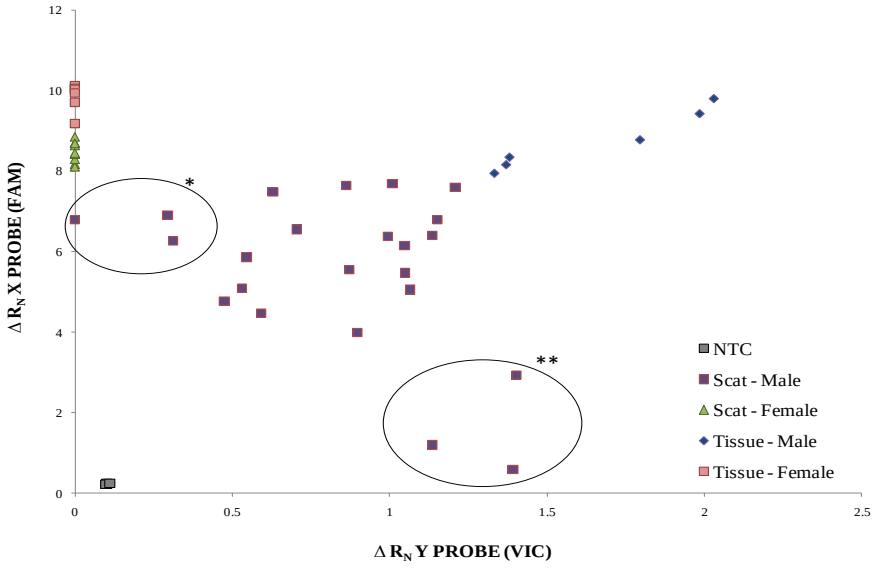


Figure 17: Sex typing of scats using TaqMan® MGB probes. Male scats with low fluorescence for the ZFY probe are circled (*), along with those with low fluorescence for both probes (**).

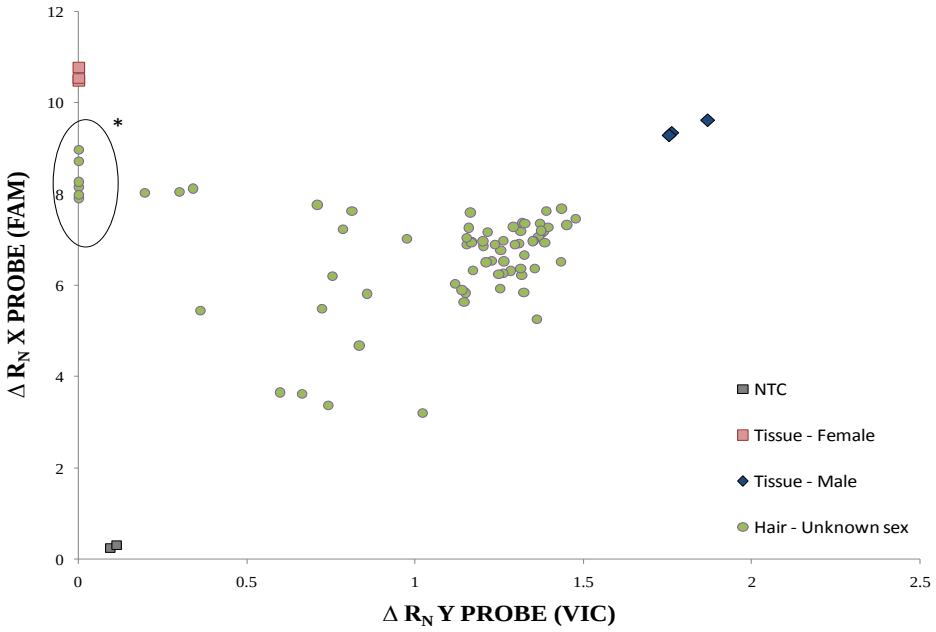


Figure 18: Sex typing of hair samples using TaqMan® MGB probes. Three unknown sex hair DNA extracts were identified as female on the basis of amplification of the ZFX allele only (*).

Assay optimisation

The probes were reordered with the dyes swapped, labelling ZFX with VIC dye and ZFY with 6-FAM to account for the difference in fluorescence intensity of each reporter dye used to label the TaqMan MGB probes, in an attempt to bring the C_T values for ZFX and ZFY closer together. Each newly synthesised probe was then combined at a range of different concentrations (50, 100, 150, 200 and 400 nM) to test the variability of C_T values with reporter dye concentration (Table 6). The lowest C_T values were obtained with the highest probe concentrations, although the values were not significantly different across probe concentrations, except at the extreme values (e.g. 50 nM VIC: 400 nM FAM). The C_T values for ZFX and ZFY were close at the original assay conditions of 200 nM of each probe (X, 26.30; Y, 26.14), an improvement on the ΔC_T values with the previous X-FAM and Y-VIC assay. The ZFY probe was labelled with FAM for all future applications as the detection of the male-specific Y chromosome is the basis of

gender determination in mammals and therefore a failure to accurately detect the ZFY allele would lead to false assignment of unknown samples as female.

Table 6: Optimisation of the sexing assay TaqMan® MGB probe concentration. The C_T values for the original probe concentrations of 200 nM each are shown in bold.

		X (VIC) probe (nM)				
		50	100	150	200	400
Y (FAM)probe (nM)	50	X 27.62 Y 26.78	26.03 26.19	25.61 26.64	24.67 26.03	24.55 26.67
	100	X 28.20 Y 26.65	27.06 26.72	26.60 26.78	26.61 26.30	25.77 27.64
	150	X 27.05 Y 26.22	27.31 26.22	26.64 26.27	26.15 26.54	25.79 26.81
	200	X 28.51 Y 26.32	27.05 26.17	26.79 26.07	**26.30** **26.14**	25.66 26.25
	400	X 28.10 Y 25.06	26.21 25.24	26.63 25.29	26.32 25.56	25.95 25.72

Cross-species application of the TaqMan® MGB sex typing assay

Due to the conserved nature of the zinc finger gene region amplified in the ZF exon assay, sex identification was possible for other species, with amplification for otter (X, 24.37; Y, 21.51), stoat (X, 26.97; Y, 26.96), mink (X, 27.06, Y, 22.47), badger (X, 30.00; Y, 27.87), stone marten (X, 23.54; Y, 27.10), fox (X, 32.16; Y, 31.10) and cat (X, 31.58; Y, 29.54) at 10^{-1} dilutions of tissue DNA. Otter, badger and stone marten were known males and were therefore accurately sexed. Some cross-reactivity of the probes was observed for wood mouse and pygmy shrew tissue DNA extracts (X, no amplification; Y, 43.20 and X, 34.10; Y, 38.40 respectively). The C_T values were high but this may have been related to poor DNA extract quality. A new assay was designed to target the more variable zinc finger intron sequence to be more specific to mustelids to prevent the amplification of exogenous sequences. The ZF Intron sex typing assay was applied and validated in the population census in Chapter 5.

3.4. Discussion

Species identification

Real-time PCR analysis proved to be a highly efficient molecular approach for the identification of pine marten hair and scat samples. The PM2/FOX TaqMan probe assay was designed to distinguish pine marten and fox as the scats of these species are often confused, especially at extremely low population densities (Davison *et al.* 2002) when accurate species identification is even more crucial. The assay was applied to 56 hair and scat samples in this study and 53 samples (44 pine marten and 9 fox) were identified to species with the PM2 and FOX TaqMan probes. This success rate (95%) was much higher than previously obtained using conventional PCR and sequencing techniques (53%, Davison *et al.* 2002). O'Mahony *et al.* (2006) used the PM2/FOX assay to reliably identify pine marten scats in the National Pine Marten Survey 2005-2006 and achieved a similarly high amplification success rate (95%). This may reflect the small size (60-bp) of the PCR product and the sensitivity of the fluorescent probe based assay.

Although scat collection is the standard survey method for pine marten, hair collection using specially designed traps is a promising alternative survey method (Statham 2005; Lynch *et al.* 2006). A species typing assay should work reliably with both sample types. In this study 90% of the hair samples were identified as pine marten using the PM2 TaqMan probe, but cross-reactivity with stoat DNA was problematic as stoats have been recorded in hair-tubes previously (Statham 2005). The PM2 probe should have been specific as the SNP mismatch between the two species was located in central third of the probe (Anonymous 2004). Stoats are unlikely to leave a large sample of hair due to their small size in comparison to the diameter of the hair-tube but the PM3 probe was developed as a pine marten species-specific TaqMan probe to ensure only pine marten genetic samples were retained for further analysis. The PM3 TaqMan probe did not

hybridise to stoat DNA as there were seven SNP mismatches between the species in the probe binding region.

Species identification was also possible by melt-curve analysis (MCA). The T_M values for identified pine marten scats did not overlap with the T_M values for fox scats. The T_M values varied between runs, but interrun variation was accounted for by comparing the sample T_Ms to the positive controls T_Ms which were simultaneously amplified in each reaction (Berry & Sarre 2007). The MCA approach has the advantage that is widely applicable to any study species (with primer redesign) without the added cost of synthesising expensive TaqMan® MGB probes. There has only been one haplotype detected so far in the Irish pine marten population (hap *p*, Davison *et al.* 2001) but variability in the fox population in the amplified region should be accounted for by sequencing more tissue samples to cover all fox mtDNA haplotypes which may be sampled among the population.

Sex identification

Accurate, rapid and non-invasive sex identification was possible based on a single nucleotide polymorphism in the final exon of the ZFX and ZFY genes. There was no detectable fluorescence for the ZFY probe for known females while there was fluorescence from both probes for male samples. The sex of the tissue samples determined by real-time PCR was in agreement with the known sex determined by conventional methods of carcass examination or standard end-point PCR by the method of Shaw *et al.* (2003). The targeted region was very short (76-bp) and facilitated the sexing of hair and scat samples. The amplification success was high at 85% for hair and 92% for scats, and is in the upper range for nuclear DNA analysis of non-invasive samples (Puechmaille *et al.* 2007; Hajkóva *et al.* 2009). There was a relatively high rate of allele dropout for the ZFY allele despite the small size of the PCR product. This was identifiable with PCR replication, which is recommended for molecular sexing (Lynch & Brown 2006) to prevent the identification of males as females due to low DNA

quantities. The ZF exon assay was also suitable for gender determination of other carnivore species due to the conserved nature of the targeted zinc finger gene (Aasen & Medrano 1990; Morin *et al.* 2005), with cross-reactivity of the probes with DNA from otter, stoat, badger, stone marten, cat, fox and mink. This assay may have broad applicability as a rapid and robust sexing method for various mammalian species.

In conclusion, real-time PCR analysis is ideally suited to the analysis of samples with low quantity and/or quality DNA as the reaction and analysis is carried out in a 96-well closed-tube assay with reduced handling and thus fewer opportunities for PCR contamination. The assays target short DNA regions (<150-bp), which resulted in high amplification success rates for hair and scat samples (>85%). The sex typing assay represents a major methodological improvement as previous methods for mustelids were based on (1) the SRY gene, which is male-specific requiring an internal control, (2) direct sequencing followed by chromatogram interpretation which can be expensive and time consuming, (3) ZF intron amplification of approximately 1 kb of nuclear DNA which can be challenging for degraded DNA samples or (4) PCR-RFLP analysis involving post-PCR manipulation with agarose gels and restriction digests (Hattori *et al.* 2003; Lynch & Brown 2006; Statham *et al.* 2007).

Chapter 4

Microsatellite analysis of Irish pine marten

4.1. Introduction

The pine marten is widely distributed across Europe, yet persecution and habitat disturbance have led to declines across the species distribution and genetic structuring between populations (Kyle *et al.* 2003). Genetic diversity and structure in Europe is also affected by the introgression of genes from American marten *Martes americana* through escapes from captivity in England and by natural hybridisation with sable *Martes zibellina* where the two species are sympatric in the Northern Ural Mountains (Davison *et al.* 2001; Kyle *et al.* 2003; Rozhnov *et al.* 2010).

The first study of European pine marten genetics focused on the analysis of mitochondrial DNA (mtDNA) sequences to reconstruct the post-glacial colonisation history of Europe (Davison *et al.* 2001). Twenty-five haplotypes were identified from sampling 139 martens from 14 countries, which split into three major lineages, Groups I to III. The Group II lineage was found in Finland and Sweden only and the Group III lineage was very divergent and found in two English martens, each representing the hybrids with *Martes zibellina* and *Martes americana* respectively. The remaining 18 Group I haplotypes were distributed across Europe, with some support for a split of this group into Group Ia and Ib haplotypes. These haplotypes had low nucleotide diversity, with single base substitutions between the haplotypes and a pattern of population expansion, which was interpreted as evidence for the colonisation of Europe from a single refugium post-glaciation. However the authors cautioned that divergent lineages may have been unsampled due to the difficulty of obtaining samples from southern Europe.

A more comprehensive phylogeographic analysis was recently conducted to clarify the results of Davison *et al.* (2001). Ruiz-Gonzalez *et al.* (2009) analysed a larger mtDNA sequence of 1,600 bp and more samples, improving the sample size for south-west Europe, Scandinavia and Russia (including sable samples). Sixty-nine haplotypes were identified for pine marten and 11 for sable, which split into two major clades, Fennoscandian-Russian and European-Mediterranean, the latter containing all pine

marten samples collected throughout the entire distribution (Schwartz *et al.* 2009). The European-Mediterranean clade is divided into two groups of haplotypes found in Central/Northern Europe or the Mediterranean regions. In addition, this improved resolution revealed the majority of the Europe was recolonized by the Central/Northern phylogroup, from a cryptic refugium in Central Europe and not in the Mediterranean (Ruiz-Gonzalez *et al.* 2009), in accordance with the paleontological data of Sommer & Benecke (2004).

Two pine marten fossil specimens collected in the south of Ireland (femur from Foley Cave, Co. Cork and a lower carnassial from Kilgreany Cave, Co. Waterford) have been carbon dated to the Neolithic period (Woodman *et al.* 1997). Older human remains also found in Kilgreany Cave date to the early Bronze Age, over 4,000 years ago. Genetic studies have detected a single haplotype (Hap *p*) so far in the current Irish pine marten population for the typically variable mtDNA control region (Davison *et al.* 2001; Statham 2005; Lynch 2006), which groups with the Mediterranean clade and not the Northern-Central European clade with pine marten from England and Scotland (Davison *et al.* 2001; Ruiz-Gonzalez *et al.* 2009). Pine marten may therefore have been deliberately introduced to Ireland by prehistoric settlers at a time, and from a location presently unknown.

The isolation of the Irish population is also reflected in the low polymorphism of nuclear DNA microsatellite markers. The number of alleles/locus (A = 1.86) and expected heterozygosity (H_E = 0.34) was the lowest in Europe, lower than the Endangered Newfoundland population of American marten (Kyle *et al.* 2003; Kyle & Strobeck 2003). This reduced variability at both mtDNA and microsatellite loci may be explained by founder effects arising from a small number of individuals contributing to the current population, which was possibly reduced further by genetic drift due to the isolation of Ireland from the rest of Europe, habitat loss and fragmentation, and direct persection.

Written historical records give some insight into the demographic history of the Irish population. Thompson (1856) compiled notes on the presence and status of the pine

marten in Ireland, which included a 16th century letter reporting on the difficulty of obtaining pine marten skins due to the extensive deforestation of the country. Records of skins exported from Ireland in the 18th century also indicate low population densities for the pine marten as some 42,000 otters were exported in the period 1697-1819 with no records of marten skins (Fairley 2001). However, the species was still regarded as being widely distributed and fairly numerous in areas with suitable cover such as well-wooded estates (Thompson 1856). The population possibly suffered further declines with the improvement in firearm technology and the development of the sporting estate in the latter half of the 19th century, as was the case in Great Britain (Langley & Yalden 1977), and several notes to the *Irish Naturalist* Journal comment on the rarity of the species (Anonymous 1893; Patterson 1894; Barrington 1910; Ruttledge 1920).

This trend continued into the 20th century until the species was protected under Irish wildlife legislation in 1976 and the first nationwide distribution survey was conducted by the National Parks and Wildlife Service between 1978 and 1980 (O'Sullivan 1983). Information on the distribution of pine marten in Ireland was obtained by the collection of characteristic pine marten faeces (scats) from suitable woodland and scrub habitat within 10km squares of the national grid which had positive records from previous surveys. This, coupled with local knowledge and historical records showed the population in the late 1970s was mainly restricted to an area west of the River Shannon, with outlying populations in the east (Co. Meath), south-east (Co. Wateford) and midlands (Slieve Bloom Mountains), and was more widely distributed as recently as 30-40 years ago (O'Sullivan 1983). The placement of strychnine poison, rather than snaring, was supposed to be the greatest contributor to the decline of pine marten in Ireland, as the poison was used to lace meat placed at the edges of woodland and in scrub areas in mountainous regions, habitat which pine marten often use for feeding.

One of the areas which appeared to have lost its marten population in the 20th century was Killarney (Co. Kerry), a mountainous region of south-west Ireland which hosts a large National Park. Following O'Sullivan's survey, pine marten from the stronghold of

the species in the west (Counties Clare and Mayo) were translocated to Killarney to the restore the population. Lynch (2006) used biometric, distributional and reproductive data, along with genetic analysis using mtDNA and the microsatellite markers of Kyle *et al.* (2003) to investigate the health of the reintroduced population in Killarney National Park. The population was found to be well-established but had a reduced genetic diversity in comparison to the source populations in the mid-west as a result of founder effects.

The current range of the pine marten in Ireland has been reassessed in the National Pine Marten Survey (NPMS) of Ireland (O'Mahony *et al.* 2007) using the same scat transect survey method employed by O'Sullivan (1983). A random sample of 183 of the 428 10km squares originally surveyed by O'Sullivan were surveyed again between 2005 and 2006. The original percentage of these squares with detection and non-detection of pine marten was 28% and 72% respectively. This was found to have increased to 64% and 36% in the NPMS, a significant range expansion in the 25 years between the two surveys. Based on the distributional data of the NPMS, population density estimates of Lynch (2006) of 0.5-2.0 pine martens per km^2 and a total area of suitable habitat available to the pine marten in Ireland of 5,811 km^2, O'Mahony *et al.* (2007) suggested the total population size of pine marten is in the range of 2,902 to 11,622 animals. The true population size is probably at the lower end of this estimate as the population density is likely to be lower in newly colonized areas and sub-optimal habitat. There is clearly a need to gather more population density estimates from various habitat types in Ireland.

Microsatellite analysis of non-invasively collected samples such as hair and scats can be used to obtain estimates of the size (Mowat & Paetkau 2002; Frantz *et al.* 2003; Frantz *et al.* 2004), and structure (Hedmark & Ellegren 2007) of populations, without live trapping or observation, enabling sampling from remote populations. However, both Statham (2005) and Lynch (2006) noted the microsatellites currently available for the pine marten were unsuitable for such analyses in Ireland and recommended a

reassessment of the genetic diversity of the entire island with a different set of microsatellites. This study therefore had the following aims:

(1) To identify a panel of microsatellite loci suitable for population genetics of pine marten in the genetically depauperate Irish population

(2) To re-evaluate the genetic diversity of Irish pine marten using the new panel identified in this study

(3) To investigate whether the suspected population bottleneck in the 20th century is reflected in the genetic composition of the current population

(4) To examine genetic structure between regional populations to identify any significant barriers to gene flow on a broad scale in Ireland.

Hypothesis 1: No genetic structure exists between regions in Ireland as a result of natural population expansion and translocations in recent decades

Hypothesis 2: Microsatellite allele frequencies differ significantly between regions as a result of genetic drift in isolated populations and the effects of natural barriers such as the River Shannon and possibly man-made barriers like major roads and towns.

4.2. Methods

4.2.1. Sample set

Leg muscle, plucked hair, ear snips, or dried skin samples were obtained from pine marten (n = 41) killed on roads in Ireland during the period 1997-2008 (Figure 19). The samples were initially stored in 96-99% ethanol at room temperature, while samples collected from 2007 onwards were stored at -50 °C in 99% ethanol. Full sample details are provided in Table 7. Tissue samples from seven French pine marten and the four captive martens from Wildwood Trust (identified as UK and Czech origin based on mtDNA sequence analysis, Statham 2005) were also genotyped to compare polymorphism with the Irish samples.

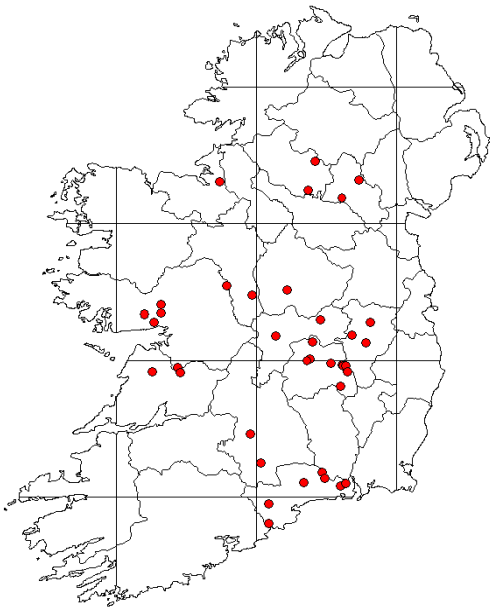


Figure 19: Geographical distribution of *Martes martes* road-kill individuals. Three samples could not be assigned to a 10 km square due to lack of information and two individuals (IRL08 and IRL09) were collected in the same locality (Table 7).

Table 7: Road-kill tissue samples used in this project. *Estimate to nearest 10 km square based on collection information. **Road Traffic Accident (RTA) date.

I.D.	Sex	RTA** Date	County	Area	Grid Reference	I.D.	Sex	RTA Date	County	Area	Grid Reference
IRL01	♂	Jun-97	Waterford	Kilclooney	S3410	**IRL32**	♂	May-05	Waterford	Youghal Bridge	X0890*
IRL02	♂		Kildare	Curragh	N7812*	**IRL33**	♂	Aug-06	Waterford	Dromana	X0994*
IRL03	♂		Unknown			**IRL34**	♀	May-07	Tipperary	Dundrum	R9645
IRL04	♀		Laois	Unknown		**IRL35**	♀	May-07	Roscommon	Kiltoom	M973471*
IRL06	♂	Dec-03	Waterford	Kilmeaden	S496130	**IRL36**	♂	Jul-06	Kildare	Blackford	S615962
IRL07	♀		Laois	Ballyfin	N3800*	**IRL37**	-	Jul-06	Laois	Ballylynan	S6589*
IRL08	♀		Kildare	Athy	S6495*	**IRL38**	♂	Apr-07	Galway	Headford	M3240*
IRL09	♂		Kildare	Athy	S6295*	**IRL39**	♂	Jun-07	Clare	Tulla	R4690*
IRL11	♂		Tipperary	Cahir	S0324*	**IRL40**	-	Jan-07	Galway	Ballygar	M795954
IRL13	♀	Apr-04	Fermanagh	Crom	H377249	**IRL41**	-	Oct-06	Roscommon		
IRL14	♀		Offaly	Tullamore	N467293	**IRL44**	-	Sep-07	Offaly	Boora Bog	N1417*
IRL15	♂	Jun-04	Waterford	Ballinamona	S604070	**IRL49**	-	Jul-07	Kildare	Prosperous	N8127*
IRL16	♂		Fermanagh	-	H429450	**IRL50**	-	Feb-07	Westmeath	Rathowen	N2251*
IRL17	♂		Monaghan	Clontibret	H7331*	**IRL51**	-		Laois	Mountmellick	N4013*
IRL18	♂	Jun-05	Waterford	Ballygunner	S644097	**IRL52**	-	Jul-07	Galway	Drimcong	M2033*
IRL19	♂	Aug-05	Waterford	Mayfield	S474171	**IRL53**	-	Mar-07	Kildare	Rathangan	N6818*
IRL27	♂	Mar-06	Sligo	Lough Gill	G7430*	**IRL54**	-	Sep-06	Laois	Ballylynan	N6591*
IRL28	♂	Jul-06	Clare	Tulla	R447949	**IRL55**	-	May-06	Laois	Ballyfin	S3699*
IRL29	♂	Aug-06	Clare	Kilnaboy	R2691*	**IRL56**	-		Galway	Headford	M3234*
IRL30	♂	Apr-07	Monaghan	Monage	H614180	**IRL58**	-	Jul-08	Galway	Dangan	M2628*
IRL31	-		Laois	Grange	S5397*						

The sex was known for some tissue samples, determined either from carcass examination or provided by the person who contributed the samples. Molecular sexing was carried out for some unknown samples by conventional PCR amplification of a region of the final intron of the zinc finger gene with the primers LGL331 (5'-CAA ATC ATG CAA GGA TAG AC-3') and LGL335 (5'-AGA CCT GAT TCC AGA CAG TAC CA-3') (Shaw *et al.* 2003). Each 50 µl reaction mix contained 0.4 µM each primer, 2 mM $MgCl_2$, 0.16 µM dNTPs, 2.5 Units of Amplitaq Gold polymerase (Applied Biosystems, cat. no. N8080245), 1X GeneAmp® PCR Gold Buffer (Applied Biosystems; 30 mM Tris-HCl, pH 8.0, 100 mM KCl) and 4 µl (*ca.* 40 ng) DNA extract. Amplification conditions were 95 °C for 10 mins followed by 35 cycles of 94 °C for 1 min, 55 ° C for 1 min and 72 ° C for 1 min, with a 10 min final extension at 72 °C. Males were identified by the presence of two bands (ZFX, *ca.* 900-bp and ZFY, *ca.* 1000-bp) on an agarose gel while only one band (ZFX) was present for females (Figure 20).

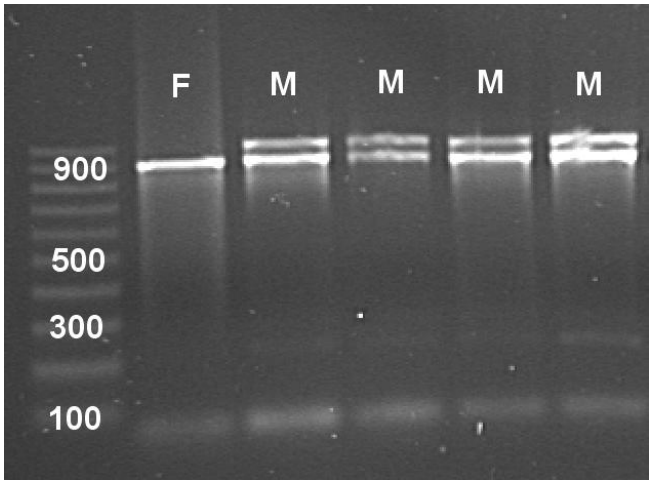


Figure 20: Sexing tissue samples using conventional PCR with the primers LGL331 and LGL335. The samples are (from left to right) IRL08 (♀), IRL06 (♂), IRL15 (♂), IRL18 (♂) and IRL19 (♂).

4.2.2. Screening for polymorphic loci

Microsatellite loci (n = 32) developed for related mustelid species were screened for cross-species utility in the European pine marten as there were no specific *Martes martes* microsatellites published prior to or during this project. Where cross-species polymorphism in pine marten was not reported, loci with the highest number of alleles in the target species were selected, with preference to primer pairs giving a product size less than 250-bp to increase the probability of microsatellite amplification from degraded DNA samples. These were originally isolated from the genomic DNA of eight mustelid species: American marten (*Martes americana*), Eurasian otter (*Lutra lutra*), Eurasian badger (*Meles meles*), American mink (*Neovison vison*), European mink (*Mustela lutreola*), Stoat (*Mustela erminea*), North American river otter (*Lontra canadensis*) and wolverine (*Gulo gulo*) as summarised in Table 8.

4.2.3. Microsatellite analysis

Microsatellite primers were initially tested unlabelled for specific and reliable amplification using pine marten tissue. Amplifications were performed in 25 µl reactions containing 2 µl (*ca.* 10 ng) DNA extract, 0.2 µM of each PCR primer (MWG Biotech, Germany), 1.25 units of standard *Taq* Polymerase (Promega, cat. no. M1661) in 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 2.5 mM $MgCl_2$ and 0.25 mM dNTP (Promega, cat. no. U1330). Amplifications were performed in Techne Flexigene thermocycler under the following conditions; 94 °C for 1 min initial denaturation followed by 3 cycles of 94 °C for 30 s, T_A for 20 s, 72 °C for 5 s; followed by 33 cycles of 94 °C for 15 s, T_A °C for 20 s and 72 °C for 5 s, with a final extension of 72 °C for 7 mins. The annealing temperature (T_A) was varied in different trials with the first 8 primers tested (Mvi39, Mvi57, Mvi2243, Mvi1342, Mer022, Lut615, RIO17 and Mel1) to find the most suitable temperature for each primer set.

Table 8: Microsatellite primers tested for cross-species amplification. Dye labels used for each fluorescently labelled forward primer are in bold. A GTTTCTT sequence was added to the 5' end of reverse primers marked with an asterisk.

Locus	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Ref
Ma1	**TAMRA-**ATTTTATGTGCCTGGGTCTA	TTATGCGTCTCTGTTTGTCA	
Ma2	**FAM-**ACCCATGAATAATGTCTTAT	*ATCTTGCATCAACTAAAAAT	
Ma5	TAAAACCAGGAAACAGATAC	AGTATGGATAAAGCACAAAC	1
Ma8	**HEX-**GTTTTCTAATGTTTCGTGTG	CAGTGGTTGACTACAAGAAA	
Ma18	TGGGTGGGTGTATTTGTGTAT	TACTCAGTGGGGAATCTGCT	
Gg7	**FAM-**GTTTTCAATTTTAGCCGTTCTG	GTTTATCTCCCTCTTCCTACCC	1
Ggu234	**PET-**TTACTTAGAGGATGATAACTTG	*GAACTCATAGGACTGATAGC	2
Ggu454	**HEX-**CTTCTTACATAGTCAATGTTTTG	TGCCATTTTCTCCAGAA	3
Mvi39	CTCACCAGGCAGGGAGC	AAAACAAGCATTCAGATCACC	4
Mvi57	GAACAGGACCAGCCCTGC	GTTGGAAATGAGGATCTCAC	
Mvi1341	**PET-**GTGGGAGACTGAGATAGGTCA	*GGCAACTTGAATGGACTAAGA	
Mvi1342	TGGGAGTGAGCGGTGAT	CTGGCCTTCAGTCAGTCTTG	5
Mvi1354	**FAM-**CCAACTGGAGCAAGTAAAT	*CATCTTTGGGAAAGTATGTTT	
Mvi2243	CGGACATTTGTTCTAAGAGGT	AGATTAACAAGCCCATGCTC	
Mvis072	**NED-**CTGCAAAGCTTAGGAATGGAGA	CCACTACACTGGAGTTTCAGCA	
Mvis075	**NED-**GAAATTTGGGGAATGCACTC	*GGCAGGATAGGATGTGAGCT	6
Mvis020	**FAM-**GGGTCAAGAGTTAGAGCCCC	GACATGGTCAGATTCCCCAC	
Mvis022	ATCAAGTCCTGCATCAGGCT	TGGGCTGTTTGTCCAGGT	
Mvi4031	GCCTTACCTCAGGCAATG	CACTTAACCAGAGGCCATCA	7
MLUT04	GGAGAGGAAACTATACCTC	CGTGTCTTGTATAGTTTTGTTCTCC	8
MLUT20	CTTATGGAGCAAAGTAACC	GTTTGTTTCCATCTTCCATCAGG	
MLUT27	**FAM-**GTGATCACAGTTCAGCTAAATGTGT	*AAGCCAGTAATGAGAACCACAAT	
RIO17	GGTTGCGAAGATAAGCAAGG	CAAGTGTTTAAAGTGTGTGTGTGT	9
Mer041	**NED-**TGTGTGATCTCTGGGAATTCTC	*GTTTCTGCTCCCCAGATAAAAGC	6
Mer022	CCATGCTTTGGGTAGGAGAA	CCTTGTTCTCAGGTGGTTGG	
Mel1	**FAM-**CTGGGGAAAATGGCTAAACC	*GCTCTTATAAATCTGAAAATTAGGAATTC	10
Mel6	AAGTCCTCCTTGCAGTTTGG	AGCAAGCTCTTGGTTCTTGG	
Mel105	**FAM-**GATATTCCCCTCCCACCACT	*CTCCAAGGGATCCTGGAACT	11
Mel10	TGCAGAAAACGATCAGGTTTC	*TCATTTTTGGCGTGTACTTCC	12
Lut604	**HEX-**TATGATCCTGGTAGATTAACTTTGTG	TTTCAACAATTCATGCTGGAAC	13
Lut615	**FAM-**TGCAAAATTAGGCATTTCATTCC	ATTCTCTTTTGCCCTTTGCTTC	
04OT14	GGTCCAAGTCCAAGCCTGCCT	TTCATATTCTTCAGGTGAATCCCAT	14

1. Davis & Strobeck (1998), 2. Duffy *et al.* (1998), 3. Walker *et al.* (2001), 4. O'Connell *et al.* (1996), 5. Vincent *et al.* (2003), 6. Fleming *et al.* (1999), 7. Anistoroaei *et al.* (2006), 8. Cabria *et al.* (2007), 9. Beheler *et al.* (2005), 10. Bijlsma *et al.* (2000), 11. Carpenter *et al.* (2003), 12. Domingo-Roura (2002), 13. Dallas & Piertney (1998), 14. Huang *et al.* (2005).

Standard microsatellite PCR protocol

It was clear that some microsatellite loci amplified more reliably than others regardless of the PCR conditions used. A standard PCR protocol for all loci was then used with a hot-start enzyme to reduce the chance of mispriming in the early stages of the reaction. All microsatellite loci were screened using the standard protocol and the final set of microsatellites were chosen based on robust amplification of a single, short, polymorphic microsatellite product. Each standard microsatellite PCR contained 1.5 mM $MgCl_2$, 0.2 µM each primer, 1 unit of Amplitaq Gold DNA polymerase, 1X GeneAmp® PCR Gold Buffer (Applied Biosystems; 30 mM Tris-HCl, pH 8.0, 100 mM KCl), 0.2 mM dNTP and approximately 10 ng tissue DNA extract in a 15 µl volume. The PCR profile was a 2-step cycling reaction as follows: 95 °C for 10 min for activation of the hot-start polymerase, followed by 38 cycles of 94 °C for 20 s and anneal/extend at 58 °C for 1 min, with a final extension of 30 min at 72 °C to promote the addition of an adenosine residue to the end of all PCR products (Brownstein *et al.* 1996).

Fragment analysis

Loci that reliably amplified a single PCR product of expected size as reported in the original primer paper, preferably with product sizes less than 250-bp, were fluorescently labelled for analysis of polymorphism (Table 9, Panel_A). Forward primers were labelled with different fluorescent dyes (6-FAM, VIC, NED, HEX, TAMRA or PET) to facilitate subsequent multiplexing of PCR products. Reverse primers were unlabelled but a 'PIGtail' sequence (5'-GTTTCTT-3') was added to the 5' end to promote full non-templated nucleotide addition (Brownstein *et al.* 1996). Table 9 also includes the subsets of loci used throughout the project for reference.

All microsatellite products were analysed on an ABI Prism 310 Genetic Analyser (Applied Biosystems, cat. no. 310-00-100/120-W). DNA fragments were electrophoresed through a polyacrylamide polymer (POP-4) in a 47 cm x 50 µm capillary, under default run conditions. Samples were diluted as necessary (typically 1 in

10, 20 or 50) in molecular grade water, determined from the intensity of PCR products on ethidium bromide stained 2% agarose gels. Each sample (1 µl) was denatured in 15 µl Hi-Di formamide (Applied Biosystems, cat. no. 4311320) with 0.2 µl GS500LIZ™ size standard (Applied Biosystems, cat. no. 4322682) for 5 minutes at 95 °C, followed by rapid cooling to 4 °C. Electrophoresis ran for 28 mins at 60 °C as standard for GeneScan applications on the ABI310.

Table 9: Panels of microsatellite primers used in the project and their application.

Locus	**Panel_A**	**Panel_B**	**Panel_C**	**Panel_D**	**Panel_E**
Application	Fluorescently labelled	Polymorphic in Irish population	Analysis of genetic structure	Individual identification Panel1	Individual identification Panel2
Ma1	•				
Ma2	•	•	•	•	•
Ma5	•				
Ma8	•	•	•		
Gg7	•	•	•	•	•
Ggu234	•	•	•		
Ggu454	•	•	•		
Mvi1341	•	•	•	•	•
Mvi1354	•	•	•	•	•
Mvis072	•	•	•		
Mvis075	•	•	•	•	
Mvis020	•	•			
MLUT27	•	•	•	•	•
Mer041	•	•	•	•	•
Mel1	•	•	•	•	•
Mel6	•				
Mel105	•	•	•	•	•
Lut604	•				
Lut615	•				
04OT14	•				

Microsatellite allele sizing

Microsatellite genotypes were analysed with GeneMapper software v. 3.7 (Applied Biosystems). The Global Southern sizing algorithm was employed as it is less sensitive to temperature variation than the Local Southern method. The 250-bp peak was excluded from the GS500LIZ size standard as recommended by Klein *et al.* (2003) along with the 35-bp and 50-bp peaks which were usually obscured by primer dimer artefacts. Samples were assigned allele calls if the peak heights were above 50 RFU (Relative Fluorescence Units) each for heterozygotes and above 80 RFU for homozygotes. Ambiguous or overloaded samples were repeated at a higher or lower dilution where necessary.

Each tissue sample was genotyped at least twice per locus if heterozygous and three times if homozygous to construct consensus multilocus genotypes. Multiple PCR replicates were used as the tissue samples were in ethanol for several years and did not always amplify a PCR product on the first attempt. The genotyping error rate per reaction was calculated by dividing the number of reactions with a genotype different to the consensus by the total number of reactions. Negative PCRs were not included.

4.2.4. Statistical analysis

Descriptive statistics

Genetic variation was assessed using the mean number of alleles per locus (A), allele frequencies, observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E) of microsatellite loci using MICROSATELLITE TOOLKIT 3.1 (Park 2001) software. In addition, allelic richness (A_R) was calculated with the program MSA (Dieringer & Schlotterer 2003) to account for differences in sample size between the sample sets from Ireland (n = 41) and France (n = 7). GENEPOP v. 4.0 (Raymond & Rousset 1995) was used to test for Hardy-Weinberg equilibrium and linkage disequilibrium for each locus. Significance was assessed with exact tests for loci with less than four alleles and with Markov Chain Monte Carlo (MCMC) sampling with 100,000 iterations for loci with

more than four alleles. The program MICROCHECKER (Van Oosterhout *et al.* 2004) was used to check for null alleles, large allele dropout or scoring errors in the dataset using 10,000 iterations. A Bonferroni correction was applied to adjust significance levels for multiple tests (Rice 1989) with the initial α level of 0.05.

Evaluating historical demography

The program BOTTLENECK 1.2. (Cornuet & Luikart 1996; Piry *et al.* 1999) was used to test for genetic evidence of a recent decline in the Irish population using allele frequencies from the sample set of 41 individuals and 13 polymorphic loci (Panel_C, Table 15), which is sufficient for both bottleneck tests used in this study (Cornuet & Luikart 1996; Luikart *et al.* 1998). The first test was based on a detection of heterozygosity excess from the rapid loss of alleles in the population during a bottleneck event. The expected heterozygosity based on actual allele frequencies in the population was compared to the theoretical H_E for a population under mutation-drift equilibrium (H_{EQ}) with the same sample size and allele number as the observed population (O'Brien *et al.* 2007), and was evaluated using the three models of microsatellite mutation (infinite-allele model (IAM), the stepwise-mutation model (SMM) and the intermediate two-phase model (TPM, 90% SMM, 10% IAM), with the Wilcoxon sign-test.

The second test was a qualitative graphical method for detecting the distortion of allele frequencies following a population bottleneck (Luikart *et al.* 1998). A population of stable effective size at mutation-drift equilibrium has a characteristic L-shaped distribution of allele frequencies with the majority of alleles at low frequency (<0.1) in the population, whereas a bottlenecked population will show a mode-shift with an increase in the number of alleles at intermediate frequency (0.1-0.2) due to the loss of rare alleles from the gene pool. A population bottleneck is inferred when there are more alleles in one or more of the intermediate allele classes than in the low frequency classes.

Population structure

The statistical power of the final set of 13 microsatellite loci (Panel_C, Table 9) to detect population structure within Ireland was assessed with the program POWSIM 4.0 (Ryman & Palm 2006) using allele frequencies from the base population of 41 individuals. The data was simulated to diverge into three subpopulations, each with effective population size of 500 after 51 generations of random genetic drift (giving a simulated F_{ST} value of 0.05) with subpopulation sizes of 21, 11 and 9 (corresponding to the regional sample sizes in this study). Significance was tested with MCMC sampling using 100,000 iterations.

Heterogeneity in allele frequencies among regions was assessed using exact and chi-square tests with the software CHIFISH (Ryman 2006). Bayesian clustering analysis was then carried out using two different software programs, STRUCTURE 2.3.1 (Pritchard *et al.* 2000; Hubisz *et al.* 2009) and GENELAND (Guillot *et al.* 2005), to investigate population subdivision in the Irish pine marten population. The analysis was carried out on the whole dataset, including the French samples as a control group (n = 7), and separately using only the genotypes of the Irish individuals, under the same parameters.

The model-based clustering method implemented in STRUCTURE was used to find the highest level of hierarchial genetic structure in the dataset by grouping individuals into the optimal number of genetic clusters (*K*) to minimise deviations from Hardy-Weinberg and linkage equilibrium. The program uses a Markov Chain Monte Carlo (MCMC) procedure to estimate P (D|*K*), the posterior probability that the data fit the hypothesis of *K* clusters. The program does not require *a priori* information about population allocation for each of the individuals in the analysis but the individuals were grouped into regional populations in this study as recommended for cases where sample size is limited, to improve the estimate of the *K* value (Hubisz *et al.* 2009). Individuals were grouped into three regions based on sampling location: West (of the River Shannon),

Mid-North and South (Figure 21). These regions were taken to represent the major core areas of pine marten distribution as reported by O'Sullivan (1983).

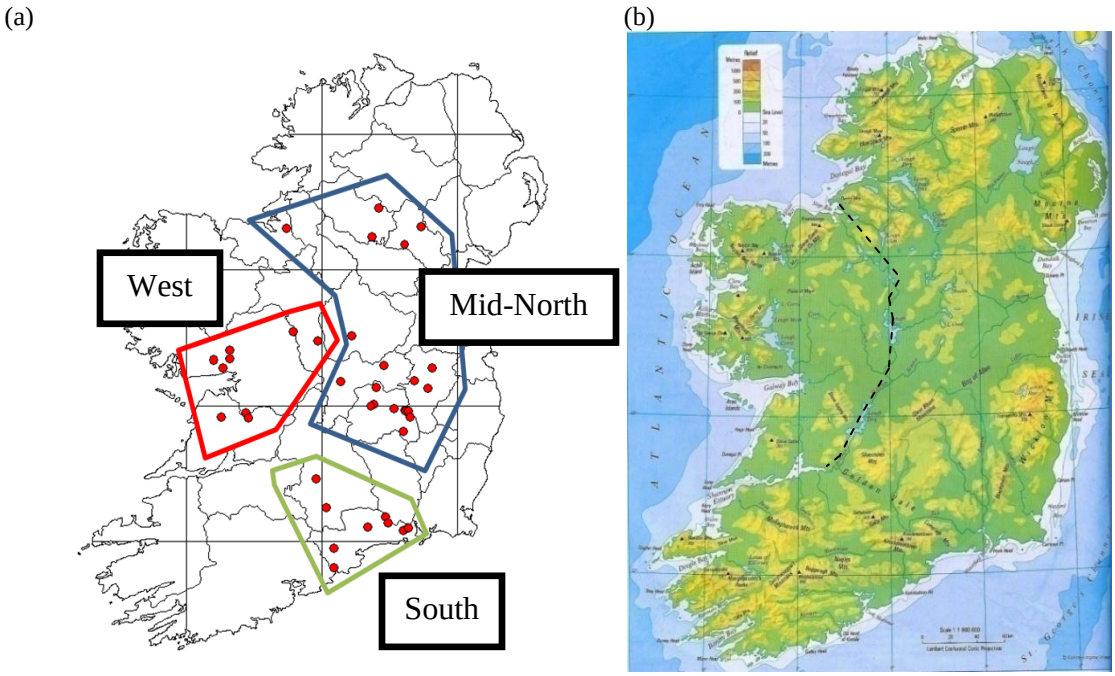


Figure 21: Regions defined for the analysis of genetic structure. Samples were assigned to regions (a) depending on which side of the River Shannon (b, dotted line) they were sampled from or on breaks in road-kill sample distribution for the southern samples.

The posterior probability P (D|*K*) that the data fit the hypothesis of K clusters was permuted with 100,000 runs as burn-in and another 400,000 runs for each simulation. Five replicates were used for each simulation of $K = 1$ to 5. The allele frequencies were presumed to be correlated between populations under the admixture model. All other parameters were left as default.

It is not always straightforward to decide the *K* value for a dataset based simply on selecting the *K* value with the highest log likelihood from the STRUCTURE output. Evanno *et al.* (2005) proposed an alternative method which calculates the second order

rate of change in the likelihood of *K*, called delta *K* (ΔK). The results of the 25 simulations in STRUCTURE (5 runs each for K = 1-5) were used as input for the program STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/struct_harvest/), which carries out the ΔK procedure automatically. The modal value of *K* was considered to represent the most pronounced genetic subdivision in the dataset (Evanno *et al.* 2005). Each individual was then assigned to a genetic cluster when the fractional membership of each individual in each cluster (Q value) was ≥ 0.8.

The second Bayesian clustering analysis carried out on the dataset was with the landscape genetics program GENELAND, which was used to estimate the number of genetic clusters in the population without the *a priori* assignment of individuals into regional populations. Sampling location (GPS coordinates) was used as prior information in the model to spatially define any subpopulations and geographic barriers between them (sampling information was not known for the French samples and was estimated). The analysis was carried out five times with the model of correlated allele frequencies using 10,000 MCMC iterations (50,000 iterations with 1 in 5 stored for analysis) with and without the French individuals. The mode of the posterior distribution of *K* values (allowed to vary from 1 to 5) was used to infer the number of genetic clusters in the dataset.

Individual identification

Probability of identity statistics were calculated using the program GIMLET v. 1.3 (Valière 2002) to evaluate the power of the microsatellite panel to identify individuals from genetic samples. The probability of identity (PI) values for each of the identified polymorphic loci (Panel_C, Table 9) were calculated using the equations for unrelated individuals (PI*ave*) and full siblings (PI*sibs*). Multilocus genotype PI values were obtained by arranging the loci in order of decreasing H_E values and multiplying the per locus PI values across loci. While both PI*ave* and PI*sibs* were calculated to find the minimum and maximum number of loci required for individual discrimination, the final microsatellite panel was selected based on the number of loci required to achieve a PI*sibs* of 0.01 as recommended by Waits *et al.* (2001). Such a low PI should ensure that the loci used could reliably discriminate between individual pine marten, even if relatives are likely to be sampled.

Parentage analysis

A simulation of likelihood-based parentage inference was conducted using using the software package CERVUS 3.0 (Kalinowski *et al.* 2007) based on allele frequencies of 41 Irish pine marten which had been typed at eight microsatellite loci (Panel_E, Table 9) to evaluate the power of the final set of polymorphic microsatellites for parentage inference. The values for the average percentage of loci typed (92%) and the error rate (2%) were constant across simulations. The number of loci (8 or 16), the number of candidate mothers and fathers (2 or 10) and the proportion of parents sampled (100% or 75%) were varied for each simulation under the assumption that the sex of the candidate parents was known. The proportion of 10,000 simulated offspring which could be assigned using log-likelihood (LOD) scores at 80% (relaxed) and strict (90%) confidence was then reported as an assignment rate to assess the power of the markers for parentage inference under the parameters of the simulation. Each simulation was run five times and the highest assignment rate for each confidence level was reported.

4.2.5. Microsatellite PCR optimisation

Primer redesign to facilitate amplification from degraded DNA samples

The primers for Mel1 and MLUT27 were redesigned to anneal closer to the microsatellite repeat region, in order to aid the amplification of microsatellite DNA from non-invasively collected samples, a technique which is commonly employed in forensics and referred to as 'miniSTR' (mini short tandem repeat) design (Butler *et al.* 2003). The original Mel1 locus (Bijlsma *et al.* 2000) was polymorphic with robust amplification, but the product size was *ca*. 270-bp. Target sequences of less than 200-bp are desirable for the analysis of nuclear DNA from low quality samples (Frantzen *et al.* 1998).

Mel1 microsatellite products from two individual pine marten which were identified as homozygotes for the original primer set (270-bp and 274-bp alleles) were purified using a Clean and Concentrator™-5 kit (Zymo Research, cat. no. D4003). DNA sequences were obtained for both strands of the microsatellite product using unlabelled primers with the BigDye® Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, cat. no. 4337455). Cycle sequencing reactions were performed with approximately 10 ng of purified template DNA, 4 µl 2.5X Ready Reaction mix, 2 µl Big Dye sequencing buffer and 0.32 µM of primer in a final volume of 20 µl. Cycling conditions were 96 °C for 1 minute, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 mins, with a 4 °C hold until purification.

Sequencing reactions were purified by ethanol/EDTA precipitation. Five microlitres of 125 mM EDTA was added to each reaction followed by 60 µl of 100% ethanol. The tubes were mixed well and incubated at room temperature for 15 mins. After refrigerated centrifugation for 30 mins at 3,000 *g* the supernatant was carefully removed from the precipitate by a brief inverted centrifugation step at 185 *g*. The DNA pellet was washed with 60 µl of 70% ethanol and centrifuged again at 3,000 *g* for 5 mins (at 4 °C). The supernatant was removed by another inverted centrifugation step at 185 *g*. The precipitate was allowed to dry for 15 minutes to remove residual ethanol. DNA pellets

were resuspended in 20 µl of Hi-Di formamide and denatured as for fragment analysis. DNA sequences were analysed in Sequence Analysis Software v. 5.2 (Applied Biosystems).

Forward and reverse sequences were aligned in SeqMan 5.05 (DNASTAR) and a new reverse primer was designed for the consensus sequence, to anneal as close to the repeat region as possible using PRIMER EXPRESS 2 software (Applied Biosystems). Although amplicons can be reduced to as little as 50-bp, there is a danger in designing primers close to the repeat region where polymorphic nucleotides may disrupt primer annealing, or allelic variants may be missed due to insertions or deletions of nucleotides outside the new amplicon range (Butler 2003). Tissue samples which were previously scored with the original primers were repeated with the shorter primer set to check for concordance. The primer set for MLUT27 was also redesigned using PRIMER EXPRESS 2 directly from sequence data provided by M. Cabria for European mink DNA (Cabria *et al.* 2007).

Multiplex PCR

Combining several loci together in a single PCR (multiplexing) considerably reduces the time and cost of microsatellite analysis. Multiplex PCR was trialled at the end of the project using the Type-It® Microsatellite PCR Kit (Qiagen, cat. no. 206241), which is specifically formulated for the co-amplification of several microsatellites without the need for PCR optimisation. Multiplexes were designed to amplify the 13 loci in Panel_C (Table 9) in three reactions based on dye colour and primer compatibility, which was checked with the software AutoDimer v. 1.0 (Vallone & Butler 2004). Primer pairs with more than 5 base pair interactions were placed in separate multiplexes where possible. Each multiplex reaction contained 5 µl of 2X Qiagen Type-It Multiplex PCR mastermix, 0.2 µM of each primer and 3 µl template DNA in a volume of 10 µl using either tissue (n = 13) or hair (n = 13) as a DNA template. Tissue DNA was normalised to 50 ng/µl before amplification. Reaction conditions were 95° C initial denaturation for 5 mins followed by 40 cycles of 95 °C for 30 s, 57 °C for 90 s, 72 °C for 30 s with a final

extension time 30 mins at 60 °C. The hair samples were also genotyped in single locus reactions for comparison (for 9 of 14 loci; Panel_D, Table 9) using the same reaction mix and amplification conditions. Fragment analysis was carried out as described in section 4.2.3.

4.3. Results

4.3.1. Screening mustelid microsatellites for reliable amplification

Thirty-two loci were screened for amplification with pine marten tissue samples (Table 8). Attempts were made to optimise the amplification conditions for each locus individually for the first eight loci tested (Mvi57, Mvi39, Mvi1342, Mvi2243, Mer022, Lut615, Mel1 and RIO17), by varying the annealing temperature between 54 °C and 62 °C (e.g. the two step annealing temperature PCR 57/55 °C in Figure 22).

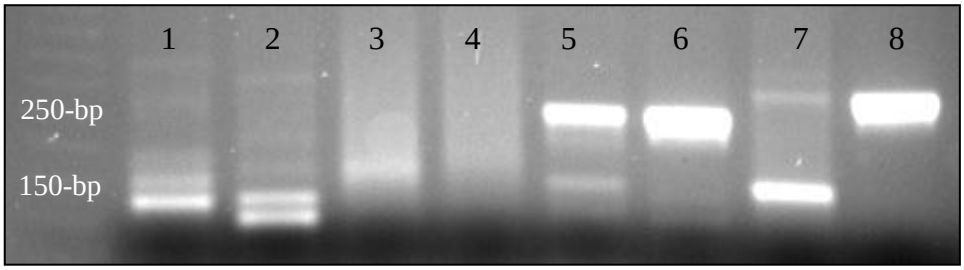


Figure 22: Microsatellite amplification with standard *Taq* polymerase. The first 8 loci tested for cross-species amplification (From 1 to 8: Mvi39, Mvi57, Mvi2243, Mvi1342, Mer022, Lut615, RIO17 and Mel1), in this case at an annealing temperature combination of 57/55 °C.

Some loci showed more robust amplification than others and were labelled for allele sizing (Mer022, Mel1 and Lut615; Figure 22). The other five loci (Mvi39, Mvi57, Mvi1342, Mvi2243 and RIO17) were difficult to amplify consistently at a range of annealing temperatures and were not labelled for analysis of polymorphism. The remaining 24 loci tested in the project were subsequently screened with a hot-start polymerase (AmpliTaq Gold). The majority of loci amplified a single PCR product of expected size according to the original primer publication (Figure 23).

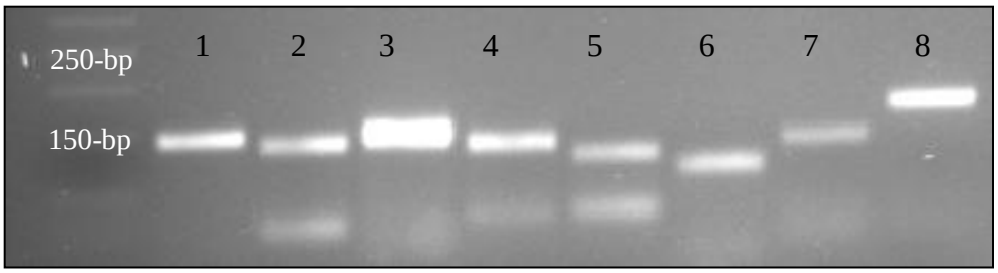


Figure 23: Microsatellite products amplified with the hot-start AmpliTaq enzyme. Microsatellite loci from 1 to 8: Ma2, Mvi1341, Gg7, Mer041, Mel105, MLUT27, Mvis075 and Mvi1354.

Both Mel10 and Ma18 were used previously in species identification protocols for pine marten (Domingo-Roura 2002; Pilot *et al.* 2007) but were found to be difficult to amplify in this study. Both primers were fluorescently labelled with green dyes (VIC or HEX) for allele sizing and while some sizing was possible with GeneMapper software, the electropherograms were difficult to interpret. Alleles scored for Ma18 for five individuals from Co. Waterford were all 148-bp. For Mel10, alleles of 153, 155, 157 and 161-bp were identified, but the allele patterns were difficult to interpret and were not consistent among replicates. Both loci were discontinued from further use, but may be useful in future studies along with the five loci above which were difficult to amplify, particularly if labelled with a stronger fluorescent dye such as 6-FAM. The optimisation of the amplification conditions for these microsatellites was not pursued further as the emphasis in this project was on the application of microsatellite analysis to non-invasively collected samples, which can be difficult to analyse, therefore the selection of loci was in favour of those which amplified consistently over a range of PCR conditions.

Twenty-five loci consistently amplified a PCR product of expected size (Table 10). Four loci (Mvis022, Mvi4031, MLUT04 and MLUT20) amplified a single product but were not labelled in favour of shorter microsatellites or other loci from the same publication (MLUT27, Cabria *et al.* 2007). The remaining loci were labelled for analysis of polymorphism. No PCR product was obtained for Mer022 after fluorescent labelling.

Twenty loci from the 32 screened in the method development were finally used to evaluate genetic diversity in the Irish pine marten population.

Table 10: Summary of microsatellite screening results. Refer to Table 8 for the individual primer sequences and reference publication.

Result	n	Primers
Single PCR product of expected size	25	Ma1, Ma2, Ma5, Ma8, Mel1, Mel6, Mel105, Gg7, Ggu234, Ggu454, 04OT14, Lut604, Lut615, Mer041, Mer022, Mvi1341, Mvi1354, Mvis020, Mvis022, Mvis072, Mvis075, Mvi4031, MLUT04, MLUT20, MLUT27
Inconsistent amplification	7	Ma18, Mel10, Mvi1342, Mvi2243, Mvi39, Mvi57, RIO17

4.3.2. Genetic diversity

The microsatellite screening revealed a low level of genetic diversity in the Irish pine marten population. Fourteen of the 20 microsatellite loci fully screened in the Irish population were variable with a maximum of three alleles each (Table 11), giving a total of 71 identified alleles in a sample of 41 individuals. However, all individuals had different multilocus genotypes. The genetic impoverishment of the Irish population was in accordance with previous studies with different microsatellites. All microsatellites amplified and 17 were variable for the samples from France (n = 7), the United Kingdom (n = 2) and the Czech Republic (n = 2). Genetic variability was higher in the European sample set (Table 12) despite the smaller sample size, which further emphasized the reduced genetic variability of the Irish population. There was a clear differentiation in allele frequencies between the Irish and French sample sets, with many private alleles (Figure 24), however the results should be interpreted with caution due small sample sizes. Further sampling in future may reveal the missing alleles. Nevertheless, 17 polymorphic loci were identified from cross-species amplification of microsatellite loci from related mustelid species in pine marten. Ten of the identified polymorphic loci were not previously characterized in the Irish population (Kyle *et al.* 2003; Statham 2005; Lynch 2006).

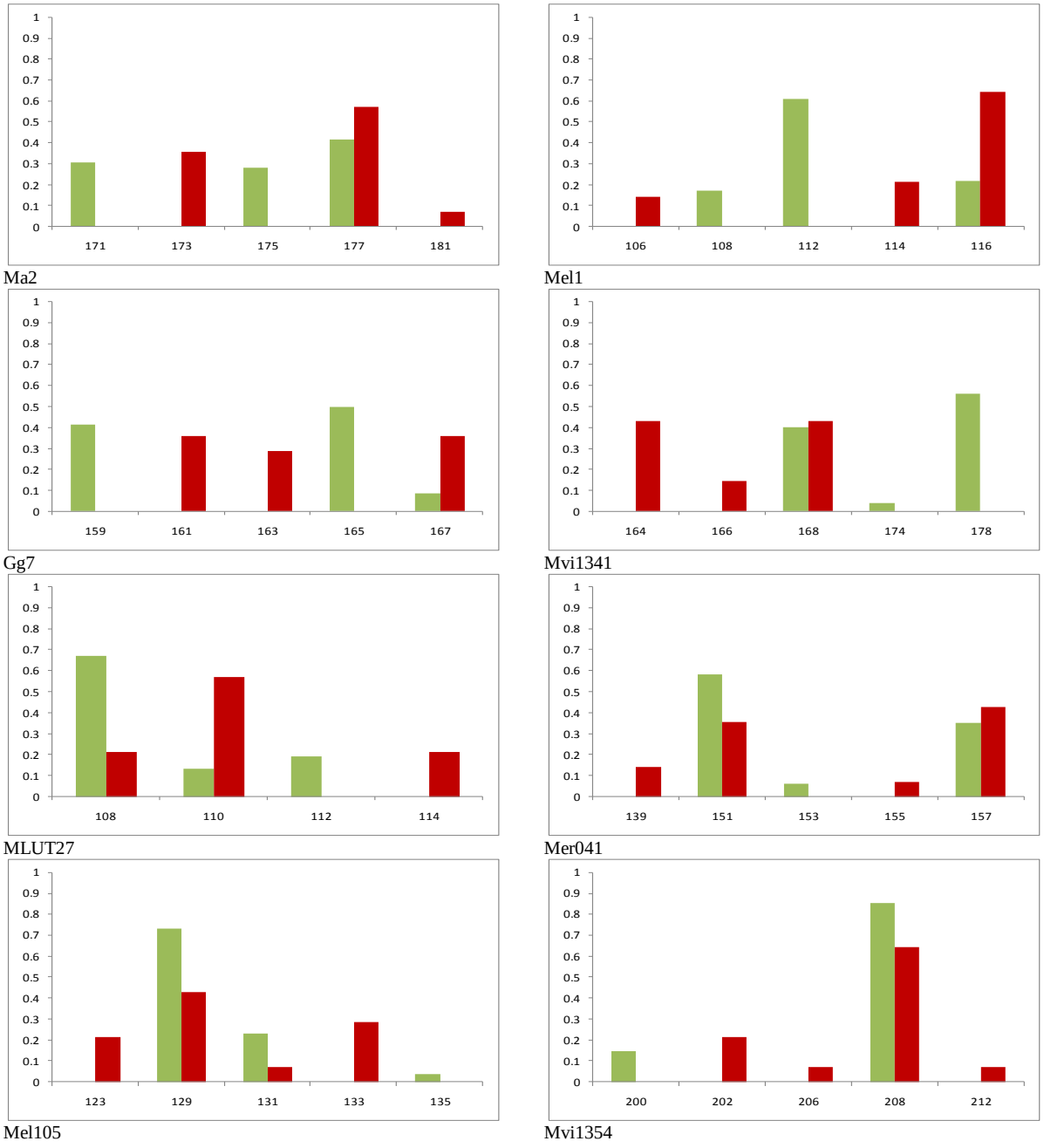
Sample	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354	Mvis075	Mvis020	Ma8	Ggu234	Mvis072	Ggu454	Ma1	Lut615	Lut604
IRL01	177/177	116/116	159/159	178/178	108/110	151/157	129/131	208/208	151/153	185/185	109/109	084/084	265/265	127/131	201/201	230/230	111/111
IRL02	171/175	112/116	165/167	178/178	110/112	151/157	129/129	208/208	151/151	185/185	109/109	084/084	263/265	127/127	201/201	230/230	111/111
IRL03	171/171	112/116	165/165	168/178	108/108	151/151	129/131	200/208	151/153	185/185	109/111	084/084	265/265	127/127	201/201	230/230	111/111
IRL04	171/177	112/112	165/165	168/174	110/110	151/157	129/131	208/208	151/151	185/187	109/109	084/090	265/265	127/131	201/201	230/230	111/111
IRL06	171/171	108/112	165/167	168/178	108/108	151/157	129/131	208/208	151/153	185/185	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL07	171/177	108/112	159/165	178/178	108/108	151/153	129/131	208/208	153/153	175/185		084/084	265/265	127/127	201/201	230/230	111/111
IRL08	171/171	112/112	159/165	178/178	110/112	151/151	129/135	208/208	151/151	185/185	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL09	175/177	116/116	159/165	168/178	108/108	151/151	129/129	208/208	151/153	175/175	109/109	084/090	263/265	127/127	201/201	230/230	111/111
IRL11	175/177	108/112	159/165	168/168	110/112	151/151	129/129	200/208	151/151	187/187	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL13	175/177	108/112	165/165	168/178	108/108	151/151	129/129	200/208	153/153	185/185	109/109	084/084	263/265	127/127	201/201	230/230	111/111
IRL14	171/175	112/112	159/165	168/178	108/112	151/151	129/131	200/208	151/151	175/185	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL15	175/177	108/112	165/167	168/168	108/112	157/157	131/131	200/208	151/151	185/185	109/111	084/084	265/265	127/131	201/201	230/230	111/111
IRL16	175/177	108/112	159/165	168/168	108/108	151/157	129/131	208/208	151/153	185/185	109/109	084/084	263/265	127/127		230/230	111/111
IRL17	171/177	112/116	159/159	168/178	108/110	151/157	129/131	208/208	151/153	185/185	109/109	084/090	265/265	127/127	201/201	230/230	111/111
IRL18	175/175	108/112	159/167	168/178	108/108	151/151	129/129	208/208	151/153	187/187	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL19	175/177	112/112	159/165	168/178	108/112	151/157	129/129	200/208	151/151	185/185	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL27	171/177	112/116	165/165	178/178	108/108	151/153	129/131	208/208	151/153	185/185	109/109	084/084	265/265	127/127	201/201	230/230	111/111
IRL28	171/177	108/108	165/165	168/174	108/112	151/153	129/131	200/200	151/153	185/185	111/111	084/084	265/265	127/131	201/201	230/230	111/111
IRL29	177/177	112/116	159/167	168/168	108/110	151/157	129/129	200/208	151/153	185/185	109/109	084/084	263/265	127/131	201/201	230/230	111/111
IRL30	175/177	112/112	159/165	168/178	108/110	153/157	129/135	208/208	151/153	185/185	109/109	084/084	263/265	127/131	-	-	-
IRL31	171/177	112/116	159/165	168/178	108/110	157/157	129/129	208/208	151/151	185/185	109/111	084/090	265/265	127/127	-	-	-
IRL32	175/177	116/116	165/167	168/178	108/108	151/157	129/129	200/208	153/153	187/187	109/109	084/090	265/265	127/127	-	-	-
IRL33	171/175	112/112	165/165	178/178	108/112	151/151	129/129	208/208	151/153	187/187	109/111	084/084	265/265	127/127	-	-	-
IRL34	171/177	108/112	159/165	178/178	108/108	151/151	129/129	208/208	151/151	185/185	109/109	084/084	265/265	127/127	-	-	-
IRL35	175/177	108/112	165/165	178/178	108/108	157/157	129/129	208/208	151/153	185/187	109/111	084/084	265/265	127/127	-	-	-
IRL36	175/177	112/112	159/159	168/174	108/108	151/151	129/129	208/208	151/151	185/187	109/109	084/084	265/265	127/127	-	-	-
IRL37	175/177	108/112	159/159	178/178	108/108	157/157	129/131	200/208	151/151	185/187	109/109	084/084	263/265	127/127	-	-	-
IRL38	177/177	112/112	159/165	168/178	108/112	151/157	129/129	208/208	153/153	185/185	109/111	090/090	265/265	127/127	-	-	-
IRL39	177/177	112/116	165/165	168/178	108/108	151/151	129/131	208/208	151/153	175/175	109/109	084/084	265/265	127/127	-	-	-
IRL40	171/177	112/112	159/165	168/178	108/108	151/151	129/131	208/208	151/151	185/185	109/111	084/084	265/265	127/131	-	-	-
IRL41	175/175	108/108	159/165	168/168	108/112	151/157	129/129	200/208	151/153	185/185	109/109	084/090	265/265	127/127	-	-	-
IRL44	171/177	112/112	159/159	178/178	108/108	151/151	129/129	208/208	151/153	187/187	109/109	084/090	265/265	127/127	-	-	-
IRL49	175/177	112/116	165/165	168/168	108/108	153/157	129/129	208/208	151/153	187/187	109/111	084/084	265/265	127/127	-	-	-
IRL50	171/175	112/116	165/167	178/178	112/112	151/157	129/135	208/208	151/151	185/187	109/109	084/084	265/265	127/127	-	-	-
IRL51	171/177	112/116	159/165	178/178	110/112	151/157	129/131	208/208	151/153	185/185	109/109	084/090	265/265	127/131	-	-	-
IRL52	177/177	112/112	159/165	168/178	108/108	151/157	129/131	208/208	153/153	185/185	109/109	084/084	265/265	127/131	-	-	-
IRL53	171/175	112/116	159/165	168/168	108/112	151/157	129/129	208/208	151/151	185/187	109/111	084/090	265/265	127/127	-	-	-
IRL54	175/177	112/116	159/159	168/178	108/108	151/157	129/131	208/208	151/153	185/187	109/109	084/084	265/265	127/127	-	-	-
IRL55	171/175	112/112	159/159	178/178	108/112	151/151	129/129	208/208	153/153	185/185	109/109	084/084	263/265	127/127	-	-	-
IRL56	171/171	112/112	159/165	178/178	108/112	151/157	129/129	208/208	151/151	175/175	109/109	084/084	265/265	127/127	-	-	-
IRL58	171/177	112/112	159/165	178178	108/108	157/157	129/131	208/208	153/153	185/185	109/109	084/084	265/265	127/131	-	-	-
Alleles	3	3	3	3	3	3	3	2	2	3	2	2	2	2	1	1	1

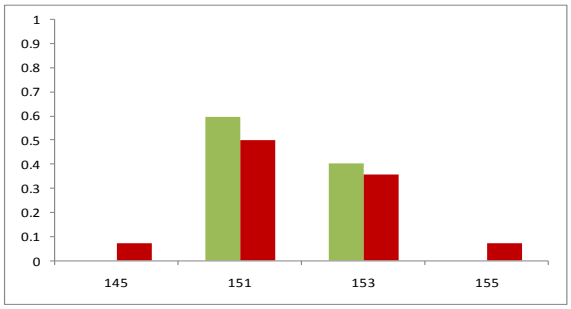
Table 11: Multilocus genotypes of Irish pine marten tissue samples**.** Sizes are alleles in base pairs. Gaps indicate genotypes which were not resolved and dashes indicate samples which were not genotyped at the loci Ma1, Lut615 or Lut604. The monomorphic loci Ma5, 04OT14 and Mel6 are not included.

Table 12: Microsatellite polymorphism for European pine marten samples. Values are allele sizes in base pairs. Gaps indicate genotypes which were not resolved. The monomorphic loci Ma5, 04OT14 and Mel6 are not included.

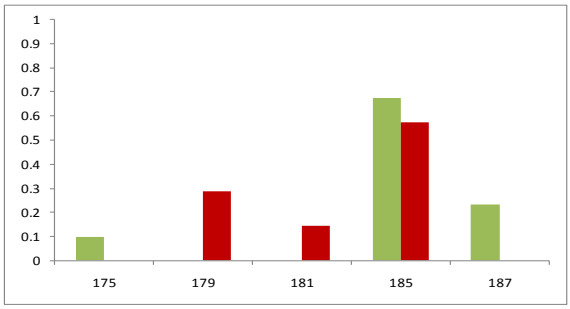
Sample	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354	Mvis075	Mvis20	Ma8	Ggu234	Mvis072	Ggu454	Ma1	Lut615	Lut604
FRANCE																	
FRA1 (hap c)	173/177	116/116	161/167	164/168	110/114	151/157	123/133	202/208	151/153	181/181	105/105	088/088	265/267	129/131	203/203	234/234	111/115
FRA2 (hap c)	173/177	114/116	163/167	164/168	110/114	151/157	129/129	202/208	151/151	179/179	105/109	084/088	265/265	127/131	203/203	230/234	111/113
FRA3 (hap c)	173/181	116/116	161/161	168/168	110/110	139/139	123/129	208/208	145/153	185/185	109/109	084/088	263/265	129/131	197/201	234/234	111/113
FRA4 (hap c)	177/177	116/116	163/163	164/166	108/110	151/155	123/133	208/208	151/151	179/185	105/109	088/088	265/265	127/127	203/203	232/232	113/113
FRA5 (hap c)	173/177	114/116	167/167	164/166	110/114	151/157	133/133	202/208	151/153	185/185	105/109	088/088	265/265	131/131	203/203	234/234	111/111
FRA6 (hap c)	177/177	106/114	163/167	168/168	108/110	157/157	129/129	208/208	151/155	185/185	105/109	084/088	263/271	131/131	203/203	234/234	111/115
FRA7 (hap c)	173/177	106/116	161/161	164/164	108/110	151/157	129/131	206/212	153/153	179/185	105/109	088/088	265/277	127/131	197/197	234/234	111/113
CAPTIVE MARTENS – WILDWOOD TRUST, UK																	
Poop (hap a)	175/177	112/116	161/169	162/164	108/108	151/151	127/127	204/204	147/147	179/179	105/109	084/084	265/265	127/131	199/199	230/234	111/111
Piddle (hap a)	177/177	110/110	169/169	162/164	108/108		127/129	204/204	151/155	179/179	105/105	084/084	265/265	131/131	199/199	230/234	111/111
Fudge (hap g)	177/179	110/110	161/163	164/166	108/110	151/151	127/131	204/206	145/151	183/183	105/109	084/084	265/265	131/131	199/199	230/234	111/111
Yorkie (hap g)	177/177	110/110	163/165	168/168	108/110	151/151	123/133	198/198	147/147	183/183	105/109	084/088	265/265	131/131	199/199		111/113
Alleles	5	5	5	4	3	4	5	6	5	4	2	2	5	3	4	3	3

Figure 24: Polymorphic microsatellite loci allele frequency distributions. Data for the Irish pine marten tissue samples are represented in green and the French samples in red. Values are allele sizes in base pairs.

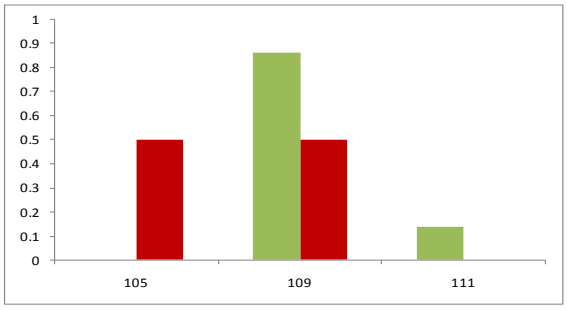




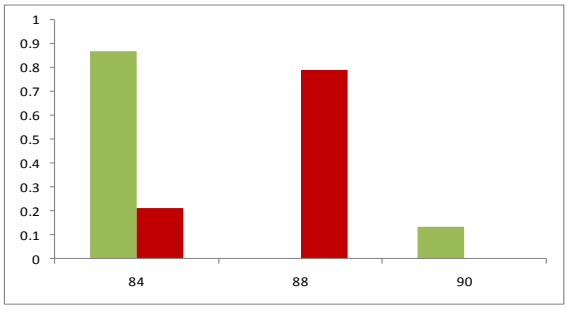
Mvis075



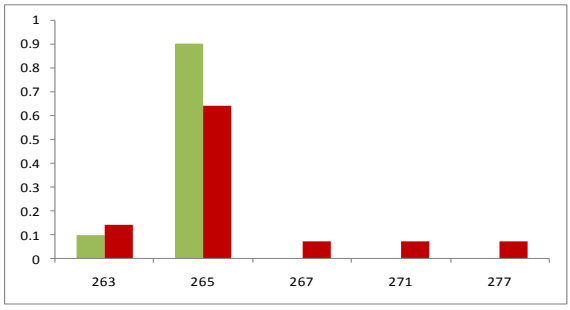
Mvis020



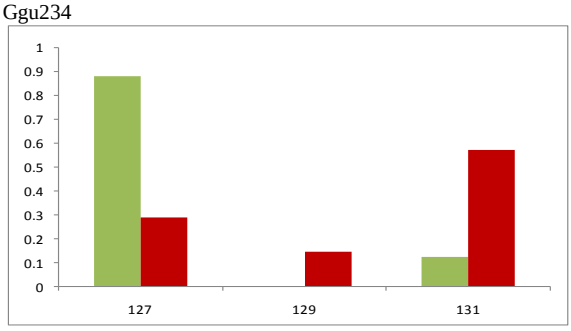
Ma8



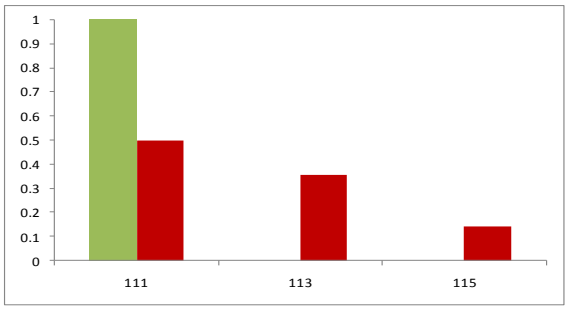
Ggu234



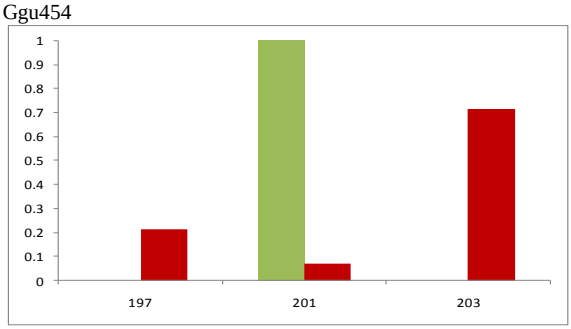
Mvis072



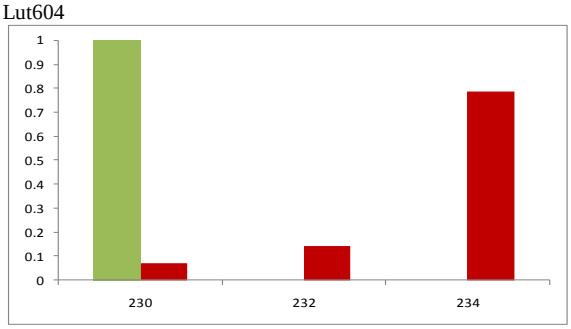
Ggu454



Lut604



Ma1



Lut615

The allelic richness and expected heterozygosity values per locus in the Irish population (AR = 2.12, H_E = 0.34) were significantly lower than the French population (A = 3.24, H_E = 0.60) across 17 polymorphic loci (Wilcoxon test, p <0.001) (Table 13). Average H_O and H_E values were similar for each population (Ireland H_O 0.33, H_E 0.35; France H_O 0.54, H_E 0.59), indicating excess homozygosity as a result of non-amplifying alleles was not a major problem in the dataset. The only locus which deviated from Hardy-Weinberg Equilibrium was Mvis020, which had a significant excess of homozygotes (p <0.001, Table 13) suggesting a null allele was present at this locus, at an estimated frequency of 0.23 (nulls, Table 13) according to the MICROCHECKER results (Van Oosterhout *et al.* 2004). This may also have been caused by scoring errors such as stuttering or large allele dropout or X-linkage, as no males were heterozygous at Mvis020 in this study. This locus was excluded from further analyses of genetic diversity and structure. There was no evidence of linkage disequilibrium (Table 14) therefore all 13 loci were retained for further analysis (Panel_C, Table 9).

Table 13: Descriptive statistics of polymorphic microsatellite loci *M. martes*.

		Ireland						France			
Locus	**Source species**	**A**	**AR**	**H_O**	**H_E**	**p**	**Null**	**A**	**H_O**	**H_E**	**p**
Ma2	American marten	3	2.99	0.73	0.67	0.56		3	0.71	0.58	0.63
Mel1	Eurasian badger	3	2.92	0.54	0.56	0.09		3	0.57	0.56	1.00
Gg7	wolverine	3	2.74	0.61	0.58	0.74		3	0.43	0.71	0.20
Mvi1341	American mink	3	2.43	0.46	0.53	0.12	0.05	3	0.57	0.66	0.69
Mer041	stoat	3	2.62	0.54	0.54	1.00	0.06	4	0.71	0.71	0.06
MLUT27	European mink	3	2.86	0.46	0.50	0.32		3	0.86	0.63	0.66
Mel105	Eurasian badger	3	2.42	0.49	0.42	0.41		4	0.57	0.74	0.27
Mvi1354	American mink	2	1.91	0.24	0.25	1.00	0.01	4	0.57	0.57	0.16
Mvis075	American mink	2	2.00	0.46	0.49	1.00	0.02	4	0.57	0.66	0.81
Mvis020	American mink	3	2.78	0.22	0.49	*0.00*	0.23	3	0.29	0.62	0.11
Ma8	American marten	2	1.90	0.23	0.24	0.55	0.02	2	0.71	0.54	0.51
Ggu234	wolverine	2	1.89	0.22	0.24	0.54	0.03	2	0.43	0.36	1.00
Mvis072	American mink	2	1.79	0.20	0.18	1.00		5	0.57	0.59	0.64
Ggu454	wolverine	2	1.86	0.24	0.22	1.00		3	0.57	0.62	0.70
Lut615	Eurasian otter	1	1.00	-	-	-	-	3	0.14	0.39	0.08
Lut604	Eurasian otter	1	1.00	-	-	-	-	3	0.71	0.65	0.72
Ma1	American marten	1	1.00	-	-	-	-	3	0.14	0.47	0.02
Overall		2.29	2.12	0.33	0.35	0.07		3.24	0.54	0.59	0.25

Table 14: Linkage disequilibrium test. Overall probability values between pairs of polymorphic loci for the linkage disequilibrium test. No pairwise combinations were significant after Bonferroni correction (adjusted nominal level of 0.0002).

Locus	**Ma2**	**Mel1**	**Gg7**	**Mvi1341**	**MLUT27**	**Mer041**	**Mel105**	**Mvi1354**	**Mvis075**	**Mvis020**	**Ma8**	**Ggu234**	**Mvis072**	**Ggu454**	**Ma1**	**Lut615**	**Lut604**
Ma2	-																
Mel1	0.605	-															
Gg7	0.979	0.955	-														
Mvi1341	0.603	0.427	0.905	-													
MLUT27	0.151	0.396	0.760	0.547	-												
Mer041	0.070	0.816	0.955	0.814	0.488	-											
Mel105	0.654	0.692	0.303	0.860	0.315	0.477	-										
Mvi1354	0.119	0.105	0.981	0.032	0.279	0.278	0.567	-									
Mvis075	0.600	0.324	0.621	0.899	0.327	0.709	0.911	0.711	-								
Mvis020	0.822	0.999	0.962	0.781	0.835	0.752	0.515	0.950	0.737	-							
Ma8	0.681	0.613	0.420	0.427	0.432	0.205	0.398	0.157	0.980	0.848	-						
Ggu234	0.368	0.334	0.898	0.752	0.876	0.613	0.358	1.000	0.667	0.808	0.861	-					
Mvis072	0.259	0.803	0.989	0.714	0.929	0.889	0.981	0.869	0.504	0.833	0.154	0.957	-				
Ggu454	0.149	0.674	0.999	0.848	0.649	0.730	0.056	0.671	0.995	0.178	0.402	1.000	1.000	-			
Ma1	0.337	0.573	0.806	0.418	0.295	0.709	0.154	0.280	0.153	1.000	0.529	1.000	0.274	1.000	-		
Lut615	0.722	1.000	0.809	1.000	1.000	0.721	1.000	1.000	0.808	0.295	1.000	1.000	1.000	0.424	1.000	-	
Lut604	0.884	1.000	0.338	1.000	1.000	0.831	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.491	1.000	0.586	-

4.3.3. Bottleneck analysis

Statistical test for heterozygosity excess

The heterozygosity excess test carried out in BOTTLENECK found evidence for deviation from mutation-drift equilibrium in the Irish population for all models of microsatellite mutation (Table 15). The exact mutational model of each microsatellite locus was not known, but was expected to lie somewhere between the two extremes (SMM and IAM) for all loci. H_E was greater than H_{EQ} for 10 of the 13 loci under the TPM with 90% step-wise mutations, leading to an overall significant bottleneck test ($p = 0.013$), which was close to significant even after correction for multiple tests (corrected α of 0.0038). This is indicative of a population which has recently lost allelic diversity due to a reduction in effective population size.

Table 15: Bottleneck analysis. The significance of the difference between the expected heterozygosity calculated from allele frequencies (H_E) and the heterozygosity expected in a population of stable effective size (H_{EQ}), assessed under the three models of microsatellite mutation.

Locus	A	He	Heq-TPM	Heq-IAM	Heq-SMM
Ma2	3	0.665	0.437	0.335	0.465
Mel1	3	0.558	0.436	0.334	0.462
Gg7	3	0.578	0.437	0.336	0.464
Mvi1341	3	0.528	0.439	0.339	0.465
MLUT27	3	0.500	0.438	0.334	0.465
Mer041	3	0.535	0.437	0.335	0.461
Mel105	3	0.415	0.439	0.340	0.463
Mvi1354	2	0.253	0.230	0.196	0.237
Mvis075	2	0.487	0.229	0.194	0.235
Ma8	2	0.240	0.232	0.193	0.238
Ggu234	2	0.235	0.231	0.195	0.235
Mvis072	2	0.178	0.231	0.196	0.236
Ggu454	2	0.217	0.228	0.196	0.237
			p = 0.013	**p = <0.001**	**p = 0.024**

Qualitative analysis of allele frequency distribution

There was also evidence of a population bottleneck with the qualitative analysis of the distribution of allele frequencies in the population (Figure 25). The shift from an L-shaped distribution curve (Figure 25, *inset*) to one with an abundance of alleles in the intermediate allele frequency classes (0.1 to 0.9, Figure 25) suggests the population has undergone a severe reduction in effective population size, that is the number of breeders was reduced significantly in the recent history of the population, within the past dozen or so generations (Luikart *et al.* 1998).

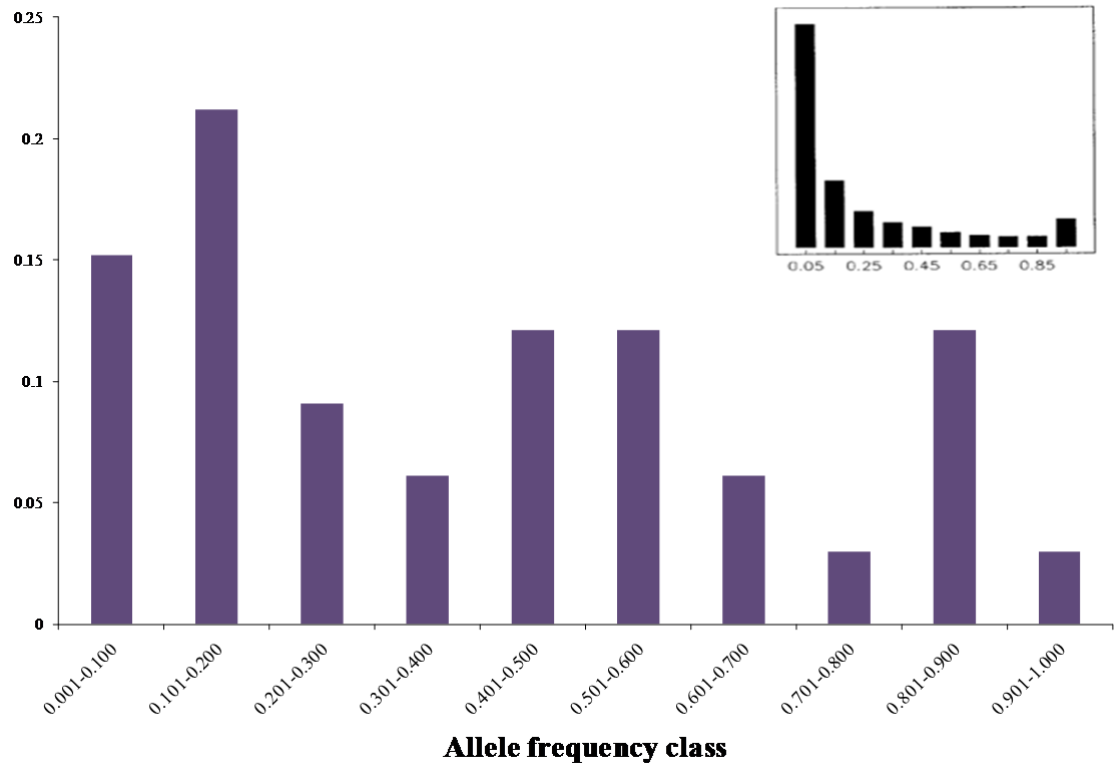


Figure 25: Allele frequency distribution in the Irish population. The shift from the typical L-shaped distribution curve (*inset*, Luikart *et al.* 1998 Fig. 1a) is indicative of a recent loss of rare alleles as a result of a population bottleneck.

4.3.4. Genetic structure

Power analysis

The POWSIM analysis indicated the 13 microsatellites used for the analysis of genetic structure had a statistical power of at least 0.98 (chi-square test 0.988, Fisher's test 0.986) to detect biologically relevant genetic differentiation (F_{ST} = 0.05) between populations (Nims *et al.* 2008). The following analyses were therefore carried out under the assumption that the 13 microsatellites used in this study were, despite low polymorphism, sufficient to provide at least a 98% probability of detecting genetic differentiation in the dataset.

Analysis of population structure

There was no significant difference in allele frequencies between the three regions Mid-North, West and South regions according to the Fisher's exact test (p = 0.419) or chi-square test (p = 0.299) implemented in the program CHIFISH.

The examination of average Ln P(D|*K*) for each value of *K* in the STRUCTURE analysis suggested the presence of two genetic clusters in the dataset (Figure 26). However, variability of likelihood values increased at higher values of *K*, as reported for other studies (Evanno *et al.* 2005; Bergl *et al.* 2007). The Evanno Δ*K* method was therefore used to identify the most likely *K* value between *K* = 2, 3, 4 and 5. The calculation of Δ*K* from the STRUCTURE output confirmed the results of the Ln P(D|*K*) analysis, with the highest value of Δ*K* at *K*=2 (Figure 26).

All individuals from the three predefined regions in Ireland (region 1, mid-north; region 2, west and region 3, south) were assigned unambiguously to the one of the clusters (q > 0.99), while all French individuals were assigned to a separate cluster (q = 0.997, region 4), as shown in Figure 27.

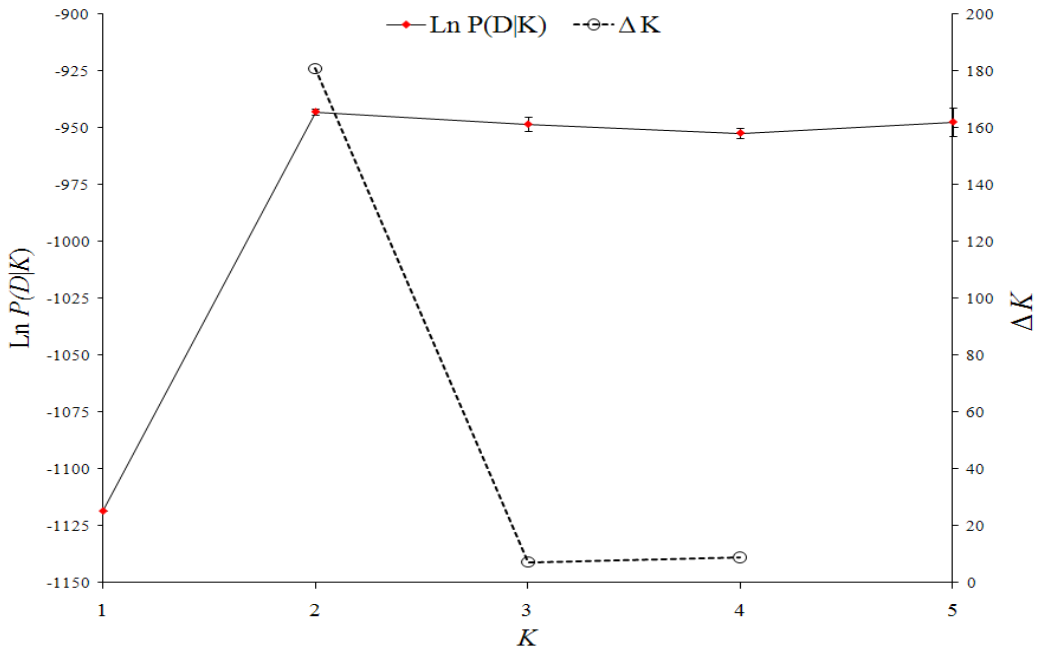


Figure 26: Number of inferred genetic clusters in STRUCTURE. The number of genetic clusters (*K*) in the dataset was inferred using both the likelihood (Ln P(D|*K*) and delta *K* Δ*K* methods.

K = 2

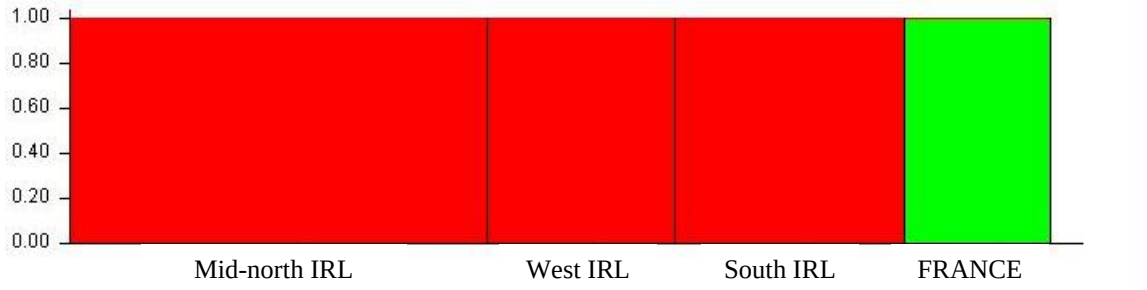


Figure 27: Individual posterior probabilities of Bayesian assignment to each of two inferred clusters (different colours) analysed by STRUCTURE.

Only one genetic cluster was identified when the Irish samples were analysed separately, with symmetric assignment of individuals to each cluster at *K* values greater than one (e.g. *K* = 2 or 3, Figure 28).

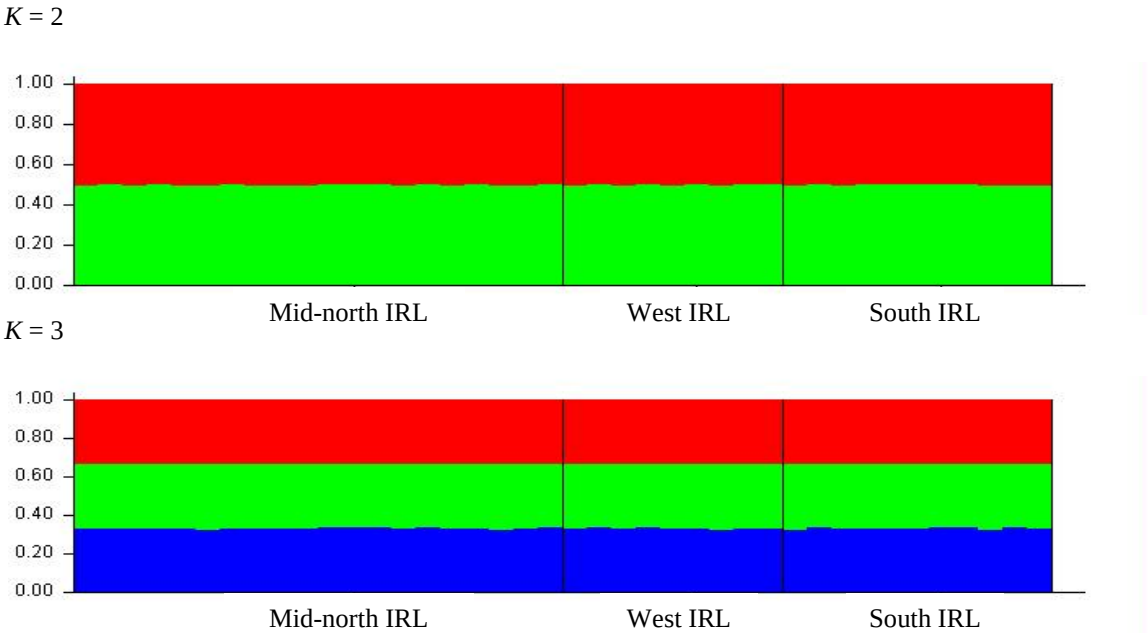
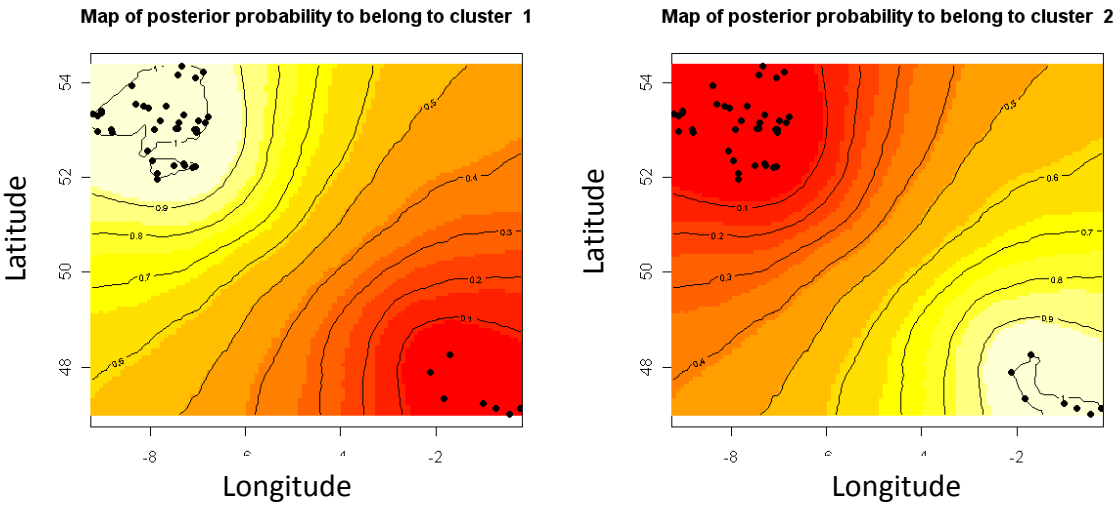


Figure 28: Individual posterior probabilities of Bayesian assignment to each of two or three inferred clusters (different colours) analysed by STRUCTURE.

The GENELAND analysis agreed with the STRUCTURE results, indicating that the most likely number of genetic clusters in the dataset was two (K = 2; Figure 29a and Figure 29b), representing the differentiation between Ireland and France. There was no further structuring within Ireland when analysed separately with all individuals assigned to the same genetic cluster based on microsatellite allele frequencies for 13 loci (Figure 29c).

(a) Cluster 1 (Irish samples)

(b) Cluster 2 (French samples)



(c) Analysis with Ireland only

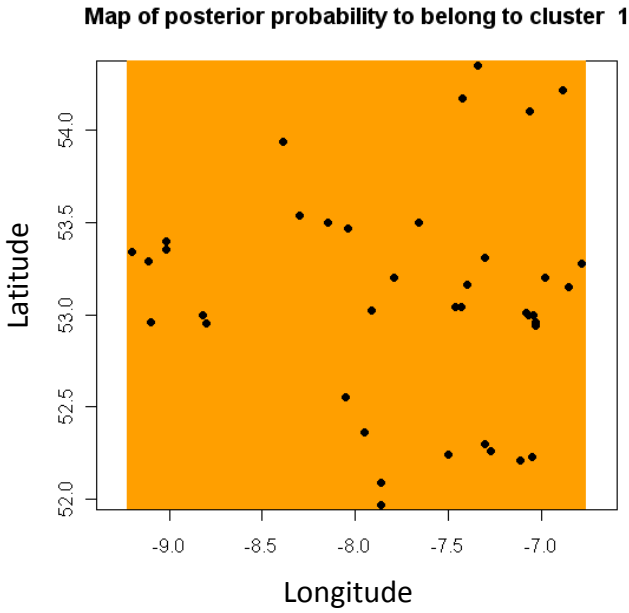


Figure 29: The number of genetic clusters identified with GENELAND. Black dots represent georeferenced multilocus genotypes with latitude and longitude coordinates. The colours represent the probability of a sample genotype belonging to the cluster, ranging from high (>0.9; light yellow) to low (<0.1; red) in increments of 0.1.

4.3.5. Individual-level analyses

Probability of identity

The probability of identity (PI) statistics based on calculations performed in GIMLET software allowed the selection of the most informative loci from those available. The PI*ave* and PI*sibs* varied per locus from 0.18 to 0.68 for PI*ave* and 0.47 to 0.84 for PI*sibs* (Table 16). Some loci were therefore more informative for individual discrimination whereas others contributed little to the overall power of the marker panel due to low polymorphism in the population.

Table 16: Probability of identity values for each polymorphic locus in the Irish population. Loci were ranked in order of increasing per locus PI*ave* values.

Locus	**PI*ave***	**Cumulative PI*ave***	**PI*sibs***	**Cumulative PIsibs**
Ma2	0.18	1.8×10^{-1}	0.47	4.7×10^{-1}
Mel1	0.24	4.3×10^{-2}	0.54	2.5×10^{-1}
Gg7	0.27	1.1×10^{-2}	0.53	1.4×10^{-1}
Mer041	0.29	3.3×10^{-3}	0.56	7.5×10^{-2}
MLUT27	0.29	9.8×10^{-4}	0.58	4.4×10^{-2}
Mvi1341	0.32	3.1×10^{-4}	0.57	2.5×10^{-2}
Mvis075	0.38	1.2×10^{-4}	0.61	1.5×10^{-2}
Mel105	0.39	4.7×10^{-5}	0.65	9.8×10^{-3}
Mvi1354	0.58	2.7×10^{-5}	0.77	7.6×10^{-3}
Ma8	0.59	1.6×10^{-5}	0.78	3.4×10^{-3}
Ggu234	0.60	9.6×10^{-6}	0.79	2.7×10^{-3}
Ggu454	0.62	5.9×10^{-6}	0.80	2.1×10^{-3}
Mvis072	0.68	4.0×10^{-6}	0.84	1.8×10^{-3}

For the Irish samples, based on the cumulative product of PI*sibs* and PI*ave* values, a multilocus genetic tag of four (PI*ave*: 3.3×10^{-3}) to eight loci (PI*sibs*: 9.8×10^{-3}) was required to achieve a low probability of identity of less than 0.01 (Figure 30). An extra locus (Mvi1354) was included in the final panel in case one of the other loci (Ma2,

Mel1, Gg7, Mvi1341, Mer041, MLUT27, Mvis075 or Mel105) did not amplify. Nine microsatellite loci were therefore used in the final panel with a PI*sibs* of 7.6 x 10^{-3}, meaning less than 1 in 100 individuals should share multilocus genotypes with the marker panel, even when a sample set includes closely related individuals.

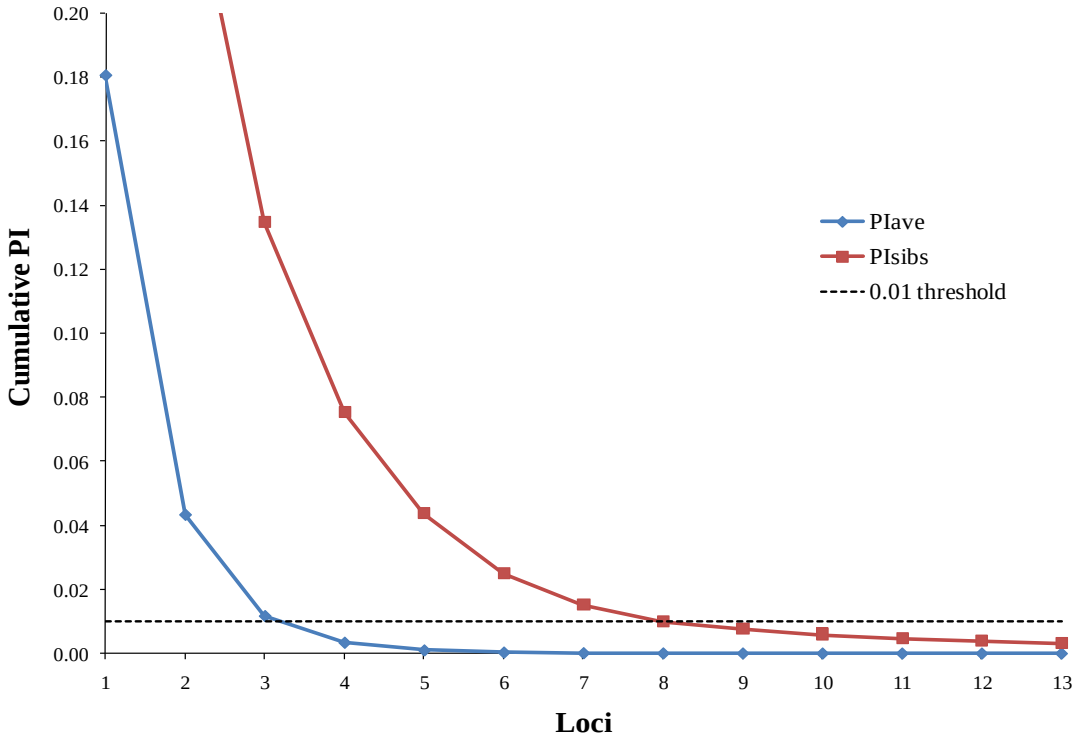


Figure 30: PI*ave* and PI*sibs* probability of identity curve for Irish pine marten. Between four and eight loci were required for individual identification (PI<0.01), depending on the likelihood of sampling related individuals.

Parentage analysis

Parentage simulations suggested that identifying the true parent from among two sampled candidates with eight loci was possible 76% of the time at the relaxed 80% confidence level, but the assignment rate declined to 23% at the strict 95% level (Table 17). The number of parentage assignments fell dramatically (1% at the 95% level) when considering 10 candidate parents which could be sampled in the population and failed completely when not all the candidate parents were actually sampled (<1% at the 95% level), which may be more likely scenarios for sampling of pine marten populations. The assignment rate did not improve significantly with the use of 16 loci (allele frequencies were replicated for the original eight loci). Therefore the microsatellites used in this study are not suitable for parentage inference in Irish pine marten.

Table 17: Assignment rates for parentage tests resolved with 80% and 95% confidence using the program CERVUS.

Number of loci	Candidate parents	Candidates sampled	Neither parent known	
			80%	95%
8	2	100%	76%	23%
8	10	100%	7%	1%
8	10	75%	3%	<1%
16	10	75%	14%	6%

4.3.6. Microsatellite analysis optimisation

miniSTR design

DNA sequences were obtained for the Mel1 microsatellite from two pine marten which were homozygous with the original primer set (with allele sizes of 270 and 274-bp). A new reverse primer (Mel1miniR: 5'-TGC TCT TAT AAA TCT GAA AAT TAG GAA TTC-3') was designed which amplified a shorter product of approximately 110-bp. Sequence analysis confirmed the repeat structure of $(GT)_n$ as shown in Figure 31. The new reverse primer sequence was conserved in the flanking sequence of both individuals IRL01 and IRL08. Tissue samples which had been genotyped with the original Mel1 primers (n = 26) were repeated with the original primer Mel1F and the new primer Mel1miniR. The genotypes obtained with both primer sets were in full agreement (examples in Table 17), therefore the shorter Mel1 primer set was used for the remainder of the project.

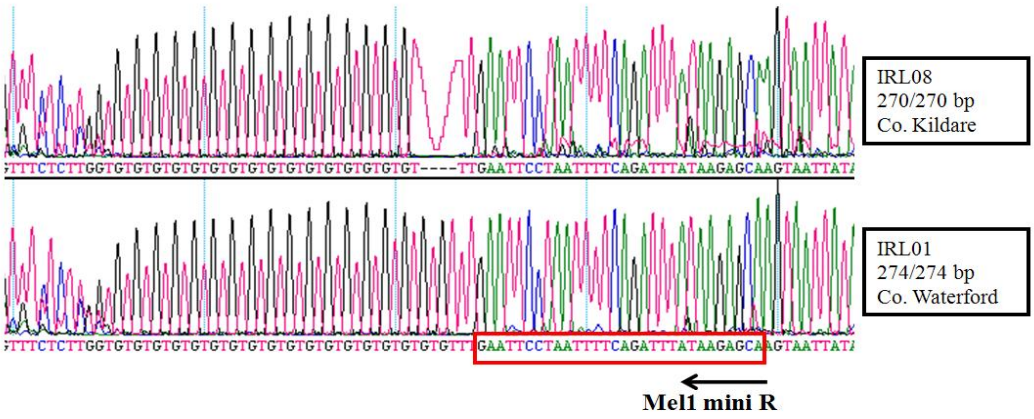


Figure 31: Mel1 microsatellite primer redesign. The microsatellite motif $(GT)_n$ differs by 2 repeat units between individuals, as expected given the size of the PCR product which was sequenced. The binding site of the new primer is boxed in red.

Table 18: Concordance of selected Mel1 genotypes obtained with the original and miniSTR microsatellite primers. Values are the size in base pairs of the PCR product.

Sample	Origin	Mel1		Mel1 mini	
IRL01	Ireland	274	274	116	116
IRL02	Ireland	270	274	112	116
IRL04	Ireland	270	270	112	112
IRL06	Ireland	266	270	108	112
FRA21	France	272	274	114	116
FRA25	France	264	272	106	114
FRA26	France	264	274	106	116

Multiplex PCR

The 13 microsatellite loci in Panel_C (Table 9) were successfully combined into three panels for multiplex amplification (Figure 32). These panels were used to obtain multilocus genotypes for tissue and hair samples. All loci were successfully amplified for 13 tissue samples (IRL40-IRL58, Table 11). The amplification success rate for 13 hair samples was comparably high (96.15%) with only seven failed reactions across 182 sample/locus combinations (Table 19). The genotype previously obtained for the hair samples from single locus reactions (Table 22) matched the genotype obtained using multiplex PCR in 79.7% of the pairwise comparisons (Table 19). Genotype inconsistencies can be attributed to sample quality as these hair samples were found to be difficult to amplify using standard single locus PCR (Chapter 5). Multiplex PCR is therefore applicable to both high quality and low quality DNA samples using the Qiagen Type-It Multiplex mastermix.

(a)

Multiplex 1	Multiplex 2	Multiplex 3
Mel1mini (110-bp)	MLUT27 (110-bp)	Ggu234 (90-bp)
Gg7 (160-bp)	Mel105 (130-bp)	Ma8 (110-bp)
Mer041 (160-bp)	Mvis075 (150-bp)	Mvis072 (260-bp)
Mvi1341 (170-bp)	Ma2 (170-bp)	Ggu454 (130-bp)
Mvi1354 (200-bp)		

(b)

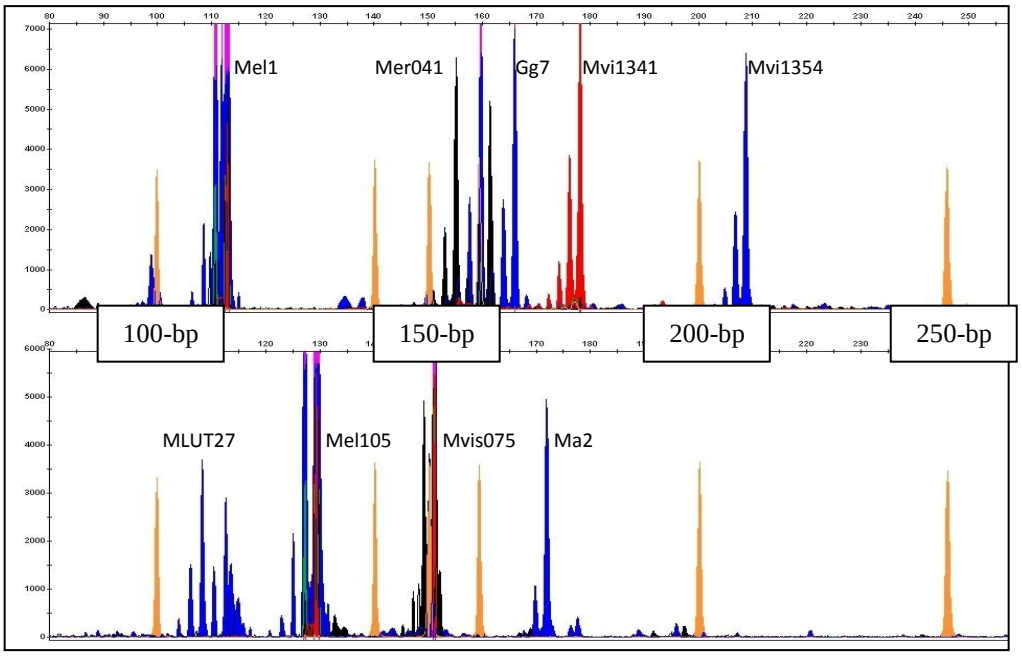


Figure 32: Panels for multiplex amplification of microsatellite loci (a) and electropherograms of the 'Multiplex 1' (top) and 'Multiplex 2' (bottom) panels (b) for IRL56 tissue DNA.

Table 19: Genotypes of hair samples amplified in single locus and multiplex reactions. A subset of hair samples collected in 2006-2007 were amplified in single locus reactions (S1 and S2) or in multiplexes (M1 and M2). Amplification failures are indicated (0) and genotype inconsistencies are highlighted in bold font.

		Multiplex 1					Multiplex 2				Multiplex 3				
Extract ID	**PCR**	**Mel1**	**Gg7**	**Mer041**	**Mvi1341**	**Mvi1354**	**MLUT27**	**Mel105**	**Mvis075**	**Ma2**	**Mvis072**	**Mvis020**	**Ggu234**	**Gg454**	**Ma8**
PWH28	M1	112/112	159/159	151/157	174/178	208/208	**0**	129/129	151/151	**0**	265/265	185/185	84/84	127/127	109/109
	S1	112/112	159/159	151/157	174/178	208/208	**108/108**	129/129	151/151	**171/175**					
PWH36	M1	108/116	165/165	151/151	178/178	208/208	**108/108**	129/129	151/151	**175/177**	265/265	185/185	84/84	127/127	109/109
	S1	108/116	165/165	151/151	178/178	208/208	**106/108**	129/129	151/151	**171/171**					
PWH48	M1	108/112	**159/159**	**151/151**	**178/178**	**208/208**	**108/108**	**131/131**	**151/151**	**171/177**	0	185/185	84/90	127/127	111/111
	S1	108/112	**165/165**	**151/157**	**168/174**	**206/208**	**104/106**	**131/133**	**0**	**169/171**					
PWH62	M1	108/112	159/165	151/153	174/178	208/208	108/108	**129/131**	151/151	171/171	265/265	185/185	84/84	0	0
	S1	108/112	159/165	151/153	174/178	208/208	108/108	**129/129**	151/151	171/171					
PWH66	M1	108/112	165/165	151/157	168/178	200/208	110/110	129/129	151/153	177/177	263/265	185/187	84/84	127/127	109/109
	S1	108/112	165/165	151/157	168/178	200/208	110/110	129/129	151/153	177/177					
PWH70	M1	112/112	159/159	**151/157**	178/178	208/208	**108/112**	129/131	151/151	171/177	265/265	185/185	84/90	127/131	109/109
	S1	112/112	159/159	**0**	178/178	208/208	**104/108**	129/131	151/151	171/177					
PWH71	M1	112/112	**159/159**	**151/157**	178/178	208/208	108/108	**131/131**	151/151	**171/171**	265/265	185/185	84/90	127/127	109/109
	S1	112/112	**0**	**0**	178/178	208/208	108/108	**129/133**	151/151	**0**					
PWH73	M1	112/112	159/165	157/157	178/178	208/208	108/108	129/129	151/151	171/177	265/265	185/185	84/84	127/127	109/109
	M2	112/112	**159/165**	157/157	178/178	208/208	108/108	129/129	151/151	**171/177**	265/265	185/185	84/84	127/127	109/109
	S1	112/112	159/165	157/157	178/178	208/208	108/108	129/129	151/151	171/177					
	S2	112/112	**159/159**	157/157	178/178	208/208	108/108	129/129	151/151	**0**					
PWH74	M1	112/112	**159/159**	151/157	178/178	208/208	108/108	**129/131**	151/151	171/171	265/265	185/185	84/90	127/127	109/109
	M2	112/112	159/159	151/157	178/178	208/208	108/108	129/131	151/151	**171/177**	265/265	185/185	84/90	127/127	109/109
	S1	112/112	**159/165**	151/157	178/178	208/208	108/108	**129/133**	151/151	171/171					
	S2	112/112	159/159	151/157	178/178	208/208	108/108	129/131	151/151	**0**					
PWH76	M1	108/112	165/165	**151/157**	168/178	200/208	110/110	129/129	**151/153**	**177/177**	263/263	185/185	84/84	127/127	109/109
	S1	108/112	165/165	**151/155**	168/178	200/208	110/110	129/129	**151/151**	**165/171**					
PWH92	M1	108/112	159/165	**151/153**	178/178	208/208	108/108	129/131	**151/153**	171/177	265/265	185/185	0	127/127	0
	S1	108/112	159/165	**0**	178/178	208/208	108/108	129/131	**151/151**	171/177					
PWH98	M1	112/112	**159/165**	**157/157**	174/178	**200/208**	**108/112**	**129/129**	151/151	**175/177**	265/265	185/185	84/84	127/127	109/109
	S1	112/112	**159/159**	**0**	174/178	**208/208**	**108/108**	**131/133**	151/151	**175/175**					
PWH115	M1	108/112	165/165	**151/157**	168/168	200/208	110/110	**129/131**	151/153	171/177	265/265	185/185	84/84	127/127	109/109
	S1	108/112	165/165	**0**	168/168	200/208	110/110	**131/133**	151/153	171/177					

Number of replicates required for microsatellite genotyping of tissue samples

Consensus genotypes obtained after repeat amplifications for each sample and locus (for samples IRL01-39 and FRA01-07) were compared to each analysed replicate (n = 1705) to identify tissue genotyping errors. Fifteen errors were detected across positive reactions, giving a per reaction error rate of 0.88%. Six of these errors were due to false alleles and nine were due to the dropout of one allele at a heterozygous locus. The allele 139 at locus Mer041 was only detected once in the dataset for individual FR03. The sample was repeated six times with the same results therefore the genotype was taken as correct. Soulsbury *et al.* (2007) found that tissue genotyping errors can occur at specific microsatellite loci and specific combinations of alleles. Ten genotyping errors occurred at 3 loci, Mer041 and Mvis075 which had allelic patterns which were difficult to score due to excess stutter bands and Mvis072 which was a relatively large microsatellite greater than 260-bp. Two allele dropouts occurred at a single locus (Mvi1341) in sample IRL13 and the other error was an instance of an 84/90 genotype in one of five replicates (84/84), which may have been due to sample mix up as no other errors were detected for that sample. Of the 657 genotypes obtained on the first PCR replicate, 649 (98.9%) matched the consensus genotype obtained by repetition. The consensus genotypes were considered to be accurate and tissue samples were genotyped once per locus for the remaining samples IRL40-IRL58 due to the low observed rate of genotyping error.

4.4. Discussion

Cross-species amplification of mustelid microsatellites

The successful cross-species transfer of mustelid microsatellites, in particular those isolated from badger, stoat and mink suggests that more polymorphic loci may be identified with future screening attempts. The microsatellites which have recently been developed for congeneric fisher *Martes pennanti* (Jordan *et al.* 2007) and stone marten *Martes foina* (Basto *et al.* 2010) may also be variable for pine marten. In addition, a new panel of microsatellites has been developed specifically for *Martes martes* after the completion of this project (Natali *et al.* 2010). These loci should certainly be tested for polymorphism in the future as specific loci can be easier to amplify and score than loci transferred from other species (Boston 2007). However, specifically isolated microsatellites may not unveil much more genetic diversity in populations which have undergone severe demographic bottlenecks (Walker *et al.* 2001; Larson *et al.* 2002; Schultz *et al.* 2009).

There was a low microsatellite genotyping error rate (0.88%) for tissue samples, with errors mainly attributed to three loci (Mer041, Mvis075 and Mvis072). These loci were more easily amplified and genotyped with the Qiagen Type-It Microsatellite Multiplex PCR Kit. An additional advantage of multiplexing microsatellite loci is the time and cost saved by combining several loci in a single PCR. Multiplex PCR was trialled at the end of the project, but the preliminary results show high success rates for both tissue (100%) and hair (96%) and suggest multiplex PCR may be successfully adopted for all genotyping in future with further optimisation, particularly for scat samples which are difficult to genotype (Chapter 5). Some of the microsatellites described here may need to be relabelled such as Mel105 or Mel1 to prevent spectral overlap, and other loci may be added to the multiplexes to replace less variable loci such as Mvi1354. Different multiplexes may also be combined after amplification on the capillary sequencer.

A new primer was designed for Mel1 based on sequence data for the flanking region obtained with the original primers for Eurasian badger (Bijlsma *et al.* 2000) to give a product less than 150-bp which should amplify more easily from degraded DNA (Bellemain & Taberlet 2004). Null alleles due to primer binding site mismatches may also be avoided by redesigning primers in this way as the new primers are based on pine marten sequence data for the region flanking the microsatellite motif.

Overall genetic variability

Contemporary genetic diversity is low in the Irish pine marten population with average heterozygosity and allelic diversity values of 0.35 and 2.29 respectively for 17 microsatellite loci. Although it is difficult to directly compare results between studies which use different microsatellites, these results are similar to those obtained in previous studies for the Irish population (H_E = 0.34; A = 1.86-2.88, Kyle *et al.* 2003; Lynch 2006), even though many more loci and individuals were examined. There were five loci (Ma1, Ma2, Gg7, Ggu454 and Lut604) in common between all studies. Only one allele has been detected for Ma1 and Lut604 so far in the Irish population while the other three loci were polymorphic in both this study and Lynch (2006). Ma2 and Gg7 were more variable in the other datasets with five and seven alleles respectively in the Lynch (2006) dataset and four alleles each in the Kyle *et al.* (2003) dataset. These differences may be due to sampling effects with different individuals included in each study and suggest that a more comprehensive sampling of the entire island in the future may reveal important areas for genetic diversity within Ireland.

The observed low genetic variability of the Irish pine marten may be due to historical founder effects and/or population bottleneck events. The higher H_E for the French individuals in this study (H_E = 0.59) supported the results of Kyle *et al.* (2003) where pine marten from continental Europe were found to have higher genetic variability (H_E = 0.58) than island populations (Kyle *et al.* 2003). A similar pattern exists between fragmented coastal *Martes caurina* (H_E = 0.37) and contiguous mainland *Martes americana* (H_E = 0.58) clades in North America (Small *et al.* 2003).

The origin of the population in Ireland is currently unknown, but suspected to be an early introduction by man (Searle 2008). The population is monomorphic for one mtDNA haplotype for the typically variable control region, and the cytochrome b gene (Davison *et al.* 2001; Statham 2005; Lynch 2006). The control region mtDNA haplotype, hap *p*, is unique to Ireland and different to the mtDNA found in Britain (hap *a*), The Netherlands and France (Davison *et al.* 2001). The closest haplotype to hap *p* is hap *i* which was found in Iberia, but also recently in museum specimens from Britain and Ireland (Dr. C. O'Reilly, pers. comm). It is possible that the population in Ireland was founded with individuals from Britain and/or Iberia in the early human settlement of the country, which was logistically possible as the species has been transported by sea to other islands (McCormick 1999) and there is evidence that swords, axes and gold jewellery were transported by sea along the Atlantic Neolithic trade routes between 4,000 and 3,500 years ago (Oppenheimer 2007, page 273). The hap *i* may have present in the UK population but lost due to the widespread decline in the species (Langley & Yalden 1977). In Ireland, hap *p* may have arisen *in situ* by mutation from hap *i* since colonization, or hap *p* may also have been present in the UK population is now lost. Studies of human Y-chromosome genetics have identified a link between south-west Europe and Ireland using SNP and microsatellite loci, associated with the spread of farming from the Near East (Hill *et al.* 2000). Deliberate or accidental transport from Britain in the Neolithic, approximately 4,600 years ago, is also the proposed mode of colonisation for the pygmy shrew (McDevitt 2008). More genetic sampling, particularly of historical and paleontological specimens, is required to resolve the question of from where and when pine marten arrived in Ireland.

Demographic fluctuations

The H_E and number of alleles in the Irish pine marten was similar to mustelid species which have undergone documented population bottlenecks such as the Newfoundland marten *Martes americana atrata* (A = 2.43, H_E = 0.45; Kyle *et al.* 2003) and the Scandinavian wolverine *Gulo gulo* (A = 2.63, H_E = 0.39; Walker *et al.* 2001). Tests

implemented in BOTTLENECK detected a significant excess of heterozygotes relative to allelic diversity for each of the three models of microsatellite mutation and the distribution of allele frequencies also showed a loss of alleles from the population. There was evidence for a recent loss of allelic diversity in the Irish pine marten population due to a population bottleneck. Furthermore, the low allelic variability and narrow allele size range for each microsatellite locus (Figure 24) is characteristic of a population founded by a small number of individuals (Jones *et al.* 2004), and thus one which already had a small effective size prior to the recent bottleneck detected in this study.

The timing and severity of the bottleneck is unknown but the BOTTLENECK test is suitable for detecting bottlenecks within the last dozen or so generations, which would be within the last 40-50 years assuming a generation interval of approximately 3 years for pine marten (Pertoldi *et al.* 2008). This would roughly coincide with a population decline associated with trapping and poisoning campaigns in the 1950s and 1960s as reported by O'Sullivan (1983). The development of agriculture over the last 30-40 years in Denmark had similar effects on the pine marten population in the Jutland peninsula, with a reduction in effective population size to only 0.2% of the historical size prior to 1970 (Pertoldi *et al.* 2008). An analysis of museum specimens from the Irish population prior to the 1950s and from early in the 19th century would help to quantify the historical genetic diversity of the Irish population and thus ascertain the severity of bottleneck events.

A loss of genetic variability may reduce a population's ability to respond by natural selection to new environmental conditions (Allendorf & Luikart 2007) and affect reproductive output due to the expression of deleterious alleles with the increased homozygosity of bottlenecked populations (Seymour *et al.* 2001; Luenser *et al.* 2005). Aguilar *et al.* (2008) measured neutral and functional genetic variation in sea otter *Enhydra lutris* populations in California to examine the effects of near-extinction due to the fur trade on genetic variability in the species. Neutral genetic variation was assessed at 14 microsatellite loci isolated from other mustelid species and revealed an average of 2.2 alleles per locus which is similar to this study. Sea otters also had a reduced number

of alleles at Major Histocompatibility Complex (MHC) loci, which are typically highly variable in mammalian species. These results raised concerns about the link between observed low genetic diversity and the fitness of sea otter populations as mortality rates from novel protozoal parasites and other disease agents in the environment were high (Aguilar *et al.* 2008).

A lack of neutral genetic diversity as measured by microsatellite markers cannot be taken as evidence for a genome-wide lack of genetic variability (Vali *et al.* 2008). For example, the San Nicolas Island fox *Urocyon littoralis dickeyi* which inhabits a small island off the coast of California was genotyped at 18 microsatellite loci and only one allele per locus was detected, yet the heterozygosity of four MHC loci was similar to other Channel Islands with larger effective population sizes (Aguilar *et al.* 2004). For threatened species with such low genetic diversity, a genome-wide assessment of genetic variability may be more appropriate.

European bison *Bison bonasus* were once widely distributed across Europe but dramatic population declines lead to their extinction in the wild in the early part of the last century. Previous molecular analyses of European bison revealed low diversity at microsatellites and MHC loci. Single nucleotide polymorphisms (SNPs) are the most abundant polymorphism in the genome, with one every approximately 500-bp (Allendorf & Luikart 2007). Pertoldi *et al.* (2010) used a huge panel of over 52,000 SNPs to screen the genome of European bison, two sub-species of American bison *Bison bison* and domestic cattle *Bos taurus*, the genome from which the SNP panel was developed. Only 929 of the SNPs were variable in European bison, which confirmed the depauperate nature of the genome, but provided a wealth of information which may be used in the management of the species in the long-term.

Population structure

Genetic variation was relatively homogenous across the range of the pine marten in Ireland with no significant differences in microsatellite allele frequencies between

regions (CHIFISH analysis) and only one genetic cluster present in the dataset according to spatial and non-spatial Bayesian clustering analysis with GENELAND and STRUCTURE. The inclusion of spatial data in the GENELAND analysis increased the power to detect structure under these circumstances (Latch *et al.* 2008) but still no major divisions in gene flow were detected. The failure to detect genetic structure was not likely to be due to low power as the microsatellites used in the analysis had a simulated power of at least 98%, and did detect significant structure between the Irish and French samples.

These results suggest there is sufficient migration of individuals between populations within Ireland to maintain the species as a single panmictic population. Pine marten are certainly capable of covering large distances as a female marten tagged and released in the west of Ireland was hit by a car 65 km away three weeks later (Birks 2002) and male pine marten in the breeding season can travel an average of 10 km in a single night (Schröpfer *et al.* 1997). Movements may be facilitated by the increased afforestation in Ireland in the past few decades as forest cover has almost doubled in the last 30 years and pine marten are known to use thicket stage conifer plantations (O'Sullivan 1983; O'Mahony *et al.* 2006). Habitat fragmentation may not pose a significant barrier to gene flow as fields and roads are not avoided (Pereboom *et al.* 2008), and pine marten are known to utilise alternative habitats such as scrub and rocky areas, hedgerows, riparian woodland and agricultural landscapes (O'Sullivan 1983; Birks *et al.* 2004; Pereboom *et al.* 2008; Balestrieri *et al.* 2009).

High levels of gene flow between populations within Ireland were also reported for Leisler's bat *Nyctalus leisleri* (Boston 2007), mountain hare *Lepus timidus hibernicus* (Hamill *et al.* 2007), pygmy shrew *Sorex minutus* (McDevitt *et al.* 2009b) and red deer *Cervus elaphus* (McDevitt *et al.* 2009a). Official and unofficial human-mediated movements of pine marten between regions may have contributed to the apparent lack of structuring, as reported for red deer (McDevitt *et al.* 2009a). An example of such a translocation took place in the 1980s where pine marten from the west of Ireland were moved to reinstate the population at Killarney National Park (KNP) in the south-west,

on the other side of the River Shannon (Lynch 2006). There were no individuals from the south-west region included in this analysis but Lynch (2006) found the allele frequencies in the KNP individuals were a subset of the founder individuals from the mid-west, indicating the KNP population had indeed been extirpated before translocation.

There may be structuring when considering the sexes separately as the road-kill samples used were mainly males and may have been mid-dispersal when killed. This can be investigated in the future by carrying out assignment tests separately for each sex with larger sample sizes, however Kyle *et al.* (2000) found no such pattern of sex-biased dispersal for the American marten in Canada. Mitochondrial DNA cannot be used as a marker for the investigation of genetic structure within Ireland as there has only been one haplotype detected so far in the contemporary population. This preliminary analysis should be expanded upon in future to enable the early detection of barriers to gene flow, as the lack of genetic structuring may be caused by recent isolation and there can be a delay in the detection of a signature of genetic differentiation after a fragmentation event (Hamill *et al.* 2007).

Tasmanian devils (*Sarcophilus harrisii*) are marsupial carnivores with a similar demographic history and genetic diversity (H_E 0.39-0.47; A 2.7-3.3) to the Irish pine marten population. Genetic variability was supposed to be a result of a founder effect post-glaciation exacerbated by restriction to an island and repeated bottlenecks over the last 150 years (Jones *et al.* 2004). No significant genetic structure was detected throughout much of Eastern Tasmania and all individuals are genetically similar at MHC loci (Jones *et al.* 2004; McCallum 2008). The emergence of a novel infectious cancer in 1996, Devil Facial Tumour Disease, threatens the species with extinction and has already resulted in a local population decline of up to 90%, with projected extinction of the species in five to ten years (McCallum 2008). The case of the Tasmanian devil is unusual as the infectious agent is a mammalian cell line, but it highlights the importance of maintaining genetic diversity in island populations in the face of unknown environmental change.

Genetic sampling from remote areas can be facilitated by the analysis of scats, which are collected as part of national distribution surveys. Hair sampling may be used for sampling in remote areas where trails are not well defined for scat sampling and where territories may not be marked with scats due to low population densities. The polymorphic microsatellites identified in this study have sufficient power to discriminate between individuals for the estimation of population size through non-invasive sampling, with a low probability of less than 0.01 for two individuals having the same multilocus genotype. However, simulations carried out in this study showed that parentage inference is not possible with the current panel of microsatellites due to the difficulty of distinguishing kin from non-kin. The use of non-invasive genetic sampling for monitoring pine marten population status is described in Chapter 5.

Chapter 5

Application of the suite of molecular techniques to census free-ranging pine marten populations

Related publication:

Mullins J, Statham MJ, Roche T, Turner PD, O'Reilly C (2010) Remotely plucked hair genotyping: A reliable and non-invasive method for censusing pine marten (*Martes martes*, L. 1758) populations. *European Journal of Wildlife Research*, **56**, 443-453. Doi:10.1007/s10344-009-0332-x.

5.1. Introduction

Reliable data on presence and abundance is vital for developing management strategies for the conservation of rare and elusive species yet can be logistically difficult to obtain (Foran *et al.* 1997; Piggott & Taylor 2003b). Pine marten distribution is typically assessed by collecting characteristic scats from defined transects in suitable habitats (Strachan *et al.* 1996; O'Mahony *et al.* 2006; Ruiz-Gonzalez *et al.* 2008), but recent studies have indicated that few of the total number of scats in the environment are actually deposited on forest trails (Roche 2008), and the relationship between scat density and local population abundance is unclear (Birks *et al.* 2004). Hair-trapping is an alternative survey method which has huge potential for surveying marten populations as it is more accurate and sensitive (Statham 2005) and can be used in remote areas where the standardised collection of scats from trails is difficult (Lynch 2006).

Genetic analysis of field signs can improve the accuracy of species identification (Davison *et al.* 2002) and yield additional information on population size, sex ratio, spatial organisation, genetic diversity, social organisation and recruitment (Taberlet *et al.* 1997; Hung *et al.* 2004; Prugh *et al.* 2005; Hedmark & Ellegren 2007). There have been no published accounts of using non-invasive genetic techniques to census European pine marten populations, except some mention in Posłuszny *et al.* (2007). However, these methods have been successfully applied to other mustelid species such as the American marten (Foran *et al.* 1997; Mowat & Paetkau 2002), Eurasian otter (Hung *et al.* 2004) and wolverine (Hedmark & Ellegren 2007).

The primary aim of this study was to investigate the reliability of the panel of microsatellites developed in Chapter 4 to identify individuals in small populations, given the low background genetic variability in the Irish population. This study also assessed the feasibility of using the full suite of genetic methods developed in the project to census free-ranging marten populations by genotyping hair and scat, and compared the results of the non-invasive census with direct estimates of abundance via live trapping. Possible future improvements to the methodology are discussed.

5.2. Methods

5.2.1. Study area

The study area in east Waterford, Ireland is bordered by the River Suir, south-east coastline and the Comeragh Mountains. Forest cover is patchy and consists mainly of small plots of coniferous forest (Figure 33). Curraghmore Forest (52.25° N, 7.3° W) near Portlaw village is one of the largest forests in the area at approximately 10 km^2, over 75% of which is conifer high forest (Coillte 2009). Curraghmore Forest is connected to the forests of the River Suir Valley through Tower Hill Forest to the north (Figure 34), although part of Curraghmore Forest south of Tower Hill still belongs to the privately owned Curraghmore Estate and was inaccessible for this project. Historical records from Curraghmore Estate confirm the presence of a breeding population (Ussher 1898), which was still extant in the eighties (O'Sullivan 1983). In the last decade pine marten were killed on several minor roads and detected from hair-tube sites (Smiddy & Berridge 2002; Statham 2005), indicating that pine marten are widely dispersed in the study area.

The second site was Gardenmorris Wood (52.16°N, 7.3°W) which is located 12 km south of Curraghmore and is also managed by Coillte forestry company (http://www.coillte.ie/) for commercial timber. The total area of the forest is about 0.9 km^2. Pine marten were previously recorded in Gardenmorris where the gamekeeper for the Gardenmorris Estate reportedly shot three in 15 years (Ruttledge 1920). The site was selected for a population census as genetically-verified pine marten scats were found in Gardenmorris in 2007, confirming their presence in the isolated site (Peter Turner, pers. comm.)

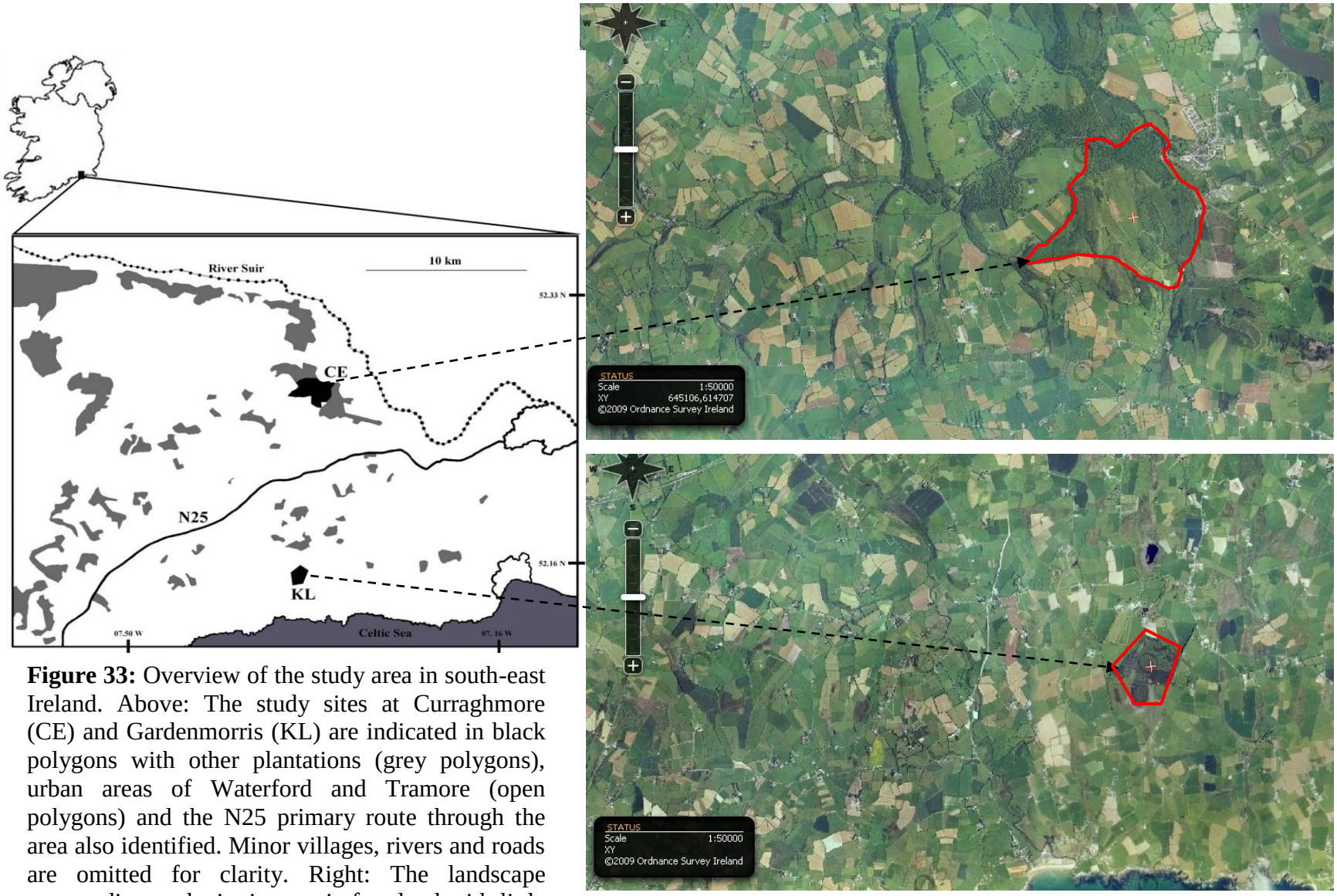


Figure 33: Overview of the study area in south-east Ireland. Above: The study sites at Curraghmore (CE) and Gardenmorris (KL) are indicated in black polygons with other plantations (grey polygons), urban areas of Waterford and Tramore (open polygons) and the N25 primary route through the area also identified. Minor villages, rivers and roads are omitted for clarity. Right: The landscape surrounding each site is mosaic farmland with little forested habitat. Images ©2009 Ordinance Survey Ireland.

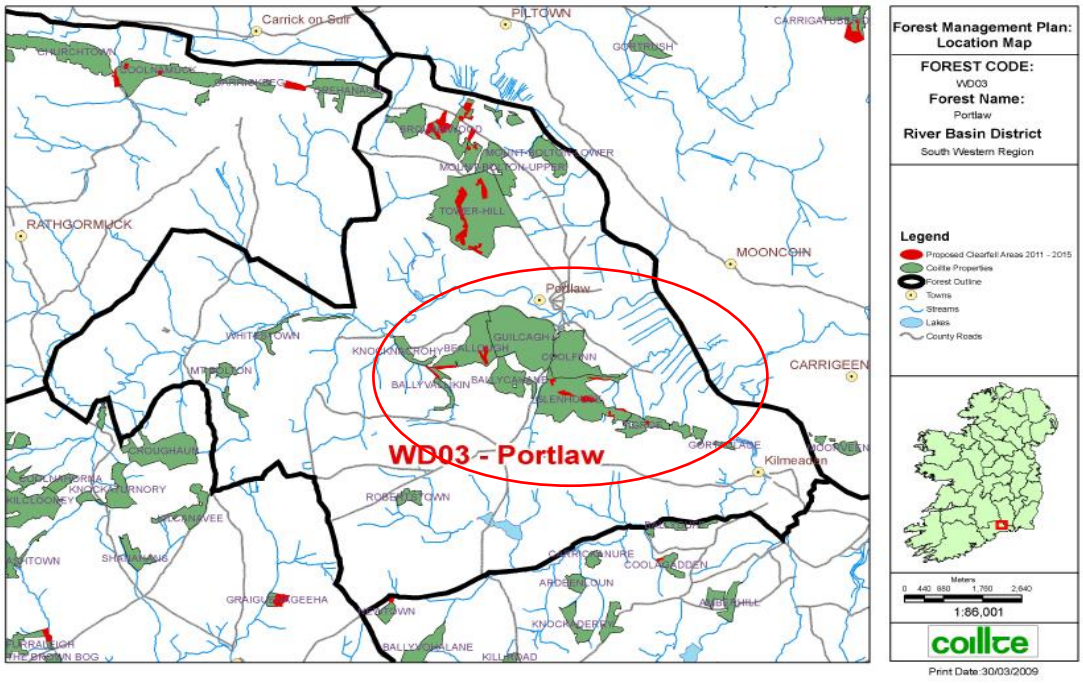


Figure 34: Forestry in the area surrounding the main study site at Curraghmore Forest (circled in red). Portlaw Management Plan 2011-2015 (v. 2.0, downloaded from www.coillte.ie on 28/05/09. ©2009 Coillte Teoranta.

5.2.2. Sample collection

Scats

During field surveys, all scats putatively from pine marten or red fox were collected as they were subjected to genetic species identification. The exact location of each sample was recorded using a handheld GPS device (e.g. Garmin E-Trex). Each sample was transferred to a separate plastic Ziploc bag and labelled with the date and location. All scats were frozen at -20 °C to -70 °C until DNA extraction.

Remotely-plucked hair

Hair was plucked non-invasively and remotely from individuals using hair-tubes designed by Peter Turner, Waterford Institute of Technology. Each hair-tube was constructed from PVC drainage pipe (118 x 250 cm) with a section (3 cm) removed longitudinally to give stability against the tree trunk. The hair-tubes were attached vertically to trees approximately 1 m above the ground, off the forest track out of public view (Figure 35a). The tubes were secured in place with 1.5 mm galvanised wire threaded around the tree through holes drilled in the tube. A cap was fitted to the top of the tube to keep the tube interior dry and to prevent bait removal without contact with the glue patches situated at the opposite end. Raw chicken wing was used as bait, which was skewered with wire and fixed in place inside the tube just under the cover (Figure 35b). Marmalade was also smeared inside the tube and a commercial marten lure (Cumberland's Northwest Trappers Supply, PO Box 408, Owatonna, MN, USA, http://www.nwtrappers.com) was applied to a tree nearby as an extra attractant.

(a) (b)

Figure 35: Hair-tube design. Hair-tube in place (a), with bait secured at the top of the tube (b) along with glue patches inside the tube entrance to remove dorsal hair while the tube visitor removes the bait.

A patch of a glue-based mouse trap (Solway Feeders, http://www.solwayfeeders.com, cat. no. 3248) was used for snagging hair. The glue patches (10 x 14 mm) were held in the tube by attachment to corrugated plastic (20 x 25 mm), with both components held together and stuck inside the tube with double-sided Scotch™ pressure-sensitive adhesive tape. The inside of the each tube was wiped clean before applying the patches to ensure they remained in place while in contact with the animal. The patches were placed inside the tube on the side opposite the chicken bait to remove dorsal hair. Patches with hair were placed in 20 ml plastic screw-cap tubes and were stored under the same conditions as the scat samples.

Hair plucked from live trapped individuals

Live trapping was carried out as part of a radio-telemetry study not reported here. The opportunity was taken to obtain genotypes from live trapped marten for reference against genotypes obtained from non-invasive surveys. Hair-tubes were used to pre-bait each site for live trapping in order to assess visitation rates to increase trapping success and to avoid leaving the cage traps in place for longer than was necessary (Figure 36). The sites were pre-baited for two days and the traps were set on the third day. Wire cage traps (23 x 23 x 74 cm, Tomahawk Live Trap Co., Tomahawk, WI, USA) were used for live trapping. A bed of straw was placed inside the traps, which were covered with small branches and leaf litter to provide warmth, protection and camouflage from the public. Traps were baited with raw chicken in the trap and cheese at the entrance. As the trapping took place in a public forest, each trap had a sign attached detailing the purpose of the study and a warning that there may be a wild animal inside. A hair sample was plucked from the tail of each individual using the same glue patches which were used in the hair-tubes.

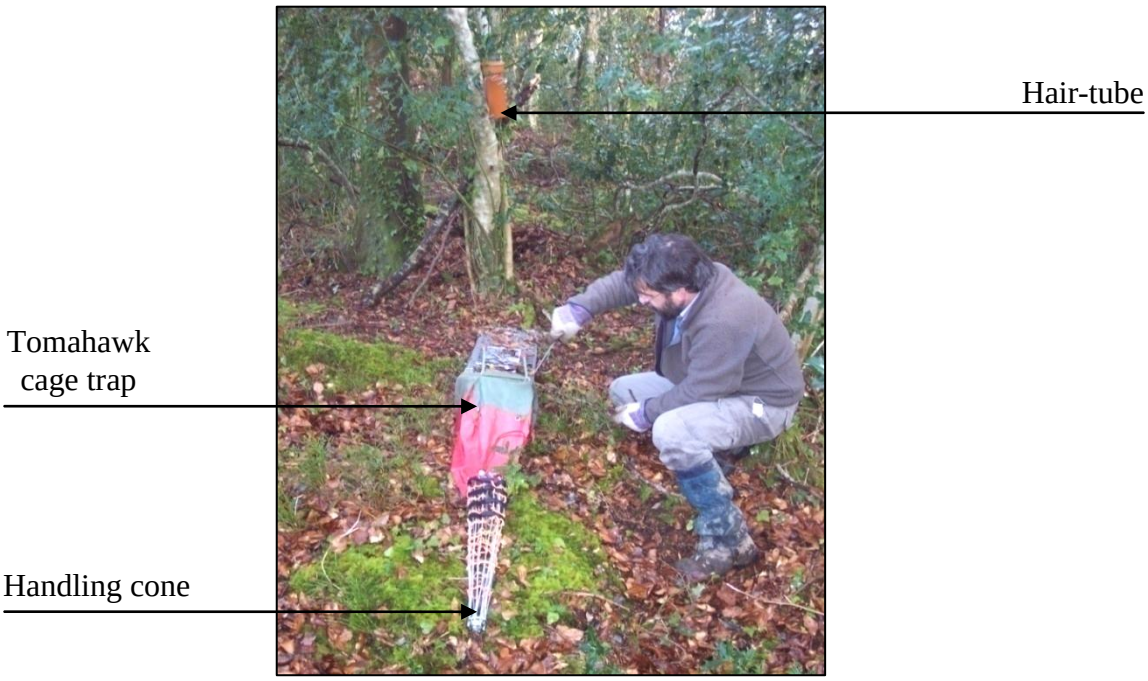


Figure 36: Site set up for live trapping. Hair-tubes were used to pre-bait trap sites. Cage traps were placed underneath the same trees as the hair-tubes and baited open for a couple of nights before being set for capture. Trapped martens were moved to the handling cone before being placed under anaesthetic for collaring.

5.2.3. Curraghmore Forest census 2006-2007

Sample set

The final microsatellite panel (Panel_D, Table 9, pg 99) was initially tested on an archived sample set of 119 plucked hair samples and 107 scats which were collected from February 2006 to March 2007 as part of a parallel study of the presence and gender distribution of pine marten in Curraghmore Forest (Roche 2008). All scats were previously species typed as pine marten in a real-time PCR reaction with the PM2 probe and pine marten hair samples were identified with the PM3 probe. This study covered

the Coillte forest management units of Guilcagh, Tigroe, Coolfin and Glenhouse with 16 hair-tubes (Figure 37).

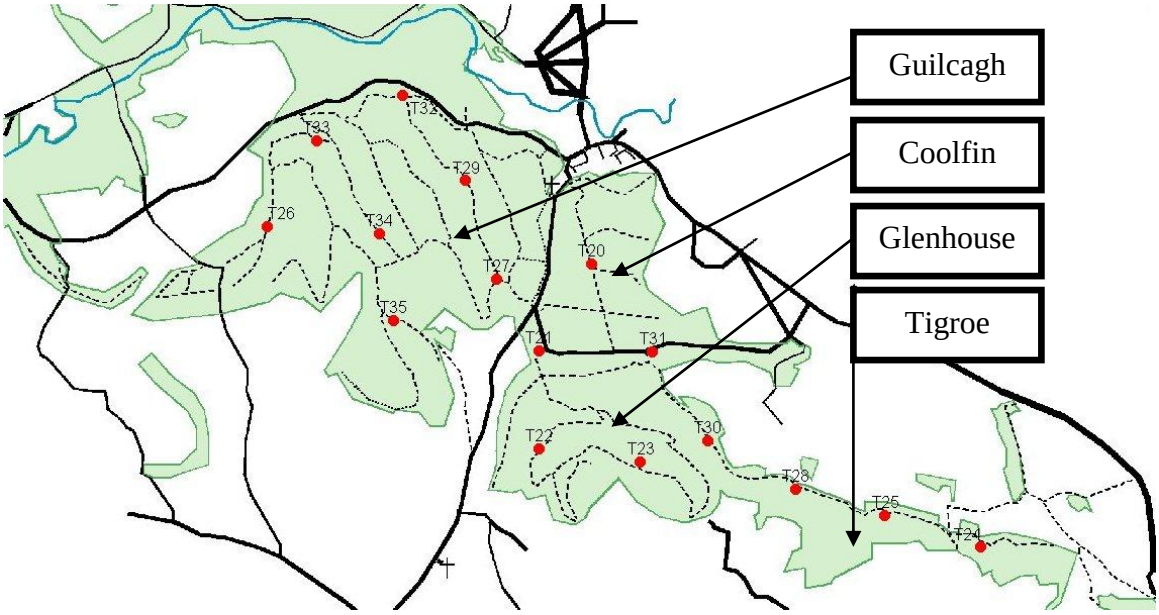


Figure 37: Hair tube locations for the 2006-2007 sample set in Curraghmore forest.

The 2006-2007 sample set was not originally intended for genomic DNA analysis and therefore was not collected and stored under optimal conditions. Hair and scat samples were collected once a month upon re-baiting the tubes for the following month. All scats were frozen at -50 °C, though they may have been in the field for up to a month before collection. Hair was stored at room temperature until DNA extraction (1-10 months after collection). However, the sample set was considered to be useful for microsatellite analysis as the coverage in space in time provided an opportunity to monitor the movements of individuals in Curraghmore and compare the number of pine martens identified from plucked-hair and scats.

Microsatellite analysis

Low amplification success rates were anticipated for microsatellite analysis, therefore a screening step was carried out where all DNA extracts were amplified once with the

reduced-size microsatellite locus Mel1mini (110-bp). Only those samples which amplified in the first PCR were replicated. Replicates were then compared and samples with matching replicate genotypes were taken to the next round of genotyping. This subset of extracts was then amplified once at each of the remaining eight microsatellite loci in Panel_D (Table 9). Amplification success per locus was calculated to give an index of the DNA quality of the sample set and to identify problematic loci.

Live trapping was carried out in Guilcagh in February 2008 as part of a pilot study in preparation for a radio-tracking project planned for summer 2008. A sample of hair was plucked from the tail of each captured individual and genotyped at 20 microsatellite loci (Panel_A, Table 9) in single locus reactions to compare the genotyping success rate of hair samples freshly plucked from live individuals with remotely-plucked hairs from the 2006-2007 census set. Each locus was typed twice if heterozygous and three times if homozygous to construct consensus genotypes.

5.2.4. Curraghmore and Gardenmorris Census 2008

Census design

Hair and scat samples were collected from Curraghmore (Guilcagh management unit, 2.5 km^2) and Gardenmorris to carry out a non-invasive population census using the finalised molecular techniques of species, sex and individual identification. Hair-tubes were set-up in both sites in April-May 2008. The tubes were pre-baited in April to assess the visitation rates. Pre-bait hair samples were collected every 1-7 days (Table 20). The population census was carried out in May once at least half of the sites had captured hair. For the census, hair-tubes were set out in a grid pattern and baited for seven sampling sessions; daily in Curraghmore and every 2-3 days in Gardenmorris. Each grid cell was 500 x 500 m, with 10 hair-tubes in Curraghmore (1/cell) and eight (2/cell) in Gardenmorris (Figures 38 & 39). The density of hair-tubes was doubled in Gardenmorris

in order to provide enough samples for genetic analysis. Scats were also collected from the trails between the tubes with the aim of comparing the number of individuals identified from genotyping plucked hair and scats. All scats were cleared from the transect at the start of the census so that each sample was as fresh as possible.

Table 20: Schedule of non-invasive sampling sessions for the 2008 population census.

Sampling session		Dates of sample collection	
		Gardenmorris (8 hair-tubes)	Curraghmore (10 hair-tubes)
Pre-bait	1	06/04/08	08/04/08
	2	10/04/08	15/04/08
	3	22/04/08	19/04/08
	4	25/04/08	22/04/08
	5	28/04/08	23/04/08
Census	1	01/05/08	07/05/08
	2	05/05/08	08/05/08
	3	08/05/08	09/05/08
	4	11/05/08	10/05/08
	5	13/05/08	11/05/08
	6	15/05/08	12/05/08
	7	17/05/08	13/05/08

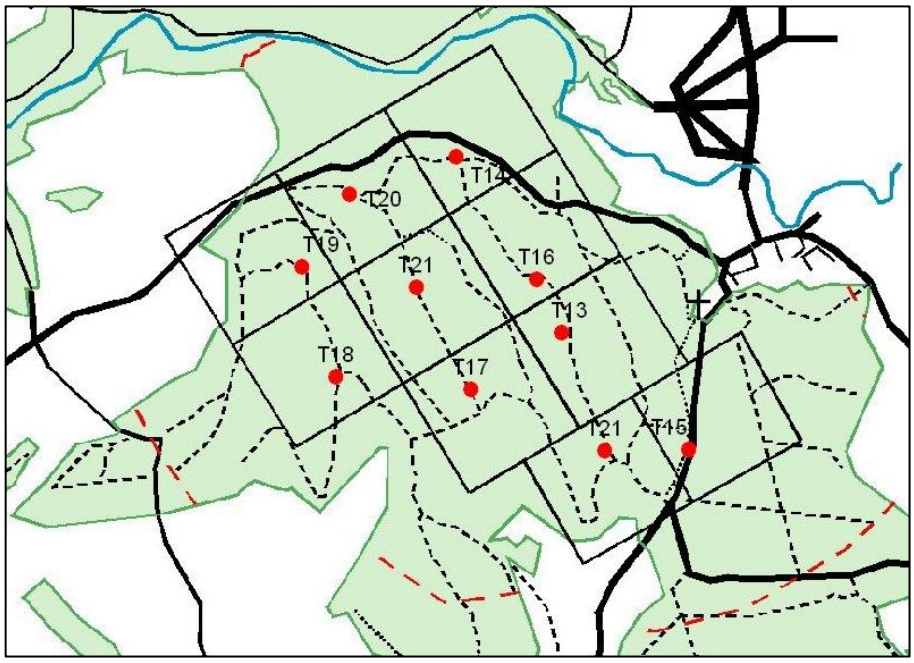


Figure 38: Tube layout in Curraghmore Forest for the 2008 census.

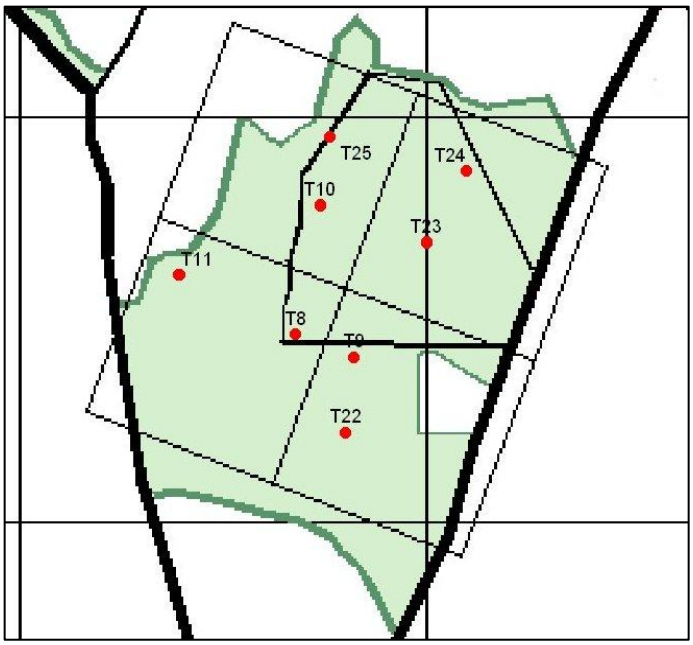


Figure 39: Tube layout in Gardenmorris for the 2008 census.

Genetic analysis

All extracts were species typed with the PM3 real-time PCR species typing assay. Extracts giving no amplification with the PM3 assay were not analysed further. All extracts were then tested in duplicate with a ZF Intron real-time PCR sex typing assay. This assay was designed to target variable introns in the ZFX and ZFY genes in order to avoid the use of conserved DNA sequence regions (as per teh ZF exon assay), which may be problematic when analysing scats, amplifying prey DNA and leading to false sex typing results (Murphy *et al.* 2003). The assay targeted short regions of 84-bp and 136-bp respectively. Sequence alignments are shown in Figure 40.

Each real-time PCR contained 7.5 µl TaqMan® MGB Universal PCR mastermix, 1 µl (0.5 µM) of each primer MMXF (5'-GGC AGA GCA ACC CTG TCA TAA-3'), MMXR (5'-GGG CCT GAG GTT GGT ACC ACC A-3'), MMYF (5'-GCA TTG GGC TCC CTG CT-3') and MMYR (5'-AGA TAT CCA AAT ACA TGT GGC TTT AAA TG-3') and 0.6 µl (0.2 µM) of each probe MMX (5'-VIC-CCT GGT CTG AAA ACT-MGB-3') and MMY (5'-6FAM-TGT GTC TCT CTC TGT CAA-MGB-3') with 2 µl of undiluted template DNA. The PCR cycling conditions were the same as for the ZF exon

assay (page 74). Males were identified by amplification for the ZFY probe whereas females were identified by ZFX amplification only. Extracts with late C_T values were checked for possible ZFY allele dropout (e.g. ZFX amplification only, C_T >40).

Figure 40: Alignment of sequences for the ZF intron assay. Alignments of the ZFX and ZFY gene introns with primers underlined and probes (grey shading) indicated. All oligonucleotides were multiplexed together in a single reaction for sex typing.

Reliability of microsatellite genotypes

The eight loci in Panel_E (Table 9, page 99) were used to identify individuals as Mvis075 was difficult to amplify and score from the samples in the 2006-2007 set, with excess stutter patterns and low amplification success rates. These loci still had a combined PI*sibs* of 0.011, giving a low probability of two individuals having the same multilocus genotypes. Each extract was initially amplified twice at all loci using the standard amplification conditions (section 4.2.3.), with 45 cycles. Any sample which failed to amplify at least four loci on the first attempt was not genotyped further. A variation of the comparative multiple tubes approach was used to construct multilocus

genotypes (Frantz *et al.* 2003). Reactions were added stepwise until either each allele had been seen twice for heterozygotes, three times for homozygotes, or five reactions had been carried out (negative reactions included).

Amplification success rates per locus and per sample were calculated based on the first round of PCR for comparison with the 2006-2007 sample set. The consensus multilocus genotypes after multiple replications for the hair-tube survey were compared with the first two PCR replicates carried out to calculate microsatellite genotyping error rates using the software GIMLET v. 1.3 (Valière 2002). Genotyping errors were identified as allele dropout (amplification of one allele at a locus known to be heterozygous from the consensus) and false allele amplification (amplification of an extra allele at a locus known to be homozygous from the consensus genotype). The per locus error rates were reported to identify any particularly error prone microsatellite loci which may need to be replaced in future analyses.

Minimum population size estimate

All consensus genotypes were entered into the GENALEX program (Peakall & Smouse 2006) and replicated genotypes were grouped automatically with the 'multilocus matches' option. Unique genotypes that were observed only once in the sample set were likely due to errors in genotyping and were grouped with other samples if there was a match at the most informative locus. Finally, the number of unique genotypes was taken as the minimum population size and the sex of each individual also gave an estimate of the sex ratio in the population. The area of the forest trapped was used to estimate population density. For the Gardenmorris site, the entire forest area was just under 1 km^2, but the Curraghmore trapped area of 2.5 km^2 was adjacent to a private forest which was inaccessible (north of the study area outlined in red, Figure 33). The forest was bordered on all other sides by roads and given the short duration of the census, the study population was regarded as closed to immigration or emigration. Approximate home ranges were plotted for the duration of the census as 100% minimum convex polygons (MCP) around all the hair-tubes which each individual visited.

Live trapping

Live trapping was carried out in Guilcagh in June 2008 to collar individuals for a separate radio-tracking project. This provided an opportunity to compare both invasive and non-invasive methods for identifying the number and sex of the pine marten in the area in a relatively short time frame. The trapping sites were as for the hair-tube sites in the population census, with the hair-tubes *in situ* but not baited in the interim four week period. The hair samples plucked from live captures were genotyped at all 14 loci in Panel_B (Table 9) for reference.

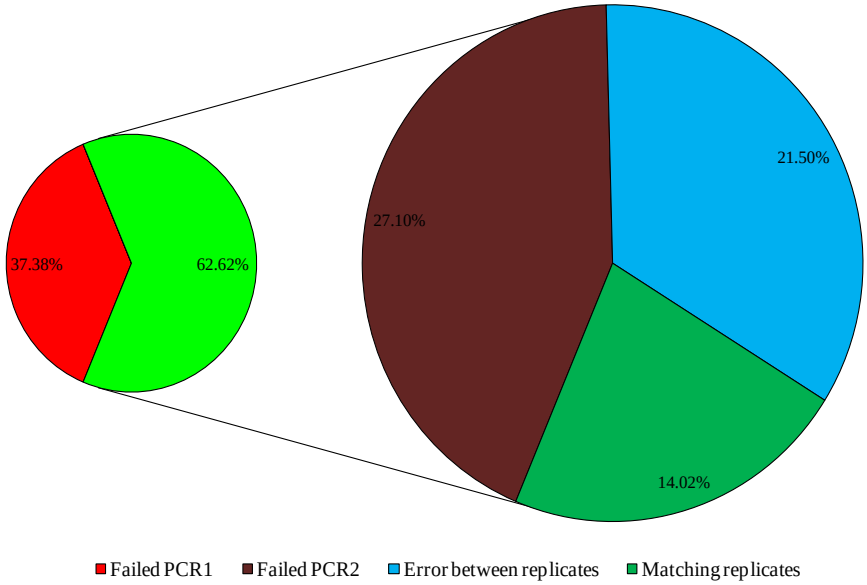
5.3. Results

5.3.1. Curraghmore Forest population census 2006-2007

The entire sample set of 119 hair and 107 pine marten DNA extracts was screened with the microsatellite Mel1mini. About a third of the samples failed to amplify microsatellite DNA in the first PCR (scats: 37.38%; hair 30.25%). Some of the samples which amplified on the first attempt failed to amplify in the second PCR (a further 27.10% of scats and 10.92% of hair samples), indicating the samples contained very low quantities of amplifiable DNA (Figure 41). Fifty-four (45.38%) hair samples had matching genotypes between reactions whereas the figure was much lower for scats with only 15 of 107 scats (14.02%) (Tables 21 and 22).

Overall the hair samples appeared to be more successful and reliable than the scat samples for individual identification, but the entire dataset was poor quality. Eight different genotypes were identified for the Mel1mini locus. Three genotypes were found in both the hair and scat samples (108/112, 112/112 and 108/116) a total of 31, 18, and 15 times respectively. Two hair samples had the 112/116 genotype while the 108/108, 110/112 and 110/110 genotypes were identified from one scat each, giving a provisional estimate of at least seven individuals in the dataset. However, the 110 allele was not previously found in the Irish population from tissue samples and may have been a PCR artefact. No further microsatellite analysis was carried out on the scat samples from the 2006-2007 set due to low amplification success and high genotyping error rates.

(a) Scat (n = 107)



(b) Plucked hair (n = 119)

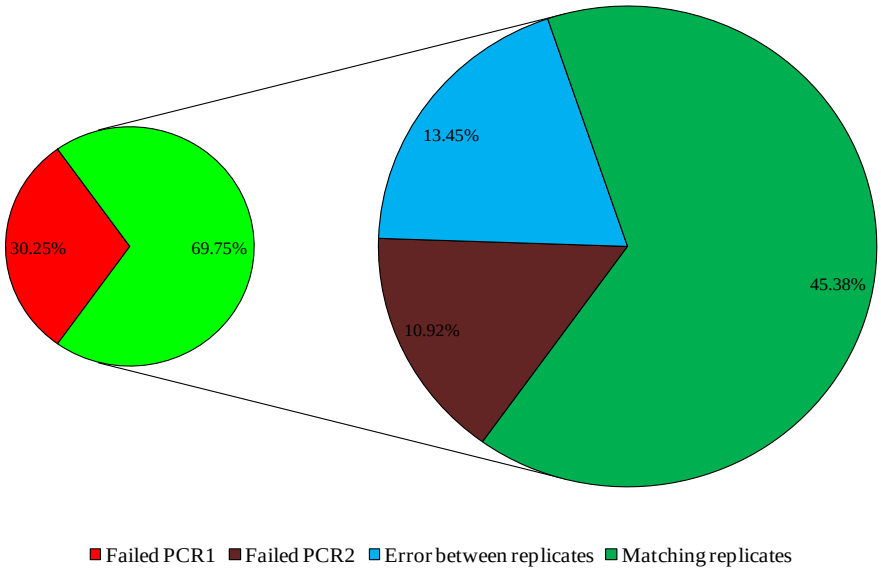


Figure 41: Microsatellite amplification success and genotyping error rates for archived hair and scat samples. Percentages were calculated for two replicates of Mel1mini microsatellite PCR.

Table 21: Genotypes of 119 pine marten hair extracts screened with the microsatellite locus Mel1mini.

EXTRACT	Rep1	Rep2	Consensus	EXTRACT	Rep1	Rep2	Consensus
PWH01	0			PWH61	108/112	108/112	108/112
PWH02	108/112	108/112	108/112	PWH62	108/112	108/112	108/112
PWH03	108/112	108/112	108/112	PWH63	108/112	108/112	108/112
PWH04	0			PWH64	112/112	112/112	112/112
PWH05	108/108	0		PWH65	108/112	108/112	108/112
PWH06	0			PWH66	108/112	108/112	108/112
PWH07	0			PWH67	108/112	108/112	108/112
PWH08	0			PWH68	112/116	112/116	112/116
PWH09	0			PWH69	108/112	108/112	108/112
PWH10	108/108	0		PWH70	112/112	112/112	112/112
PWH11	0			PWH71	112/112	112/112	112/112
PWH12	0			PWH72	108/112	108/112	108/112
PWH13	108/116	116/116	ERROR	PWH73	112/112	112/112	112/112
PWH14	0			PWH74	112/112	112/112	112/112
PWH15	0			PWH75	108/112	108/112	108/112
PWH16	0			PWH76	108/112	108/112	108/112
PWH17	112/112	112/112	112/112	PWH77	0		
PWH18	108/112	108/112	108/112	PWH78	108/112	108/108	ERROR
PWH19	112/116	112/116	112/116	PWH79	108/112	108/112	108/112
PWH20	108/116	108/116	108/116	PWH80	0		
PWH21	108/116	108/116	108/116	PWH81	0		
PWH22	0			PWH82	112/112	112/112	112/112
PWH23	0			PWH83	0		
PWH24	108/116	108/116	108/116	PWH84	108/116	108/116	108/116
PWH25	112/112	112/112	112/112	PWH85	0		
PWH26	108/116	108/116	108/116	PWH86	104/104	108/112	ERROR
PWH27	112/112	112/112	112/112	PWH87			
PWH28	112/112	112/112	112/112	PWH88			
PWH29	108/108	0		PWH89	108/112	0	
PWH30	108/112	0		PWH90	108/112	108/112	108/112
PWH31	108/112	108/112	108/112	PWH91	108/112	108/108	ERROR
PWH32	112/112	0		PWH92	108/112	108/112	108/112
PWH33	0			PWH93	108/112	108/112	108/112
PWH34	112/112	110/110	ERROR	PWH94	112/112	112/112	112/112
PWH35	0			PWH95	108/116	0	
PWH36	108/116	108/116	108/116	PWH96	108/112	108/112	108/112
PWH37	108/112	108/112	108/112	PWH97	108/108	0	
PWH38	0			PWH98	112/112	112/112	112/112
PWH39	108/116	108/116	108/116	PWH99	108/112	108/112	108/112
PWH40	112/112	0		PWH100	108/112	108/112	108/112
PWH41	108/116	108/116	108/116	PWH101	108/112	108/112	108/112
PWH42	106/106	0		PWH102	110/112	0	
PWH43	108/116	108/116	108/116	PWH103	108/116	116/116	ERROR
PWH44	108/112	110/110	ERROR	PWH104	110/112	0	
PWH45	0			PWH105	108/116	108/116	108/116
PWH46	0			PWH106	108/116	108/116	108/116
PWH47	0			PWH107	0		
PWH48	108/112	108/112	108/112	PWH109	0		
PWH49	108/112	0		PWH110	108/112	108/112	108/112
PWH50	108/116	108/116	108/116	PWH111	108/108	108/112	ERROR
PWH51	108/114/116			PWH112	0		
PWH52	110/110	108/110	ERROR	PWH113	108/116	108/116	108/116
PWH53	0			PWH114	0		
PWH54	116/116	108/108	ERROR	PWH115	108/112	108/112	108/112
PWH55	108/108	110/112	ERROR	PWH116	112/112	108/110/116	ERROR
PWH56	112/112	108/112	ERROR	PWH117	108/116	108/116	108/116
PWH57	0			PWH118	108/112/116		
PWH58	0			PWH119	110/116	106/108	ERROR
PWH59	108/108	110/114	ERROR	PWH120	112/112	108/112	ERROR
PWH60	0						

Table 22: Genotypes of 107 pine marten scats screened with the microsatellite locus Mel1mini.

EXTRACT ID	Rep1	Rep2	Consensus	EXTRACT	Rep1	Rep2	Consensus
PWS05	0			PWS86	102/102	104/110	ERROR
PWS06	0			PWS88	0		
PWS07	0			PWS90	106/108	0	
PWS08	0			PWS91	104/112	106/108	ERROR
PWS09	0			PWS92	0		
PWS10	108/108	108/108	108/108	PWS94	0		
PWS11	0			PWS101	110/112	110/112	110/112
PWS14	0			PWS102	112/112	110/112	ERROR
PWS20	0			PWS103	108/112	108/110	ERROR
PWS22	112/112	0		PWS105	108/112	108/112	108/112
PWS25	0			PWS107	112/112	112/112	112/112
PWS26	0			PWS108	110/112	108/112	ERROR
PWS27	112/112	0		PWS109	110/112	108/108	ERROR
PWS28	0			PWS110	110/110	112/112	ERROR
PWS29	108/108	104/106	ERROR	PWS111	110/112	112/112	ERROR
PWS30	112/112	0		PWS114	0		
PWS31	0			PWS115	108/112	110/112	ERROR
PWS32	0			PWS119	102/110/112		
PWS33	112/112	112/112	112/112	PWS121	0		
PWS35	112/112	112/112	112/112	PWS122	116/116	0	
PWS36	0			PWS125	110/112	110/110	ERROR
PWS37	0			PWS126	110/110	112/112	ERROR
PWS38	112/112	0		PWS127	110/112	112/112	ERROR
PWS41	108/110/112			PWS128	112/112	0	
PWS42	106/112	0		PWS129	110/110	0	
PWS43	112/112	0		PWS130	0		
PWS44	106/110/112			PWS131	0		
PWS45	108/112	0		PWS132	110/110	108/112	ERROR
PWS46	110/112	116/116	ERROR	PWS133	0		
PWS47	110/112	0		PWS142	116/116	0	
PWS48	108/110	0		PWS144	108/112	116/116	ERROR
PWS49	110/112	112/112	ERROR	PWS146	0		
PWS50	114/116	0		PWS147	106/108	112/112	ERROR
PWS51	0			PWS148	108/112	108/112	108/112
PWS52	112/112	0		PWS149	110/112	112/112	ERROR
PWS54	112/112	112/112	112/112	PWS150	112/112	0	
PWS55	106/112/114			PWS152	108/112	108/112	108/112
PWS58	106/108/112			PWS154	112/112	112/112	112/112
PWS60	112/112	0		PWS159	112/112	0	
PWS61	110/112	112/112	ERROR	PWS160	108/112	108/112	108/112
PWS62	112/112	106/110	ERROR	PWS166	108/112	0	
PWS66	0			PWS167	112/112	0	
PWS67	108/112	0		PWS168	110/110	110/110	110/110
PWS68	106/110/112			PWS169	0		
PWS70	108/108	108/116	ERROR	PWS170	110/112	0	
PWS76	108/112	0		PWS171	112/112	0	
PWS77	0			PWS172	106/110	0	
PWS79	104/108/112			PWS174	0		
PWS80	106/110/112			PWS180	110/112	112/112	ERROR
PWS81	110/112	0		PWS181	108/112	108/112	108/112
PWS82	112/112	0		PWS182	0		
PWS83	0			PWS183	0		
PWS84	110/112	0		PWS188	108/116	108/116	108/116
				PWS189	112/112	112/112	112/112

Hair samples which gave matching genotypes for both replicates at the Mel1 locus were genotyped further at the eight other loci required for individual identification (Table 23). Amplification at the Mel1 locus did not ensure genotypes would be obtained for the remaining loci as only 66.9% of the 408 PCRs required to complete one replicate of 9-locus genotypes amplified successfully, further highlighting the difficulties in amplifying microsatellite DNA from the set of non-invasively collected samples. Some loci were more robust to low quality samples than others, with per locus success rates ranging from 27.5% to 92.2% (Figure 42).

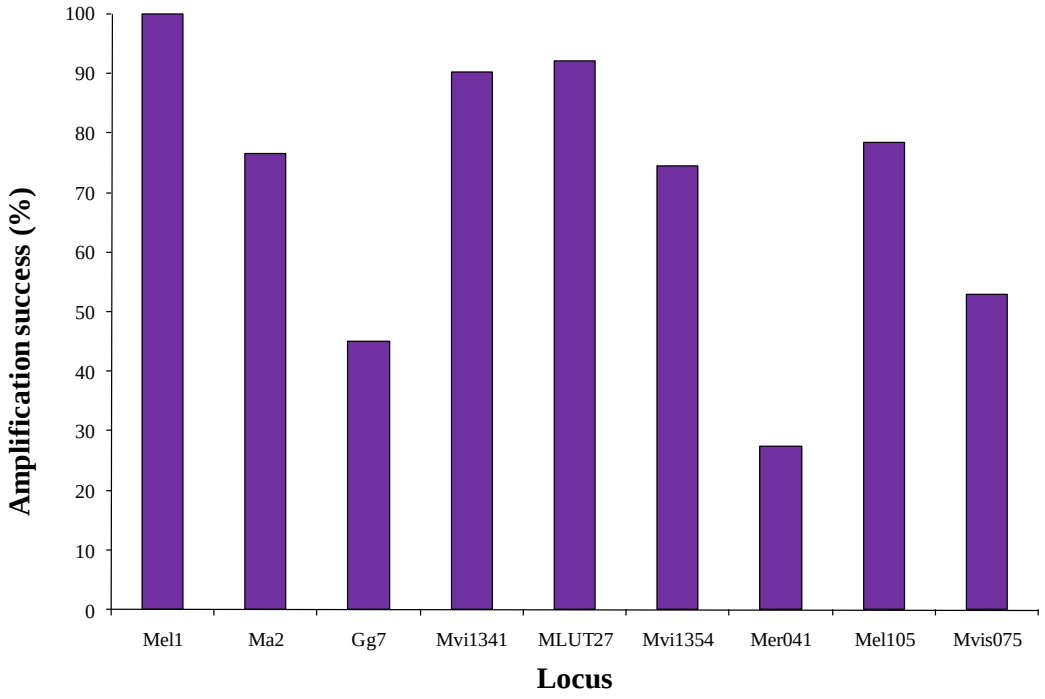


Figure 42: Amplification success rates for nine microsatellite loci amplified from plucked hair. Success rates are relative to Mel1 as this microsatellite was used to screen samples for amplification.

Table 23: Multilocus genotypes for 51 hair samples collected from Curraghmore Forest in 2006-2007. Failed amplifications are indicated (0).

	ID	Mel1	Ma2	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354	Mvis075
1	PWH02	108/112	175/177	0	168/168	110/110	0	0	0	0
2	PWH03	108/112	0	0	168/174	104/108	0	131/133	0	0
3	PWH17	112/112	169/171	159/159	0	0	0	0	204/204	0
4	PWH18	108/112	171/171	165/165	0	108/108	0	129/129	208/208	0
5	PWH19	112/116	171/171	0	168/168	106/108	0	127/133	208/208	0
6	PWH20	108/116	171/175	165/165	178/178	108/108	151/151	129/133	208/208	0
7	PWH21	108/116	173/177	0	178/178	106/106	0	0	208/208	0
8	PWH24	108/116	169/177	0	178/178	108/108	0	129/129	208/208	0
9	PWH25	112/112	0	0	174/178	108/108	0	0	0	0
10	PWH26	108/116	175/177	165/165	178/178	108/108	0	131/133	208/208	0
11	PWH28	112/112	171/175	159/159	174/178	108/108	151/157	129/129	208/208	151/151
12	PWH30	108/112	169/171	165/165	168/168	108/110	0	125/129	208/208	0
13	PWH31	108/112	169/177	0	168/168	110/110	0	131/133	200/200	0
14	PWH36	108/116	171/171	165/165	178/178	106/108	151/151	129/129	208/208	151/151
15	PWH37	108/112	0	0	168/178	110/110	0	0	200/208	0
16	PWH39	108/116	0	0	178/178	106/106	0	0	0	0
17	PWH41	108/116	175/175	0	178/178	102/102	0	129/129	208/208	151/151
18	PWH43	108/116	169/171	0	166/166	0	0	131/131	0	0
19	PWH48	108/112	169/171	165/165	168/174	104/106	151/157	131/133	206/208	0
20	PWH50	108/116	171/171	0	0	108/108	151/151	0	200/206	0
21	PWH61	108/112	0	0	174/178	108/108	0	0	0	0
22	PWH62	108/112	171/171	159/165	174/178	108/108	151/153	129/129	208/208	151/151
23	PWH63	108/112	177/177	165/165	168/178	110/110	151/157	129/129	200/208	0
24	PWH64	112/112	0	0	174/178	106/108	0	129/129	200/208	151/153
25	PWH65	108/112	165/171	165/165	168/178	108/108	151/151	129/131	208/208	0
26	PWH66	108/112	177/177	165/165	168/178	110/110	151/157	129/129	200/208	151/153
27	PWH67	108/112	171/177	165/165	168/178	108/110	151/151	131/131	200/208	151/153
28	PWH69	108/112	177/177	0	168/178	110/110	0	129/129	0	0
29	PWH70	112/112	171/177	159/159	178/178	104/108	0	129/131	208/208	151/151
30	PWH71	112/112	0	0	178/178	108/108	0	129/133	208/208	151/151
31	PWH72	108/112	169/171	0	168/168	0	0	0	0	0
32	PWH73	112/112	171/177	159/165	178/178	108/108	157/157	129/129	208/208	151/151
33	PWH74	112/112	171/171	159/165	178/178	108/108	151/157	129/133	208/208	151/151
34	PWH75	108/112	171/177	165/165	168/178	110/110	151/151	129/129	200/208	0
35	PWH76	108/112	169/175	165/165	168/178	110/110	151/155	129/129	200/208	151/151
36	PWH79	108/112	0	0	174/178	108/108	0	131/131	0	151/151
37	PWH82	112/112	0	0	0	108/108	0	131/131	0	151/153
38	PWH84	108/116	175/177	165/165	178/178	108/108	0	129/129	208/208	151/151
39	PWH87	108/112	171/171	0	174/178	108/108	0	129/129	200/200	151/151
40	PWH89	108/112	0	0	168/178	110/110	0	0	200/200	0
41	PWH90	108/112	177/177	0	168/178	110/110	0	131/133	0	0
42	PWH92	108/112	171/177	159/165	178/178	108/108	0	129/131	208/208	151/151
43	PWH93	108/112	0	0	0	0	0	0	0	0
44	PWH94	112/112	169/175	0	174/178	106/106	0	129/131	0	0
45	PWH98	112/112	175/175	159/159	174/178	108/108	0	131/133	208/208	151/151
46	PWH105	108/116	175/177	0	178/178	101/106	0	131/133	206/208	151/151
47	PWH106	108/116	171/177	165/165	178/178	101/101	0	129/131	208/208	151/151
48	PWH110	108/112	171/171	0	174/178	106/108	0	131/131	204/208	151/151
49	PWH113	108/116	171/175	0	178/178	108/108	0	123/129	206/208	0
50	PWH115	108/112	171/177	165/165	168/168	110/110	0	131/133	200/208	151/153
51	PWH117	108/116	0	0	178/178	108/108	0	0	0	0

5.3.2. Genotyping hair from live trapped individuals

Two male pine marten were captured using cage traps in Curraghmore Forest in one night in February 2008. Hair samples were taken from two of the individuals using glue patches and amplified at all 20 microsatellites previously tested for polymorphism in tissue (Panel_A, Table 9). Hair DNA amplification success rates were much higher than the 2006-2007 sample set at 97.4% across loci (110 out of 113 PCRs), with no genotype inconsistencies. The six loci which were previously identified as monomorphic in Irish pine marten (Mel6, Ma5, Lut615, 04OT14, Lut604 and Ma1) were also invariable in these two Curraghmore individuals, along with 10 of the remaining 14 loci. Individual discrimination was based on four loci (Ma2, Gg7, Mer041 and Mvi1354, Table 24). The two males shared an allele at each of these loci indicating that they were possibly first-order relatives such as parent-offspring.

The high amplification success rates and low genotyping error rates demonstrated the feasibility of using the developed techniques for a population census using freshly plucked hair, in contrast with the poor results for the 2006-2007 sample set. A more comprehensive population census using genetic analysis of non-invasively collected samples was therefore undertaken three months later in May 2008, with emphasis on the collection and genotyping of samples as soon as possible to improve success rates.

Table 24: Genotypes of two live trapped pine marten in Curraghmore forest, Co. Waterford. Values are allele calls in base pairs, loci which discriminated the two individuals in this case are shown in bold.

	Ma2	Mel1	Gg7	Mvi1341	Mer041	Mel105	Mel105	Mvis075	Mvi1354	Mvis020	Ma8	Ggu234	Mvis072	Ggu454
T130	**175**	112	**159**	174	**157**	108	129	151	**200**	185	109	84	265	127
(♂)	**177**	112	**165**	178	**157**	108	129	151	**208**	185	109	84	265	127
T131	**171**	112	**159**	174	**151**	108	129	151	**208**	185	109	84	265	127
(♂)	**175**	112	**159**	178	**157**	108	129	151	**208**	185	109	84	265	127

5.3.3. Curraghmore and Gardenmorris population census 2008

Sample collection

The visitation rates to hair-tubes increased over the duration of the prebait period in both Curraghmore and Gardenmorris (Figure 43). After the fifth pre-bait session, a hair sample had been left in 5 out of 8 hair-tubes in Gardenmorris and 9 out of 10 hair-tubes in Curraghmore. The duration of the pre-bait period (PI-P5) was 16 days in Curraghmore and 25 days in Gardenmorris. Samples were collected for the census two weeks after the pre-bait period. Overall hair capture success rates for the census were high over the seven sampling sessions (C1 to C7, Figure 43), with a total of 69 hair samples from 70 hair-tube trap nights (98.6%) in Curraghmore and 45 samples from 56 trap nights in Gardenmorris (80.3%). A hair sample was always left on the glue patches once the bait was taken and both patches were retained in the tubes.

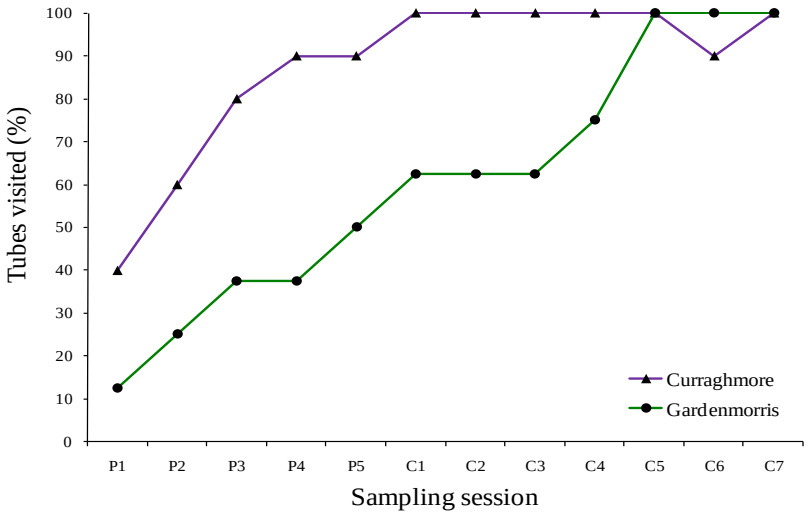


Figure 43: Increase in tube visitation rates over time during the trap pre-baiting period (P1 to P5) and the census (C1 to C7). Ten tubes were placed in Curraghmore and eight in Gardenmorris.

In most cases large tufts of hair were obtained, with at least 50 follicles per patch (Figure 44), enough for more than one DNA extraction if necessary. In many cases the hair extended beyond the glue patch, enabling the follicles to be cut from the end of the hair without contacting the glue. Forty-two scats were collected from the trails between the hair-tubes, 29 from Curraghmore and 13 from Gardenmorris. The average number of scats collected per session was 2.43±1.40 (range 1-4) in Gardenmorris and 4.14±1.77 (range 1-6) in Curraghmore.

Figure 44: Remotely plucked hair obtained during the census of Curraghmore forest.

Species and sex identification

The last three capture sessions were selected for genetic analysis as all hair-tubes were visited, apart from one site in Curraghmore Forest, providing a large enough sample size (53 hair and 22 scat samples) for analysis and a coverage of the study area. Mitochondrial DNA was amplified from 51 (96%) hair samples and 17 (90.9%) scats with the PM3 real-time PCR assay. Three of the undetermined results were later identified as fox with the PM2/FOX species typing assay. The remaining samples were either from a species other than pine marten or fox, or failed to amplify due to low DNA quantity. Nuclear DNA was amplified from 51 (96.2%) hair samples and 15 (88.2%) scats with the ZF intron sex typing assay. Real-time PCR results for the census samples are summarised in Tables 25 and 26.

Table 25: Species and sex identification of hair and scat samples collected in Curraghmore Forest in May 2008. Samples which did not amplify are indicated (U).

Session	Sample	ID	Hair-tube	PM3	ZFX	ZFY	Result[a]
C5	Hair	PWT121158	T12	27.87	32.46	33.39	PM, ♀
		PWT131158	T13	27.42	32.67	U	PM, ♀
		PWT141158	T14	28.24	32.69	33.48	PM, ♂
		PWT151158	T15	28.11	U	U	U
		PWT161158	T16	31.93	31.36	U	PM, ♀
		PWT171158	T17	29.20	35.42	36.37	PM, ♂
		PWT181158	T18	28.32	33.10	U	PM, ♀
		PWT191158	T19	27.83	33.64	U	PM, ♀
		PWT201158	no sample				
		PWT211158	T21	29.06	34.98	U	PM, ♀
	Scat	PWS11158	T17-T21	18.54	30.95	32.56	PM, ♂
		PWS21158	T17-T18	23.55	33.60	37.15	PM, ♂
		PWS31158	T17-T18	22.49	34.61	37.22	PM, ♂
		PWS41158	T17-T18	22.48	30.96	33.40	PM, ♂
		PWS51158	T18	20.34	33.06	36.75	PM, ♂
C6	Hair	PWT121258	T12	26.42	32.51	34.18	PM, ♂
		PWT131258	T13	28.00	32.67	U	PM, ♀
		PWT141258	T14	27.11	30.66	U	PM, ♀
		PWT151258	T15	30.59	34.98	36.13	PM, ♂
		PWT161258	T16	31.76	32.53	U	PM, ♀
		PWT171258	T17	27.87	33.32	35.83	PM, ♂
		PWT181258	T18	33.07	32.33	U	PM, ♀
		PWT191258	T19	27.09	31.75	32.17	PM, ♂
		PWT201258	T20	27.18	31.85	32.07	PM, ♂
		PWT211258	T21	28.37	31.96	35.36	PM, ♂
	Scat	PWS11258	T17-T21	20.43	33.11	35.46	PM, ♂
		PWS21258	T18	23.79	30.99	36.48	PM, ♂
		PWS31258	T19-T20	29.36	U	U	U
C7	Hair	PWT121358	T12	22.00	29.84	30.99	PM, ♂
		PWT131358	T13	22.18	29.85	30.67	PM, ♂
		PWT141358	T14	23.06	31.28	31.69	PM, ♂
		PWT151358	T15	24.57	31.54	32.74	PM, ♂
		PWT161358	T16	23.09	31.41	31.62	PM, ♂
		PWT171358	T17	24.80	32.85	33.20	PM, ♂
		PWT181358	T18	24.82	29.20	U	PM, ♀
		PWT191358	T19	23.49	30.44	U	PM, ♀
		PWT201358	T20	22.89	30.35	U	PM, ♀
		PWT211358	T21	22.60	30.09	30.56	PM, ♂
	Scat	PWS11358	T17-T21	31.40	U	U	U
		PWS21358	T17-T21	24.98	35.67	39.89	PM, ♂
		PWS31358	T18-T21	24.80	36.80	38.48	PM, ♂
		PWS41358	T18	22.26	37.31	U	PM, ♀
		PWS51358	T12-T13	24.03	35.81	37.26	PM, ♂
		PWS61358	T12-T15	U[b]			

[a]Pine marten (PM), male or female [b]Later identified as a fox scat with the FOX probe.

Table 26: Species and sex identification of hair and scat samples collected in Gardenmorris Forest in May 2008. Samples which did not amplify are indicated (U).

Session	Sample	ID	Hair-tube	PM3	ZFX	ZFY	Result
C5	Hair	GMT81358	T8	29.30	32.98	33.89	PM, ♂
		GMT91358	T9	23.06	31.40	32.07	PM, ♂
		GMT101358	T10	22.34	30.38	U	PM, ♀
		GMT111358	T11	27.12	32.16	33.45	PM, ♂
		GMT221358	T22	20.33	30.59	32.80	PM, ♂
		GMT231358	T23	19.85	29.31	U	PM, ♀
		GMT241358	T24	18.67	U	U	U
		GMT251358	T25	20.30	31.34	U	PM, ♀
	Scat	GMS11358	T9	28.95	32.11	34.63	PM, ♂
C6	Hair	GMT81558	T8	U	31.75	32.24	PM, ♂
		GMT91558	T9	20.87	31.99	32.42	PM, ♂
		GMT101558	T10	21.47	31.29	U	PM, ♀
		GMT111558	T11	20.94	31.05	31.34	PM, ♂
		GMT221558	T22	20.33	31.27	31.76	PM, ♂
		GMT231558	T23	21.44	30.54	30.99	PM, ♂
		GMT241558	T24	21.49	31.60	32.07	PM, ♂
		GMT251558	T25	U	32.64	33.23	PM, ♂
	Scat	GMS11558	T8	U[a]			
		GMS21558	T8	U			
		GMS31558	T8	U[a]			
C7	Hair	GMT81758	T8	23.51	31.52	32.28	PM, ♂
		GMT91758	T9	22.39	30.45	31.34	PM, ♂
		GMT101758	T10	22.09	29.65	30.21	PM, ♂
		GMT111758	T11	23.80	29.20	30.93	PM, ♂
		GMT221758	T22	21.83	28.74	U	PM, ♀
		GMT231758	T23	22.25	29.80	30.78	PM, ♂
		GMT241758	T24	22.99	29.73	31.07	PM, ♂
		GMT251758	T25	23.19	30.46	31.36	PM, ♂
	Scat	GMS11758	T9	21.71	31.85	35.14	PM, ♂
		GMS21758	T9-T22	29.36	30.07	U	PM, ♀
		GMS31758	T22	20.82	35.85	40.86	PM, ♂
		GMS41758	T25	U[a]			

[a]Later identified as fox scats with the FOX probe.

Individual identification

All 53 hair samples amplified at least one of the eight microsatellite loci used to identify individuals in the census (Panel_E, Table 9). The scat samples were not fully genotyped as preliminary amplification success was low, with a genotype obtained at the Ma2 locus for just 3 out of 8 scats in Session C7 (PWS31358, 171/171; PWS41358, 177/177; PWS51358, 175/177). Resources were targeted towards the hair samples given the poor amplification success and high genotyping error rate for scats in the 2006-2007 sample set. Overall microsatellite amplification success across the eight loci was 93.8% for the 848 PCRs which were initially carried out for the hair samples (Table 27).

Table 27: Reliability of remotely plucked hair genotyping in the population census. Amplification success (Amp%), Allele dropout rates (ADO%) and false allele amplification rates (FA%) are indicated.

Locus	**Amp (%)**	**ADO (%)**	**FA (%)**
Ma2	94	0	0
Mel1	97	0	0
Gg7	94	0	3.8
Mvi1341	95	0	0
Mer041	92	1.6	11.4
MLUT27	92	9.3	1.9
Mel105	92	1.7	0
Mvi1354	94	0	1.5
Mean	**93.8**	**1.6**	**2.3**

Two of the samples were excluded from further genotyping due to low amplification success rates (6% and 31% of replicates) across loci. The genotype reliability was also high as only 15 errors were recorded from 848 reactions (nine allele dropouts and six false allele amplifications), giving a per reaction error rate of 0.018. Genotyping error rates varied per locus. No mismatches were detected between PCR replicates for Ma2, Mel1 and Mvi1341. The majority of inconsistencies were attributed to Mer041 and MLUT27.

The amplification success for the 2008 census was higher than the 2006-2007 census set across seven microsatellite loci (Figure 45). There was a significant improvement in the amplification success for the loci Gg7 and Mer041 with the 2008 sample set, presumably due to the higher quality DNA samples.

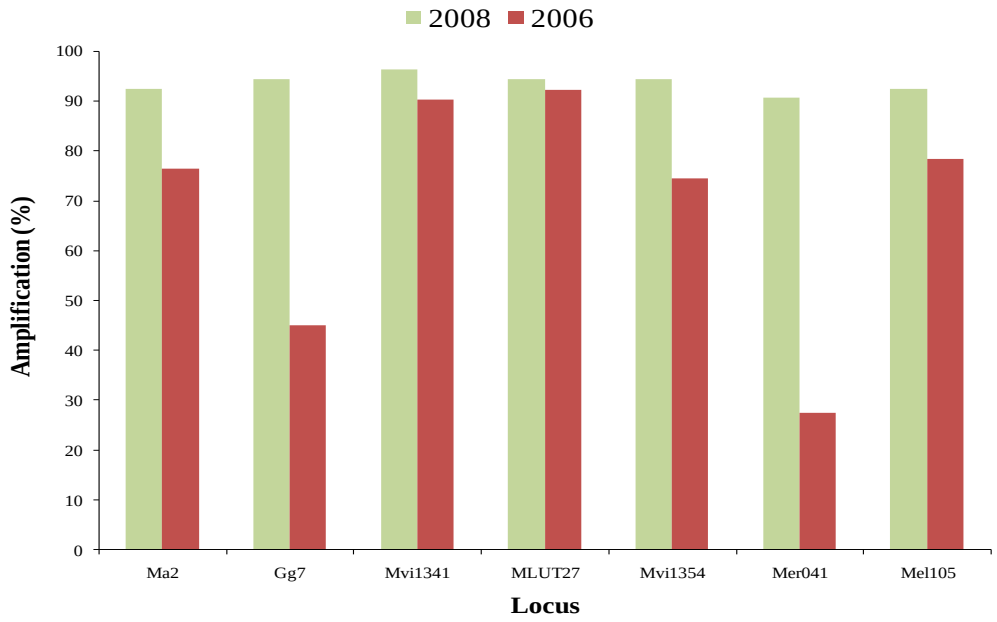


Figure 45: Microsatellite locus amplification success rates for the 2006-7 and 2008 datasets.

Considering all replicates required to complete multiple tubes replication (n = 1102 PCRs), where a correct genotype was counted as 1 and an incorrect genotype or failed amplification was counted as zero, the global quality index of the 2008 census data was high at 0.92. This value is an indicator of the quality of a non-invasive genetic dataset, with a value of 1 equal to 100% genotype reliability (Miquel *et al.* 2006).

Nine unique multilocus genotypes were identified from the set of 51 hair samples after multiple-tubes genotyping. Two pine marten were identified in Gardenmorris (1 male and 1 female) and seven (5 males and 2 females) in Curraghmore (Table 28). Three individuals were captured only once in the three sampling sessions in Curraghmore.

Such multilocus genotypes are typically repeated to rule out genotyping errors (Mowat and Paetkau 2002). However, individual CE05 was different from the other six individual genotypes in Curraghmore at the most informative locus (Ma2) and was also live trapped in February 2008 (male T130, Table 25).

Table 28: Unique genotypes identified in the population census. The number of times each genotype was captured in the hair-tube survey (n) varied per individual. Consensus genotype mismatches are shown in italics. Note that CE02a and CE02b are counted as different individuals due to different sex typing results.

ID	Sex	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354	n
Curraghmore										
CE01	♂	171/175	112/112	159/159	174/178	108/108	151/157	129/129	208/208	11
CE02	♀	171/177	108/112	165/165	168/178	108/110	151/153	129/131	200/208	5
CE02a	♂	171/177	108/112	165/165	168/178	108/110	151/153	129/131	200/208	1
CE02b	♂	171/177	108/112	159/165	168/178	108/110	151/153	129/131	200/208	1
CE03	♀	177/177	108/112	165/165	168/178	110/110	151/157	129/129	200/208	6
CE04	♂	171/171	112/116	165/167	168/174	108/108	151/153	129/129	200/208	3
CE05	♂	175/177	112/112	159/165	174/178	108/108	157/157	129/129	200/208	1
Gardenmorris										
GM01	♂	171/177	112/112	159/165	168/178	108/110	157/157	129/131	208/208	16
	♂	171/177	112/112	159/165	168/178	108/110	*155*/157	129/131	208/208	1
	♂	171/177	112/112	159/165	168/178	108/110	*151*/157	129/131	208/208	1
GM02	♀	171/171	112/112	159/165	178/178	108/108	151/157	129/131	208/208	5

CE02a and CE02b were not captured in other surveys. The similarity of the two genotypes to each other and to CE02 suggested either the three individuals were related or genotyping error was responsible for the mismatches with CE02. However, CE02a and CE02b were genotyped as males, while CE02 was female (Table 28). CE02a was also different at one allele, 159 at the Gg7 locus. While it is recommended that some level of mismatch is allowed at microsatellite loci for individual identification to avoid

biasing population size estimates (Creel *et al.* 2003), differences in sex typing results have biological significance and are usually counted as different individuals (Hajkova *et al.* 2009). The sex typing was repeated for CE02a and CE02b, to give a total of four PCR replicates, all of which were identified as male, with relatively low C_T values (average C_T values for ZFX/ZFY were 33.13/34.88 for CE02a and 33.00/35.84 for CE02b). In addition, if these samples were a mixture of hair from CE02 and one of the three males then three alleles should be observed for at least one of the microsatellite loci. The two genotypes were therefore assumed to be close relatives of CE02. Given the time of the year, and the collection of the samples from hair-tubes CE02 previously visited, it was possible these two males were actually cubs of the female CE02, travelling with her or independently within her territory.

Plotting individual home range

Minimum convex polygons (100%) were drawn around the locations of the hair-tubes each individual pine marten visited (Tables 29 and 30) to plot the minimum home range for each individual during the survey period (Figures 46 and 47). Although these home ranges most likely did not represent the true size of each territory, the plot gives extra validity to the genotyping results as all samples which were assigned to an individual were found within a small area, realistic of a home range size and not scattered randomly throughout the forest. Although the number of samples genotyped for each marten and the duration of the study period was probably not enough to get an accurate picture of home range, the typical pattern of intrasexual territoriality was detected with the territories of each sex being separate and those of opposite sexes overlapping to some degree (Schropfer *et al.* 1997).

Table 29: Hair-tubes visited by each individual during the census of Curraghmore Forest.

Hair-tube	Sampling session			Visiting individuals
	C5	**C6**	**C7**	
T12	CE01	CE01	CE01	1
T13	CE02	CE02	CE01	2
T14	CE01	CE02	CE05	3
T15	CE01	CE01	CE01	1
T16	U	CE02	CE01	2
T17	CE01	CE02b	CE01	2
T18	CE03	CE03	CE03	1
T19	CE03	CE04	CE03	2
T20	no sample	CE04	CE03	2
T21	CE02	CE02a	CE04	3
N	3	6	4	

Table 30: Hair-tubes visited by each individual during the census of Gardenmorris Forest.

Hair-tube	Sampling session			Visiting individuals
	C5	**C6**	**C7**	
T8	GM01	GM01	GM01	1
T9	GM01	GM01	GM01	1
T10	GM02	GM02	GM01	2
T11	GM01	GM01	GM01	1
T22	GM01	GM01	GM02	2
T23	GM02	GM01	GM01	2
T24	GM01	GM01	GM01	1
T25	GM02	GM01	GM01	2
N	2	2	2	

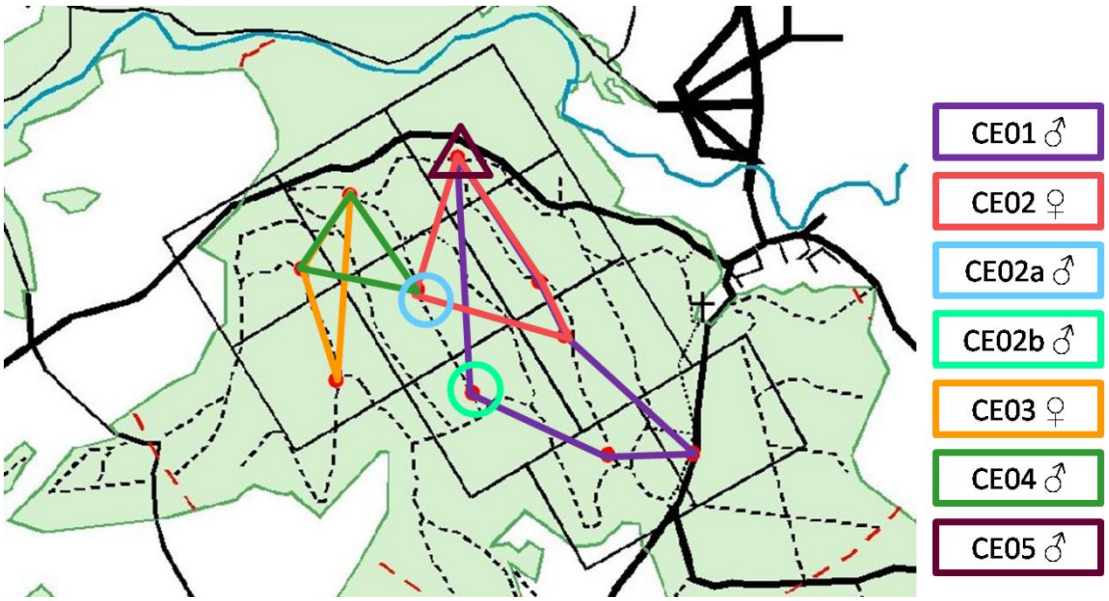


Figure 46: Individual home ranges in Curraghmore Forest. Minimum Convex polygons (100%) were drawn around the hair-tube sites (red filled circles) which each pine marten visited during the census.

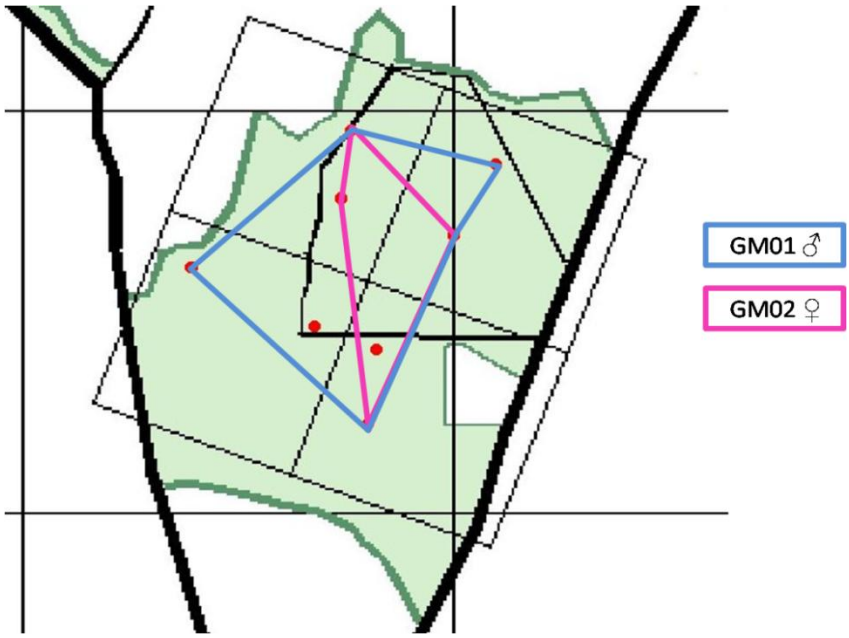


Figure 47: Individual home ranges in Gardenmorris Forest.

5.3.4. Minimum population size estimate through live capture

Five pine marten (three males and two females) were captured in one night (10 trap nights) in Curraghmore in June 2008. The number and sex of the captured individuals matched the results of the non-invasive census (the juveniles were not captured). The microsatellite genotypes of the live captured individuals confirmed the same animals were trapped with invasive and non-invasive methods (Table 31). The genetically determined sex of all hair samples which were associated with an individual CE01-CE05 identified from plucked hair matched the known sex of the individual from live trapping, thereby validating the real-time PCR ZF intron sex typing assay.

One of the females (F410) showed signs of recent lactation, and was later spotted with two kits during a radio-tracking session (Pete Turner, pers. comm.). The sex of these kits could not be determined but these field observations may explain the identification of two unique male genotypes (CE02a and CE02b) in the non-invasive census which were similar to the female CE02 (Table 28).

Table 31: Unique multilocus genotypes from remotely plucked hair (normal font) compared to reference genotypes of live trapped individuals (bold font) in Curraghmore forest.

Individual	Sex	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354
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
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
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
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
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

5.4. Discussion

Non-invasive genetic sampling of pine marten populations

Non-invasive genetic methods have been widely applied to studies of mustelid species (Mowat & Paetkau 2002; Frantz *et al.* 2003; Hung *et al.* 2004; Hedmark & Ellegren 2007), but for the European pine marten *Martes martes*, have generally concentrated on species identification (Domingo-Roura 2002; Vercillo *et al.* 2004; Statham *et al.* 2005; Ruiz-Gonzalez *et al.* 2008) and molecular sexing (Lynch & Brown 2006; Statham *et al.* 2007). The reliable amplification of microsatellite markers from biological samples is a prerequisite for identifying individual pine marten in order to characterise the size and structure of populations within the landscape. Preliminary results indicated that this would be difficult to achieve as only 44% and 16% of the hair and scat samples respectively had matching microsatellite genotypes between PCR replicates for the 2006-2007 sample set, despite the small size (110-bp) of the Mel1mini locus used to screen the samples.

The age of the samples was probably the most important factor contributing to the poor results for the 2006-2007 sample set. DNA degrades rapidly when exposed to environmental conditions such as humidity, UV radiation and decomposer organisms (Santini *et al.* 2007). Scats should be collected as soon as possible for optimal results, preferably within one week of deposition (Piggott 2004; Hajkova *et al.* 2006; Arrendal *et al.* 2007; Santini *et al.* 2007). The scats collected during the repeat survey in 2008 were less than three days old as the tracks were cleared of scats before the start of the census in both the Curraghmore and Gardenmorris study sites, yet microsatellite DNA amplification success was still low (37.5%) in comparison to plucked hair (93.8%). This is in contrast with the 88% success rate for nuclear DNA amplification from scats with the real-time PCR sex typing assay, and the 91% success rate for mitochondrial DNA amplification for the species typing assay. Scats are a reliable source of DNA for non-invasively assessing pine marten presence and distribution but further adjustments to

microsatellite genotyping protocols are required before they can be confidently used to estimate population size.

Sample collection is the first and possibly the most crucial step in non-invasive genetics (Lampa *et al.* 2008). Scat DNA degradation may be reduced by collecting the samples directly into the preservation agent S.T.A.R. buffer, which can then be kept at room temperature for short term storage up to five days, or at -20°C for longer term storage until DNA extraction. A suitable alternative would be storage in 70 or 95% ethanol (Frantz *et al.* 2003; Santini *et al.* 2007). DNA extraction should also be carried out as soon as possible as amplification success can be reduced by as much as 20% with two weeks of storage at -20°C (Lampa *et al.* 2008). The season of collection can also have a significant effect on DNA amplification success rates due to differences in diet or weather conditions (Maudet *et al.* 2004; Piggott 2004; Arrendal *et al.* 2007) and should be taken into consideration when planning future non-invasive sampling projects.

Scats are an important source of DNA for monitoring pine marten populations as they are collected without disturbing the population and can yield additional information on diet, breeding status and parasite load (Kohn & Wayne 1997). The future optimisation of scat DNA genotyping protocols would be a significant contribution to the study of these elusive mammals. The scat DNA amplification success rate (37.5%) reported here is only based on a small number of samples amplified at a single locus. This percentage would probably improve with a larger sample set but an average of approximately 60% is to be expected (Hajkova *et al.* 2009) using standard PCR protocols. Hedmark & Ellegren (2006) achieved an amplification success rate of 91% for wolverine scats with a modification of the pre-amplification method originally devised by Piggott *et al.* (2004). Two PCR replicates were carried out for each of four multiplex groups (amplifying 18 loci in total) in the first step, followed by single locus PCRs in the next step using the multiplex amplifications as template DNA. The multiplex pre-amplification approach is worth evaluating for pine marten scats as other studies have also improved genotyping success with the two-step PCR protocol compared to standard single locus PCR (Bellemain & Taberlet 2004; Lampa *et al.* 2008).

Remotely plucked hair was a more reliable DNA source than scats for genetic tagging of individual pine marten. The amplification success rates were high for both mitochondrial (96.2%) and nuclear DNA (93.8-96.2%) for hairs collected during the 2008 population census. Genotyping errors can have a strong negative impact on the integrity of non-invasive genotyping by inflating population size estimates through the creation of unique genotypes which do not exist in the population under study (Creel *et al.* 2003). Each sample genotype was determined from an average of 2.7 PCR replications per locus using a modification of the comparative multiple-tubes genotyping approach of Frantz *et al.* (2003) to aid in the detection of erroneous genotypes. The overall error rate was low at 1.8% across 848 reactions and the final multilocus genotypes exactly matched those of individuals live captured in an independent survey. Preliminary trials have indicated that several loci can be easily amplified in multiplex reactions (Chapter 4, section 4.3.5) which would reduce genotyping costs further, enabling genetic methods to be more easily integrated into large-scale monitoring projects in the future. Genomic DNA was successfully extracted from hair using the Chelex® 100 protocol but future optimisation of the extraction protocol may improve success rates further for lower quality samples. McNevin *et al.* (2005) suggested the Chelex resin should be added to the digestion buffer after the Proteinase K and not simultaneously, as the Chelex will sequester calcium ions which are required for full enzyme activity. Alternatively, additional calcium ions could also be included in the extraction buffer to prevent the full sequestration of calcium by the Chelex resin (Pfeiffer *et al.* 2004).

Recent non-invasive genetic studies for badgers (Frantz *et al.* 2004) and otters (Anderson *et al.* 2006) have also found hair to be more reliable for microsatellite DNA analysis than scats. Scats remain useful for assessing pine marten presence, distribution and diet in traditional surveys but plucked hair genotyping may be more cost-effective for intensive surveys as a result of requiring fewer reactions per sample to obtain reliable microsatellite genotypes. The field and molecular methods described in this study are readily transferrable to other marten species with some minor modifications to the primer sequences and possibly the hair-tube dimensions. The hair-tube designed for use

in this study and others (Statham 2005; Roche 2008) has been used in pilot studies to survey American marten *Martes americana atrata* in western Newfoundland (B. Hearn, pers. comm.), and pine marten and stone marten *Martes foina* in central Italy (F. Vercillo, pers. comm.).

Population census

Nine unique multilocus genotypes (seven in Curraghmore and two in Gardenmorris) were identified from a total of 51 hair samples, which was taken as the census population size during the study period, giving a population density 2.0-2.8 marten/km^2 (2 marten/km^2 when considering adults only, see below). Molecular sex identification by real-time PCR amplification identified five males and two females in Curraghmore, while the two Gardenmorris individuals were a male and female. Not all individuals had equal catchability, which is to be expected for any type of trap (Krebs 2006). Six individuals visited hair-tubes on more than one occasion, within and between sampling sessions, which suggests hair sampling may be used in future to estimate pine marten population size with capture-mark-recapture (CMR) analysis using computer programs such as CAPTURE (Mowat & Paetkau 2002), or CAPWIRE (Miller *et al.* 2005) which specifically deals with non-invasive genetic CMR where an individual can be captured on more than one occasion within a sampling session, unlike live trapping CMR.

Three individuals (CE05, CE02a and CE02b), all males, were identified from only one hair sample in the census survey period. The male CE05 (detected from hair-tube T14 in session C7, Table 29) was also present in the same area of Curraghmore Forest in February 2008, three months prior to the census, according to the genotype obtained from live trapped marten T130 (Table 24). This animal was also captured in June and July 2008 in Tomahawk wire-mesh traps. CE05 appears to reside in the territory of CE01 who was captured 11 times, based on the available locations for both animals from non-invasive genetic sampling (Figure 46).

The two other males (CE02a and CE02b) which only visited one hair-tube during the course of the study were identified as possible offspring of the female CE02 as the CE02a genotype differed only in sex while CE02b differed in sex and by one allele at the Gg7 locus (Table 28). These multilocus genotypes were obtained by multiple tubes PCR replication and three additional lines of evidence support the definition of these two genotypes as real individuals in the study population. Firstly, the real-time PCR sex identification test was considered accurate as the results of all sex identification for the remotely collected hair samples matched the known sex of the live trapped individuals with the same genotype. Second, both CE02 and CE02a visited the same hair-tube in different sampling sessions (Table 29) and CE02 was live captured in June 2008 at the same hair-tube site (T17) visited by CE02b in the May 2008 census (Table 29). Finally, CE02 had extended nipples from recent lactation when captured in June and was later observed with two almost fully grown kits in July 2008.

Population status

Genetic diversity is regarded as a major component of biodiversity which needs to be conserved, along with ecosystem and species diversity. Laikre *et al.* (2008) defined conservation genetic monitoring as the "systematic, temporal study of genetic variation within particular species/populations with the aim to detect changes that indicate compromise or loss of such diversity". This study demonstrated the accuracy of remotely plucked hair genotyping for identifying individual pine marten. However the sampling methods must be reliable over time to be used as a monitoring tool to gather temporal information on the magnitude and distribution of genetic diversity (Laikre *et al.* 2008).

Thirteen multilocus genotypes from the 2006-2007 set (Table 19) were compared to individual genotypes identified in the 2008 census (Table 28) to identify any possible matches. The hair samples PWH28 (male: C_TX, 38.53; C_TY, 37.47) collected in October 2006 and the sample PWH66 (female, C_TX, 34.32; C_TY, undetermined) collected in April 2006 matched the individuals CE01 and CE03 which were detected from live traps

and hair-traps in Curraghmore in 2008 (Table 32). Genetic tagging can therefore be used to ascertain the status of a pine marten population. In this case these two animals were resident in the same area of Curraghmore Forest in 2006 and 2008 (Figure 48).

Table 32: Individuals identified as resident in Curraghmore Forest in 2006 and 2008. Data taken from Table 23 for the 2006-2007 dataset and compared to the genotypes of individuals identified in the 2008 population census.

Extract ID	Captured	Ma2	Mel1	Gg7	Mvi1341	MLUT27	Mer041	Mel105	Mvi1354
PWH28	2006	171/175	112/112	159/159	174/178	108/108	151/157	129/129	208/208
CE01	2008	171/175	112/112	159/159	174/178	108/108	151/157	129/129	208/208
PWH66	2006	177/177	108/112	165/165	168/178	110/110	151/157	129/129	200/208
CE03	2008	177/177	108/112	165/165	168/178	110/110	151/157	129/129	200/208
PWH74i	2006	171/177	112/112	159/159	178/178	108/108	151/157	129/131	208/208
PWH74ii	2006	171/171	112/112	159/159	178/178	108/108	151/157	129/131	208/208
PWH74iii	2006	171/171	112/112	159/165	178/178	108/108	151/157	129/133	208/208
PWH74iv	2006	0	112/112	159/159	178/178	108/108	151/157	129/131	208/208
GM02	2008	171/171	112/112	159/165	178/178	108/108	151/157	129/131	208/208

The hair sample PWH74 was collected in May 2006 (female, C_TX 35.52; C_TY, Undetermined), in the part of Curraghmore which was not surveyed in 2008 (Coolfin, Tigroe and Glenhouse, Figure 36). This sample had a genotype very similar to the female GM02 which was identified in the 2008 census 12km away in Gardenmorris Forest. The remaining 6 polymorphic loci (Mvis072, Ggu234, Ma8, Mvis020, Mvis075 and Ggu454) were also genotyped in a multiplex reaction (Table 19) and the two genotypes (GM02 and PWH74) differed by just one allele at locus Ggu234. These two females identified through hair capture may have been different individuals but very closely related, becoming genetic shadows of each other with the low genetic variability in the population. However, it is possible to monitor such movements with genetic tagging of pine marten using remotely plucked hair samples in the future with systematic surveys.

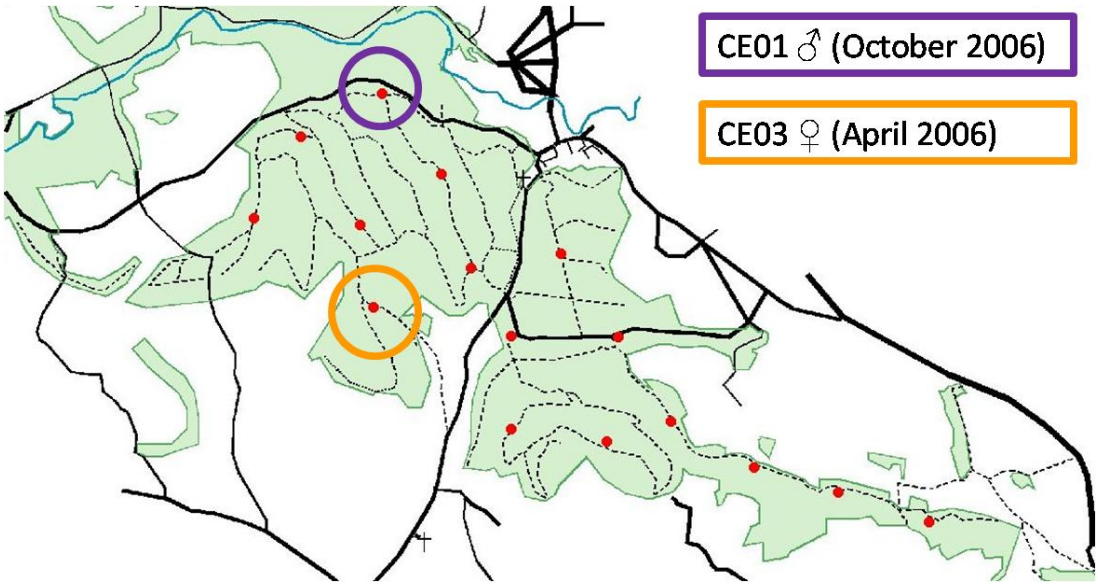


Figure 48: Home range fidelity for two individuals in Curraghmore Forest. CE01 and CE03 were also identified from the 2006-2007 sample set from hair-tube sites (red filled circles) close to the capture site in the 2008 census (Figure 46).

The combination of the 2006 and 2008 hair-tube survey results indicates the population in Curraghmore forest is relatively stable and of high density, with individuals resident in similar territories from one year to another. Fewer females were detected than males, but it is unclear whether this represents a lack of sampling due to the smaller size of the female territories or an actual sex-bias in the population. A female (CE06) was live trapped in July 2008 in the home range of the male CE01 which was not detected in the previous hair sampling or live trapping sessions. CE06 was probably resident during the surveys as it was also present in the same forest in 2009 (C. O'Reilly, unpublished data).

Sampling design is an important factor to consider for adequate sampling of the population. In this study a fixed site design was used with a 500 x 500 m grid size. Tube visitation rates increased in both sites in the pre-bait period to 100% by the end of the census period. There was a scat placed in a prominent place at hair-tube T18 for the final three days of the census, presumably to mark the site. This kind of trap habituation may bias estimates of population size and can be reduced by moving hair-tube sites between

sampling sessions. Boulanger *et al.* (2006) detected 15-25% more females with a 64 km^2 grid unit in which hair-sampling stations were moved each session compared to a 25 km^2 grid fixed site design for a bear *Ursus arctos* census in Alberta, Canada. A moving site design is more expensive but it captures more individuals, particularly when females have smaller home ranges than males, as is the case for pine marten.

The importance of Curraghmore Forest as a core population for pine martens in Co. Waterford and possibly other populations further afield is evident. Two individuals were killed at Fiddown Bridge which crosses the River Suir and presence has been detected from other forests along the Suir Valley in previous studies (Statham 2005). As such, the population at Curraghmore is an important source of individuals for the colonization and establishment of smaller, sink populations such as Gardenmorris, and the population should be monitored to ensure its survival. Population dynamics can now be more thoroughly investigated in future with non-invasive genetic sampling, which has proven to be accurate and robust, once technical limitations are overcome.

Chapter 6

General Discussion

6.1. Summary

As a carnivore, forest-dweller and furbearer, the European pine marten *Martes martes* has suffered either directly or indirectly at the hands of man for several centuries through woodland clearance and persecution. Little is known about the Irish pine marten population apart from distributional data collected in two surveys conducted 25 years apart in 1978-1980 (O'Sullivan 1983) and 2005-2006 (O'Mahony *et al.* 2007). The first survey found that the pine marten was widely distributed at the early part of the last century but changes in agricultural practice in the 1950s had a significant negative impact, causing the loss of regional populations and the restriction of the species largely to a stronghold in the west of Ireland. A similar pattern exists for the marten populations in Northern Ireland (Tosh *et al.* (2007). The most recent survey for the Republic fo Ireland revealed the pine marten is currently recolonising former areas with the help of legal protection, translocations and significant afforestation in the years between the surveys.

These surveys are dependent on the location and accurate characterization of pine marten scats, which can be difficult to distinguish from other carnivore species such as the red fox, particularly when the pine marten is at low densities (Davison *et al.* 2002). Surveying for scats is further complicated by the fact that a large number may not be located on forest trails at all (Roche 2008). Researchers have overcome these difficulties by using genetic identification of scats to confirm species and the use of hair-sampling devices for mapping species distribution in areas where scat location is difficult (Statham 2005; Lynch 2006). While the methods previously used for genetic species and sex identification were successful, the identification of individual animals for population size estimation and intra-specific genetic structure analysis was hampered by low polymorphism of the available microsatellite loci (Statham 2005; Lynch 2006), the genetic marker commonly used for the purpose. This project therefore set out to find a set of microsatellite markers suitable for DNA fingerprinting of European pine marten, with particular reference to the Irish population.

Firstly, in Chapter 2, a novel method was developed for extracting DNA from scats which used a specially formulated buffer for preserving faecal samples. Target DNA amplification success, assessed by real-time PCR, was high at 96%. Real-time PCR targets a much smaller PCR product than conventional PCR techniques, which increases the chance of obtaining a result for hair and scat samples which typically have low DNA concentrations. The method was also used to extract DNA from scats of varying ages for the National Pine Marten Survey (NPMS) in combination with species identification by real-time PCR, with a similar success rate of 95% (O'Reilly *et al.* 2008), a considerable improvement on previous molecular analysis of pine marten scats using conventional PCR techniques (53%; Davison *et al.* 2002).

In Chapter 3, novel real-time PCR methods for species and sex identification were tested with hair and scat samples collected from captive and wild martens. Again the DNA amplification success rates were high at 95% for species identification of both sample types and only slightly lower for sex typing at 85% and 92% for hair and scats respectively. After some optimisation and redesign of the primer and probe combinations, both assays were shown to be specific to pine marten (species test) and either the ZFX or ZFY genes (sex test). The sex typing assay was further modified later in the project, targeting a more variable intron sequence to reduce the chance of amplifying non-target DNA from the scats. Again, high amplification success rates of 88-96% were obtained for hair and scat samples and the specificity of the assay was verified with DNA samples from known sex individuals.

Chapter 4 describes the screening of 32 mustelid microsatellite loci for amplification and polymorphism in pine marten. Twenty loci were selected to assess genetic variability in 41 individual pine marten collected as casualties from Irish roads. Allelic diversity (2.29) and heterozygosity (0.34) values were low in comparison to continental European populations and a genetic signature of recent decline in effective population size was detected, which may be associated with the impact of changes in farming practices in the mid-twentieth century. A preliminary analysis of genetic structure suggests movement and gene flow is occurring between the core populations in Ireland. The Irish population

should be treated as a single unit in terms of management until a more extensive inventory of the magnitude and distribution of genetic diversity can be carried out to properly assess the genetic status of the Irish pine marten.

Non-invasive genetic sampling (NGS) is the only viable option for broad geographical sampling of pine marten populations in Ireland and other countries where genetic samples are not readily available due to the absence of a fur harvest. Statistical analysis showed eight of the most variable microsatellite loci were sufficient to discriminate between individuals sampled non-invasively in the field, despite the low background genetic variability in the population. A pilot non-invasive sampling study was then conducted in Curraghmore Forest and Gardenmorris Wood, Co. Waterford to estimate census population size. Species and sex were determined by real-time PCR and individual identification was carried out by genotyping each sample at eight microsatellite loci. The scat genotyping was abandoned due to poor success rates but the amplification success rate for microsatellites from plucked hair was high at 94%. In Curraghmore Forest five males and two females were identified, five of which (three males and two females) were also caught in live traps in the same site one month later. The two males which were not live trapped were possibly offspring of one of the females based on their genotypes and personal observations of two kits with one of the captured females in later surveys, but parentage could not be associated with confidence due to the low variability of the marker set.

This study demonstrated that NGS is a feasible, accurate and robust method for tagging individuals, but samples (particularly scats) should be analysed as soon as possible after collection from the field. Improvement of scat genotyping rates would represent a major coup for genetic monitoring as scat collection is the standard survey method for pine marten in several countries. Plucked hair was a more reliable source of DNA for microsatellite analysis and may be the most cost effective means to estimate population size, particularly in areas where scats are difficult to locate. The hair-tubes and molecular techniques used in this project will find use in conservation genetic studies of other populations of pine marten in Europe and in other species of arboreal mammal

with some modification of the tube dimensions and primer and probe sequences, and careful selection of microsatellite loci to suit the particular aim of the study.

6.2. Future directions

Habitat fragmentation is possibly the biggest threat to the survival of wildlife as it leads to the increased isolation and decreased area of habitat patches, which may render local populations vulnerable to regional extinction (Bright 1993). In Ireland, the forest cover was estimated at 69,000 ha or 1% of the total land area at the end of the 19th century (DAFF 2007). Significant afforestation took place in the 20th century resulting in a current total forest cover of 10%. This figure increases to 14% when small woodplots, hedgerows, individual trees and scrub habitats are included, yet 63% of the total land area in Ireland is under grassland, cropland, paved roads and built land. Furthermore, the majority of the forest estate is represented by even-aged conifers planted below 300m elevation in the last two decades.

These major changes to the structure of Ireland's forests no doubt had a significant effect on the distribution and population dynamics of the pine marten and other forest mammals. Recent survey data suggests that although some regional extinction of pine marten populations seems to have occurred in the last century, the population is recovering and recolonising former parts of its range in Ireland with the help of this increased afforestation among other factors (O'Mahony *et al.* 2007). However, Bright (1993) cautioned that the threat of habitat fragmentation should not be ignored simply because it has occurred in the past, as the critical thresholds of habitat fragmentation with which mammal populations can survive may only now have been reached. Factors which may lead to the sensitivity of a species to habitat fragmentation (Bright 1993) include:

- *Population density* – low population density may lead to the loss of populations in isolated fragments, but can be tempered by the immigration of individuals from neighbouring fragments.
- *Dispersal rates* – Low dispersal rates or short dispersal distances can slow the recovery of populations post-fragmentation and may be strongly influenced by the nature of the matrix habitat between the fragments. The presence of hedges or other corridors may be particularly important for facilitating dispersal in metapopulations.
- *Habitat selection* – Species which can utilize edge or matrix habitat may be less sensitive to fragmentation than habitat specialists, to a certain critical level of habitat fragmentation.
- *Vulnerability to chance events* – Small populations, particularly slow breeders, are more vulnerable to variability in the number or sex of offspring, or unpredictable environmental events such as disease outbreaks and natural disasters.

It is now possible to gather empirical data on some of the above factors for pine marten populations based on the methods developed in this project. For the estimation of population density, microsatellite analysis of hair samples collected remotely and non-invasively from free ranging pine marten during a defined census period provided an estimate of the minimum number of individuals present in two forests in a mosaic landscape. Absolute population size estimates may be obtained in future, using the same field and molecular techniques but with the application of capture-mark-recapture (CMR) statistical models. Traditional CMR methods are based on the capture and marking of individuals in the first sampling session, followed by recapture in subsequent sessions, with the ratio of the marked to unmarked individuals giving an estimate of census size (Nc). In non-invasive genetic sampling (NGS) individuals are "marked" by their genotype, which is supposed to be permanent (but may be misread due to genotyping errors), and "recaptured" in other sessions, or within the session, by the

genotyping of more non-invasively collected samples. Several statistical models are available for estimating Nc from NGS data, from one or several sampling sessions in open or closed populations, which were recently reviewed by Luikart *et al.* (2010).

It is possible to estimate Nc for very large areas with intensive sampling effort and use of the appropriate statistical models, for example grizzly bear *Ursus arctos* Nc was estimated over 33,480 km^2 of the Northern Contintental Divide Ecosystem in Montana, U.S.A by hair sampling (Kendall *et al.* 2009). The estimation of Nc from different habitats in the other core areas of population distribution in the west and midlands can be used to refine the only currently available population size estimate for Ireland of 2,905-11,622 individuals (O'Mahony *et al.* 2007), which was based on density estimates from a single study area. In the future, monitoring the effective population size (Ne) may be possible, enabling the early detection of bottlenecks and genetic structuring (Schwartz *et al.* 2010). Ne is the size of the ideal population that will result in the same amount of genetic drift as the observed population (Allendorf & Luikart 2007), and is a more reliable predictor of a populations ability to evolve in response to environmental changes than either the Nc or the genetic diversity of a population (Luikart *et al.* 2010).

The genetically depauperate nature of the Irish pine marten reported in this study and others (Davison *et al.* 2001; Kyle *et al.* 2003; Statham 2005; Lynch 2006) for microsatellites and the control region of mitochondrial DNA has potentially negative consequences for the population in terms of ability to adapt to future environmental changes. However, these loci are widely assumed to be neutral with respect to selection and therefore can only be used as a proxy for adaptive genetic variation and not a direct measure of the fitness of a population. Two other types of genetic marker which could be utilised in the future to study ecologically meaningful genetic variation are candidate genes such as the Major Histocompatibility Complex (MHC) which are associated with the ability to respond to pathogens and the initiation of an immune response (Piertney & Webster 2010), and Single Nucleotide Polymorphisms (SNPs) which are biallelic, of short amplicon size and abundant in the genome. A comprehensive picture of genome-wide genetic variability may be obtained through SNP assays, which have been

developed for non-model wildlife species (Sacks *et al.* 2009), but the availability of sequence data for the entire genome of *Bos taurus* enabled Pertoldi *et al.* (2010) to assay almost 53,000 SNPs for *Bison bonasus*. Such analyses may be possible in the near future for *Martes* species, especially with the use of next-generation sequencing technologies to obtain vast amounts of DNA sequence data.

If overall genetic diversity is low in the population, pine marten may be vulnerable to the introduction of novel diseases or parasites in the environment, regardless of any increase in range and abundance. Furthermore, reproductive potential may be affected by inbreeding and manifest in males as testicular abnormalities, for example in koalas (Seymour *et al.* 2001) and Florida panthers *Puma concolor coryi* (Mansfield *et al.* 2002). Although only limited live trapping was carried out in this study, two of the three males captured in Curraghmore appeared to have only one testicle descended (Appendix II). The prevalence of such abnormalities should be monitored in future in live trapped and road-killed individuals.

It is essential to quantify the degree of landscape connectivity as gene flow between local populations maintains genetic variation and spreads potentially adaptive genes (Segelbacher *et al.* 2010). No significant genetic structuring was identified in this study, which suggests the dispersal capability of pine marten is sufficient to homogenise allele frequencies between populations. However, Schwartz & McKelvey (2009) recommend that some information should be collected on patterns of local population spatial autocorrelation before applying landscape-level genetic analyses as strong-within population correlation can reduce the strength of between population differentiation. This study relied on the opportunistic collection of road-kill samples. Non-invasive genetic sampling may improve future analyses by allowing sampling in local populations and places where roadkill samples are less frequent. The addition of the new markers of Natali *et al.* (2010) may increase the power to delineate fine scale genetic structuring and give insights into the types of habitat which facilitate short and long-distance dispersal (Worthington-Wilmer *et al.* 2008). Different habitats have different permeability to movements, however at small spatial scales forest cover, fields,

hedgerows and built-up areas appear to act neither as corridors nor barriers to marten dispersal (Pereboom *et al.* 2008). On the landscape scale, genetic approaches can possibly be used in the future to predict, localize and implement conservation corridors (Segelbacher *et al.* 2010). This information could then be used as input for computer models which can enable researchers to give practical guidelines on the management of the landscape for pine marten conservation by evaluating changes in the genetic composition and structure of populations in response to environmental changes, both in the short and the long-term (Schwartz *et al.* 2010). Clearly there is much optimism and scope for the future conservation of pine marten populations in fragmented landscapes.

Appendix

Appendix I: Pine marten tissue sample information. French pine marten tissue was received from A. Bifolchi, University of Angers, France.

I.D.	Sex	RTA Date	County	Area	Collector
MM01	♂	Jun-97	Waterford	Kilclooney	P. Turner
MM02	♂		Kildare	Curragh	K. Buckley
MM03	♂		Unknown		D.P. Sleeman
MM04	♀		Laois	Unknown	J. Corcoran
MM06	♂	Dec-03	Waterford	Kilmeaden	M. Statham
MM07	♀		Laois	Ballyfin	J. Corcoran
MM08	♀		Kildare	Athy	G. O'Brien
MM09	♂		Kildare	Athy	G. O'Brien
MM11	♂		Tipperary	Cahir	G. O'Brien
MM13	♀	Apr-04	Fermanagh	Crom	A. Murray
MM14	♀		Offaly	Tullamore	
MM15	♂	Jun-04	Waterford	Ballinamona	P. Turner
MM16	♂		Fermanagh	-	A. Murray
MM17	♂		Monaghan	Clontibret	Peatlands Trust
MM18	♂	Jun-05	Waterford	Ballygunner	M. Statham
MM19	♂	Aug-05	Waterford	Mayfield	M. Statham
MM27	♂	Mar-06	Sligo	Lough Gill	J. Corcoran
MM28	♂	Jul-06	Clare	Tulla	P. Turner
MM29	♂	Aug-06	Clare	Kilnaboy	R. Crimmins
MM30	♂	Apr-07	Monaghan	Monage	J. Roche
MM31	-		Laois	Grange	A. Moran
MM32	♂	May-05	Waterford	Youghal Bridge	P. Smiddy
MM33	♂	Aug-06	Waterford	Dromana	P. Smiddy
MM34	♀	May-07	Tipperary	Dundrum	J. Breen
MM35	♀	May-07	Roscommon	Kiltoom	J. Fox
MM36	♂	Jul-06	Kildare	Blackford	J. Fox
MM37	-	Jul-06	Laois	Ballylynan	D. Buckley
MM38	♂	Apr-07	Galway	Headford	P. Stuart
MM39	♂	Jun-07	Clare	Tulla	P. Stuart
MM40		Jan-07	Galway	Ballygar	P. Smiddy
MM41		Oct-06	Roscommon		R. Carden
MM44		Sep-07	Offaly	Boora Bog	P. Stuart
MM49		Jul-07	Kildare	Prosperous	G. Farrell
MM50		Feb-07	Westmeath	Rathowen	L. Manning
MM51			Laois	Mountmellick	G. Rochford
MM52		Jul-07	Galway	Drimcong	B. Buckley
MM53		Mar-07	Kildare	Rathangan	E. Fenlon
MM54		Sep-06	Laois	Ballylynan	D. Bridgette
MM55		May-06	Laois	Ballyfin	E. Kirwan
MM56			Galway	Headford	P. Stuart
MM58		Jul-08	Galway	Dangan	P. Stuart

Appendix II: Biometric data for pine marten captured in Curraghmore Forest.

The weights and measurements in the table below were recorded from five pine marten which were live trapped in June 2008 in Curraghmore Forest, Co. Waterford. The values for both males and females were within the range obtained previously for pine marten in Killarney National Park (Lynch 2006).

Animal	Sex	Body Weight (kg)	Body Length (cm)	Tail Length (cm)	Hind Foot (cm)	Age from Tooth wear	Observations
F410	♀	1.05	43	19	8.5	Young adult	Probable litter
F433	♀	1.15	45	19	8.5	Young adult	
Lynch (2006)	♀	0.8-1.35	33-50	15-26	8.0-9.8		
M325	♂	1.67	54	22	10	Mature adult	One testicle?
M279	♂	1.70	48	22	9.5	Mature adult	
M395	♂	1.83	53	23	9.5	Young adult	One testicle?
Lynch (2006)	♂	0.85-2.05	33-54	16-30	8.5-10.5		

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