NON-INVASIVE GENETIC MONITORING OF PINE MARTEN (*MARTES MARTES*) AND STONE MARTEN (*MARTES FOINA*) IN AND AROUND THE NIETOPEREK BAT HIBERNATION SITE, POLAND.

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by

John Power

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Under the supervision of

Dr. Catherine O'Reilly and Dr. Peter Turner.

Department of Chemical & Life Sciences

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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.

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ABSTRACT

Międzyrzecz Fortified Front (MFF), NW Poland, was built by the Germans during World War II. The middle section of the MMF, the "Central Sector Wysoka", consists of a line of tunnels that connect to above ground fortifications. After World War II, the system was deserted, and due to its use by hibernating bats from Central Europe, it became designated as a NATURA 2000 site. It is now one of the ten largest bat hibernation sites in the European Union, with 38,500 bats belonging to 10 different species (Four species listed under Annex II of the Habitats Directive) recorded during the January 2015 bat census. The surrounding area consists of fragmented forest and farmland that supports pine marten and stone marten populations. During recent bat censuses, marten scats have been found in the tunnels as well as circumstantial evidence that marten have been predating on the hibernating bats. In order to assess the potential impact, (if any) of marten at this site, a non-invasive survey was conducted to collect marten scats. The aims of the study were to conduct molecular analysis that identified the species, sex and individual from scat samples. Secondly, a molecular based dietary study was conducted on scats to identify the presence of bat DNA in the scats.

Scat and hair tube surveys were used to collect samples for molecular analysis. DNA was extracted from these non-invasively collected samples. Existing TaqMan® MGB probe based qPCR assays were used for species identification and sex determination. Bat species-specific primers were designed to identify bat DNA in the scats using a SYBR® Green Dye qPCR assay. Microsatellite analysis was used for individual animal identification.

Results confirm that both pine marten and stone marten are present in the tunnels. The scat distribution of both species is similar above and below ground with only pine marten scats being found in the north of the survey area. Evidence of both species has been found in the tunnels throughout the year and not only when the bats are hibernating. Scats from multiple individuals of both species have been identified in the tunnels indicating that tunnel use is by more than one or two individuals. Scat densities in the tunnels are more associated with access points than with higher bat densities. Dietary analysis has confirmed the presence of bat DNA in scats from both marten species, with DNA from *Myotis daubentonii* and *Myotis myotis* occurring with the highest frequency. While the degree of marten consumption of bats appears high the impact on the resident bat population at present appears low.

1 INTRODUCTION

1.1 Background

Constructed in the 1930s and during WWII the Miedzyrzecz Fortified Front (MFF) was designed to defend Germany against any march on Berlin from the east. Originally part of Germany it is now located in the Lubusz Voivodeship of Poland. It lies approximately halfway between Berlin and Poznan (Figure 1.1).

The Central Sector “Wysoka”, starting south of Staropole, and ending on Lake Kursko, (Figure 1.1) is approximately 15 km long, and is studded with extra fortification objects connected by an underground tunnel system. This was due to the lack of large forests and lakes in this area that would provide natural terrain defences. The entire bunker system was never fully completed and this factor, coupled with an inexperienced group manning it meant that when the Russians did begin their invasion there was minimal resistance from the MFF (Miniewicz & Perzyk, 1993). As a result a large portion of the tunnel system remains intact. After the war the Russian, and then the Polish armies made use of the MFF but when they abandoned it, the system was discovered by migrating bats. In 1981 the Polish Minister of Forestry and Wood Industry proclaimed a part of the Miedzyrzecz Fortified Front a nature reserve called "Nietoperek". After protests, among fears that the bat reserve might be used as a nuclear dumping site the then Minister of Environment Protection and Natural Resources declared the MFF a protected site to be used both as a bat reserve and as a tourist attraction. Only qualified guides were to be permitted access to a defined tourist route and gratings were erected to minimise disruption to the bats in the remainder of the system. “Nietoperek” is now a NATURA 2000 site - PLH 080003 (G.E.P.D, 2012) with four species of bats on the Habitats II directive (EU Council, 2012) hibernating there each year. These species are *Myotis myotis, Myotis bechsteinii, Myotis dasycneme* and *Barbastella barbastellus.*

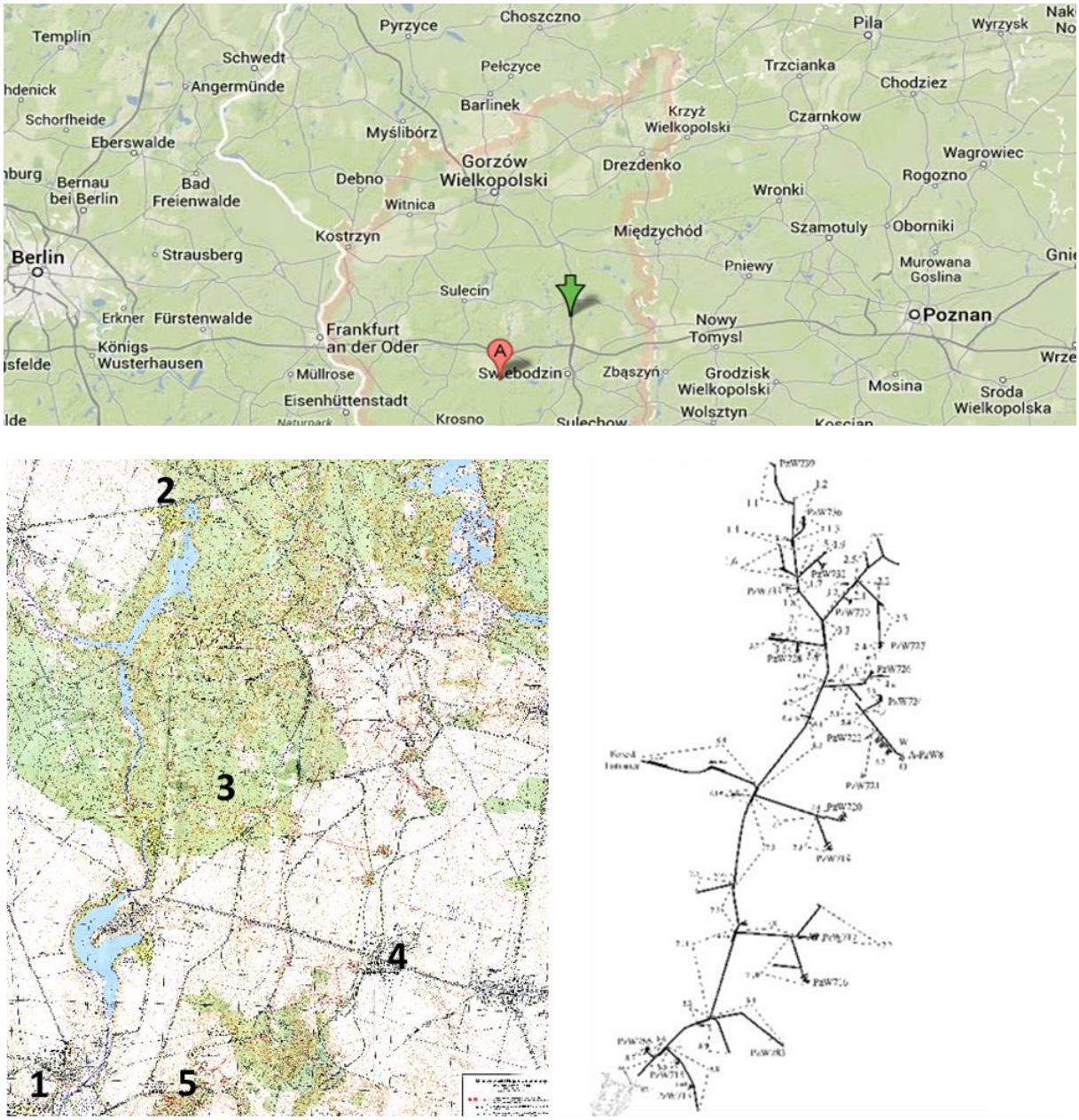


Figure 1.1 Location of MFF Central Sector including a map of the above ground area and a map of Nietoperek bat reserve. 1. Staropole 2. Kursko. 3 Forest entrance 4. Entrance at the museum in Pniewo 5. Entrance at Boryszyn.

For the purpose of this report the MFF Central Sector will be referred to as "the tunnels" or "tunnel system" as only a portion of the MFF is covered in the survey area. The entire tunnel system connecting the above ground fortifications is divided into 9 sections and these sections are further divided into sub-sections allowing the counting of each species of bat, and their location in the system as part of an annual census. There were over 38,500 bats of 10 different species found in the tunnels in January 2015 (R.E.P.D., 2015). The area above the tunnels is inhabited by both pine marten and stone marten. On previous bat censuses,

signs attributed to pine marten or stone marten have been seen down in the tunnel system. These include the presence of scats, as well as scratch marks from claws visible up the sides of some of the tunnel walls below places where bats are known to be hibernating.

The area above the tunnel system comprises fragmented forest, small villages and arable land. Directly above the bat reserve there are two main wooded areas, one in the north which contains an entrance to the tunnels large enough for military trucks (Figure 1.2) to use and one above the southern sections which contains two tourist entrances near Boryszyn and Pniewo (Figure 1.1).

Figure 1.2 Entrance to tunnels in the Northern Forest.

The southern forest is the smaller of the two and is a mixed wood forest. In most areas there is a dense understory similar to that previously found suitable for use by pine marten (Birks *et al*. 2005). The northern forest is larger, is also a mixed wood forest, and appears much more managed resulting in a less dense understory but there are areas with dense cover on the ground that would possibly be suitable as pine marten habitat. The arable land between these fragmented woodlands consists of maize, wheat, sugar beet and meadowland with crop rotation in evidence. Unlike Ireland and the UK there are no hedgerows dividing the fields in

this area. There are however, tracks between fields which have trees either side providing potential wildlife corridors. The entire above ground survey area covers 29 km^2.

Raised concrete anti-tank defences, known as "dragon's teeth" follow the line of the tunnels above ground. These defences connect the wooded areas with the arable land and provide a potential raised platform where marten may deposit scats (Figure 1.3)

Figure 1.3 Raised anti-tank defences following the line of the tunnels above ground.

1.2 Mustelidae

The Mustelidae are a family within the order Carnivora and are described by Koepfli, *et al*. (2008) as a species-rich group with 59 species classified into 22 different genera with 6 subfamilies. These subfamilies are the Mustilinae (weasels, ferrets, wolverines, martens and minks), Lutrinae (otters), Melinae (badgers), Mellivorinae (honey badger), Taxidiinae (American badger) and Mephitinae (skunks) (Wozencraft, 1993). While most have a long-thin body, Mustelids display wide variation both in morphology and ecology from the forest dwelling badger to the semi-aquatic otter. Mustelids also have a wide distribution globally with multiple genera found on different continents (as well as having been introduced into New Zealand) (I.U.C.N, 2013).

1.2.1 Martes

One of the mustelid genera is the genus *Martes* which comprises 8 species: the stone marten *M.foina*, European pine marten *M.martes*, yellow throat marten *M.flavigula*, Japanese marten

M.melampus, American pine marten *M.americana*, Nilgiri marten *M.gwatkinsii*, fisher *M.pennanti* and the sable *M.zibellina* . In the genus *Martes* there are 3 subgenera. These are; the subgenus *Martes* which includes *M.foina, M.martes, M.zibellina, M,melampus* and *M.americana*, the subgenus *Pekania* which has only one member, *M.pennanti*, and the subgenus *Charronia* which includes *M.flavigula* and *M.gwatkinsi* (Nowak, 2005). Proulx & Aubrey (2014) suggest that according to recent phylogenetic studies, *M.pennanti* may be more closely related to the wolverine and tayra *Eira barbara* which lead them to suggest a sub-family encompassing the genera *Martes, Pekania, Gulo*, and *Eira* and proposed that the term "Martes Complex" be used to describe the 11 species that comprise these 4 genera.

While the pine marten and stone marten both belong to the same subgenus they are not the most closely related species in it. According to Marmi, *et al*., (2004), the pine marten and stone marten divergence event took place 3.1-2.2 million years ago while the pine marten and other *Martes* species divergence took place much later than this. The pine marten and sable are more closely related. It has been found that a high degree of hybridisation occurs between the sable and pine marten where they are sympatric in areas of Russia (Rozhnov *et al*. 2010) and the offspring is known as a "kidas". The offspring tend to maintain reproductive isolation however and the reproductive rate of both populations' decreases in these areas of hybridisation leading Kassal & Sidorov, (2013) to propose this phenomenon as a barrier to either species penetrating very far into the others range. It is possible, given the opportunity, that the pine marten and American marten can hybridise (Davison *et al*. 2001). Even though they are sympatric over a large part of Continental Europe there is no documented evidence as yet that the pine marten and stone marten hybridise (Davison *et al*. 2001).

The pine marten and stone marten are two of eight mustelid species that occur in Poland. The others are; weasel (*Mustela nivalis*), polecat (*Mustela putorius*) Eurasian badger (*Meles meles*), Eurasian otter (*Lutra lutra*), stoat (*Mustela erminea*) and the non-native American mink (*Mustela vison*) (Jamrozy, 2008).

Pine marten may be classified into three main haplogroups based on comparison of the d-loop region of their mitochondrial DNA (mtDNA). These are; Mediterranean, Central Northern European and Fennoscandian Russian (Ruiz-Gonzalez *et al*. 2013a). Based on a comparison of the same d-loop mitochondrial DNA target in stone marten there is also marked differences in haplotypes found in China, the Northern Iberian Peninsula and the rest of

mainland Europe (Nagai *et al*. 2012) but there have been limited studies on stone marten phylogeography compared to other mustelids (Vergara *et al.* 2015).

1.2.2 Pine marten (Martes martes, Linnaeus, 1758)

The pine marten is slightly smaller than a domestic cat. It has a brown coat which becomes darker and heavier during the winter months. The fur along the tail and limbs also becomes darker in winter. The throat patch of a pine marten is a cream to yellow colour and allows the identification of individual animals by direct observation. Pine marten have fur on the soles of their large paws which also have semi-retractable claws. These claws along with its long tail allow great agility when climbing trees and while pine marten prey mostly on the ground they may hunt squirrels in trees. A male pine marten weighs 1.60-2.15 kg which is about a third larger than its female counterpart (Nowak, 2005).

As the name suggests pine marten are usually found in forests with forest structure being an important factor (Salek *et al.* 2014). However in the absence of suitable forest understory pine marten may utilise a variety of other habitat types including rocky outcrops, scrubland, hedgerows and even domestic buildings (Balestrieri *et al*. 2008; Pereboom *et al*. 2008). It has recently been suggested that pine marten may be less forest specialised than previously thought with an increasing number of pine marten observations in non-forested areas as a possible result of habitat fragmentation (Pereboom *et al*. 2008; Caryl *et al*, 2012). In their study on linking habitat characteristics with pine marten genetic diversity Mergey *et al.*, (2012) found no obvious association between patterns of forest habitat characteristics and genetic variability and suggest a greater behavioural plasticity in pine marten habitat selection than previously expected allowing them to adapt to different habitat types. Pine marten will however choose resting sites, even in a highly fragmented landscape, in forested areas and Larroque *et al*., (2015) found that 98% of the resting sites they recorded for pine marten were located in forested areas suggesting a specialisation in habitat resting site preference. While it has been shown that pine marten will venture into open areas they tend to stay close to forest cover suggesting pine martens exhibit a certain dependence on the presence of trees for cover in the locality (Pereboom *et al.* 2008).

The pine marten is an introduced species to the island of Minorca in the Mediterranean and in this competitor and predator-free environment Clevenger (1994) found the pine martens to be

habitat generalists. On Sardinia it has also been shown that pine marten were not restricted to mature wooded areas. While present in forested areas they were also found close to anthropic areas (Murgia *et al*. 1995).

Pine marten show a high fidelity to their home ranges (Zalewski & Jedrzejewski, 2006). Male territories will overlap with one or more female territories. Pine marten may have a number of resting sites, for use during the day, which are scattered throughout their home range. It has been shown that more than one pine marten may use the same resting site if they have overlapping ranges but not if they are of the same sex (Zalewski, 1997). Pine marten ranges vary seasonally and also with habitat and food availability (Zalewski *et al*. 1995). Male resting sites will cover a larger area than those of females In Bialowieza National Park average home range size for males is 2.23 km^2 and 1.49 km^2 for females (Zalewski *et al*. 1995). This data is in agreement with the findings of Larroque *et al.,* (2015) who also found the size of the ranges of both sexes will be smaller in winter than in summer. Females ranges being largest in April due to the increased energy requirement from kit rearing (Zalewski & Jedrzejewski, 2006). This study also summarised snow tracking data across Europe and found pine marten density increased during mild winters and low seasonality areas, that ranges increased in low forested locations but that the population in Bialowesa was at high density and the pine marten ranges were smaller than average due to the high degree of forest cover. In fragmented landscapes pine martens range size will be larger with a positive correlation between home range size and degree of fragmentation (Balharry, 1993a). Female pine marten preferentially choose their den site in high cavities of trees (Zalewski, 1997). In the absence of trees or ideal den sites pine marten will choose denning sites in sub-optimal locations including rocks and abandoned buildings (Birks *et al.* 2005).

In spring, Zalewski, (2001) found male pine marten to be most active between 8 pm and midnight while during summer activity is bimodal with activity peaks between 6-10 pm and 2-4 am. Female pine marten activity patterns differed to those of males with unimodal activity between 8pm and midnight during the summer and a bimodal rhythm in autumn /winter with activity peaks between 6-8pm and 2-6am. The length of these main activity bouts varies between season and sex with males being active longer on average. Shorter activity bouts also vary seasonally and are related to ambient temperatures with short activity bouts increasing in winter and lasting longer in females than in males (Zalewski, 2000).

1.2.3 Stone marten (Martes foina, Erxleben, 1777)

Stone marten are more associated with open areas than pine marten. They also may be found in urban environments and are considered "urban adapters" as urbanisation does not have a negative effect on the stone marten (Herr *et al*. 2009). This holds true in most of Europe but in Spain stone marten tend to be found in less human influenced environments (Lopez-Martin *et al*. 1992). Stone marten will utilise a wide range of habitats and are less reliant on forest cover than the pine marten (Nowak, 2005). Again like the pine marten, the stone marten will have multiple resting sites throughout its range which may be shared with other stone marten with overlapping territories as long as they are of opposite sex. Stone marten are more flexible than pine marten with respect to resting site location and Larroque *et al*., (2015) found stone marten resting sites in both urban areas and forested areas within their study area but with the majority of resting sites located in urban areas (83%). It is noteworthy that of these urban resting sites the majority of them had at least one side adjacent to a forested or rural area with only one of the 13 animals studied choosing a resting site that was completely surrounded by urban developments. The home ranges of stone marten will be smaller in urban areas (Herr *et al*. 2009). Territoriality is maintained in urban environments even in areas with abundant food resources and this inflexibilty may be a possible reason for this suggesting environmantal variables have limited influence over intra-sexual territoriality (Herr *et al*. 2010).

Den sites may be in out-buildings or during winter, in human occupied dwellings where they may become a nuisance. In rural or forested areas den sites will generally be situated among dense vegetation, trees, burrows or pile of brushwood (Genovesi & Boitani, 1997).

Stone marten activity patterns vary between seasons and most nocturnal activity can be seen during winter months and increased daytime activity can be seen during the summer. Crepuscular activity patterns remain consistent all year round (Posillico *et al.* 1995). As with the pine marten, the stone marten can also tolerate a high degree of habitat fragmentation but only in the presence of wildlife corridors and webs of vegetation to facilitate normal movement and ranging activity (Cervinka *et al.* 2013). Habitat fragmentation can be a problem for stone marten when the fragmentation cuts the marten off from access to a lake or river normally in it's range and Rondini & Boitani, (2002) even described open arable land as a hostile environment for the stone marten when the arable land separated the martens home range from a watercourse.

Stone marten weigh between 1.1 and 2.3 kg (Nowak, 2005). Like the pine marten they are slightly smaller than a domestic cat. Like all mustelids, sexual dimorphism is evident in both pine marten and stone marten with males being significantly larger than females (Moors, 1980). Morphological differences between pine marten and stone marten are discussed in the next section.

1.2.4 Pine marten and Stone marten: Similarities, differences and interaction

Both marten species can be either nocturnal or crepuscular and are considered opportunistic feeders. Both have long tails, pointed muzzles, dark brown eyes, and exhibit intra-sexual territoriality. In both species a males range will be larger than that of a females (Nowak, 2005). Both species are primarily solitary animals with the exception of family units like a mother and kits or during mating. As solitary animals, both species communicate primarily via olfactory cues. Like all mustelids, both species use scent marking in its various forms but scent marking behaviour is not fixed for either species with marten interchangeably using scats, anal glands, foot glands and urine to mark their territories (Hutchings & White, 2000). The reported lifespan of both species varies between countries. The expected life span of a pine marten in Ireland is between 3 and 5 years (O'Mahony, 2013) but in mainland Europe it has been quoted as up to 10 years (Gzrimek, 1990), while captive animals have been known to live for over 20 years (H. Denman, *Pers comm*). Average longevity for stone marten is 3 years with a maximum expected lifespan in the wild of 10 years (Grzimek, 1990) Like all mustelids both species exhibit delayed implantation. It has been suggested that implantation drives dispersal over winter triggering females to drive out their young in late winter. Social activity is increased during late winter and this "false heat" triggers the dispersal of young marten as a form of territorial defence (Helldin & Lindstrom, 1995). Breeding usually occurs in mid to late summer with delayed implantation occurring approximately 6 months later. Gestation is usually approximately 30 days with the young "kits" born in March/April. Both marten species are subject to intra-guild predation from foxes in areas where they are sympatric but a recent paper by Brzeziński *et al*., (2014) documents "reversed" intraguild predation where pine marten were found to have killed fox cubs in North-Eastern Poland.

While the size of both stone marten and pine marten varies regionally it does not follow Bergman's rule which is an eco-geographic principle that populations of a larger size are found in colder environments and species of smaller size are found in warmer regions (Reig, 1992). In his study of geographic variation in pine marten and stone marten morphology

throughout Europe, Reig (1992) found a north-south gradient in skull size for pine marten while there was an increase in skull size in stone marten from west to east. This trend was not evident in Italian stone marten populations however, leading de Marinas & Pandolfi, (1995) to suggest geographic isolation as a possible cause of this morphological differentiation. Reig, (1992) suggested it is possible the size of both marten species could be dependant on prey availabity but stressed this would be difficult to assess given the wide dietary niche of both species. Considering the difference in results from each of these studies it is possible that a combination of geographic location, prey availability, and geographic isolation may all contribute to a specific marten populations physical size.

The stone marten is classified as an urban adapter (Herr *et al*. 2009) and so can be found in a broad range of habitats. The stone marten may be considered eurytopic while the pine marten is considered a stenotopic species, preferring to stay clear of urban areas (Goszczynski *et al*. 2007). The stone marten has a longer tail, more elongated angular head and shorter more widely spaced ears .The stone marten's nose is light peach or grey coloured while the pine marten's nose is black or dark grey. The ears of a pine marten appear closer together and are more pointed than those of the stone marten. The stone martens paws are not as densely furred making their prints look less broad with paw pads remaining visible even in winter. Both species have claws but only the pine martens' are semi-retractable. The stone marten has shorter legs than the pine marten and so its locomotion is more similar to that of the European polecat (*Mustela putorius*) while the pine marten moves in a bounding manner similar to that of the sable. Figure 1.4 (A) and (B) show images of a stone marten a pine marten.

Figure 1.4 (A) Stone marten (*Martes foina*), (B) Pine marten (*Martes martes*).

Cranial morphometric studies have indicated both interspecific and intraspecific differences in the size and shape of the skulls and dentition in both species. Males skulls are understandably larger but also have a narrower post-orbital constriction and larger molars than females suggesting that marten males are better adapted to catching and killing live prey while females appear to prey on easier to catch food (Loy *et al.* 2004). The rostral angle was how Reig & Ruprecht, (1989) suggested to best identify the skulls of each of these species with an angle less than 54.9 degrees classed as pine marten and above this angle as stone marten. The stone marten skull design suggests a higher adaptation toward hyper-carnivory with respect to the pine marten, having a smaller head, shorter snout, coupled with narrower post-orbital constriction and lesser emphasis on cheek teeth which may be a result of selective pressure and avoidance of intraspecific competition (Reig & Ruprecht, 1989).

Other differences in dentition include the stone marten having larger carnassials and smaller molars (Wolsan *et al*. 1985). The stone marten's penis is also larger than that of the pine marten with a stone marten's baculum averaging 55.5 mm and the pine marten's averaging 37.5 mm (Gennady *et al*. 2003). Both animals have a patch of lighter fur on their neck/chest known as a bib. In the stone marten this is a much lighter cream colour and carries on from the neck down the front of both forelegs. The pine martens bib is a much darker yellow and generally does not carry on down the legs.

From Figure 1.4 it can be seen that it is relatively easy to tell these two species apart. However, the scat, or faeces from both species is identical. In areas where the two species are sympatric some researchers have used the habitat where the scat was collected to attribute species (Romanowski & Lesinski, 1991, Tryjanowsky, 1997). Pine marten are generally associated with forested areas and stone marten habitat is usually more brush covered open areas or even urban developments but species may be found in the ecotones of a highly fragmented landscape (Pilot *et al*. 2007). To overcome any potential direct competition in such areas a sequential use of shared resources is proposed by Barrientos & Virgos, (2006) where one species is active early in the night while the other becomes active closer to dawn. In an area with less habitat fragmentation only one of these sympatric species was found in any one area at the local level (Rosellini *et al.* 2008).

The niche complementarity hypothesis (Werner, 1977) states that two sympatric species must differ in at least one of the three main ecological dimensions; habitat use, diet, and activity time. Using radio tracking in their study on interspecific competition between pine marten

and stone marten in eastern France Larroque *et al*., (2015), found stone marten and pine marten differed mainly in habitat and resting site use. The stone martens preference for open habitat and proximity to human developments has been well documented (Herr *et al*. 2009 ; Herr *et al*. 2010 ; Dudus *et al.* 2014). However, in parts of Spain it was found that stone marten tend to be less associated with human developments and live in more natural habitats and it was suggested that this was due to the absence of competition from the pine marten (Lopez-Martin *et al*. 1992; Virgos & Garcia, 2002). Other competing species have been introduced (the common gennet and egyptian mongoose) to these areas of Spain however that may mimic the effect of pine marten presence and increase the competition for suitable habitat resulting in increase stone marten preference for urban areas (Santos & Santos-Reis, 2010). Contrary to this traditional view that pine marten and stone marten may co-exist in an area through habitat segregation is the research of Balastrieri *et al*., (2010) who found that in part of Northwest Italy the pine marten was expanding its range into non forested areas that were usually occupied by stone marten. In the area where the pine marten was expanding into cultivated, non-forested habitat the stone marten was no longer present.

1.3 Geographic range, distribution and conservation status of pine marten and stone marten

1.3.1 Pine marten range and distribution

The pine marten is found throughout most of Europe and parts of Asia and is indigenous as far south as Spain and Portugal, with island populations on Corsica Sardinia, Sicily Minorca and Majorca. It is found in the north as far as Finland with Lapland being the northern limit of its range (Pulliainen, 1984). It is present as far west as Ireland and eastwards as far as Iran and the Urals in Russia where some overlap occurs with its sister species the sable, where it has been suggested that hybridisation between these species prevents further overlap of one species into the others geographical range (Kassal & Sidorov, 2013). Pine marten are widespread through most of continental Europe with the exception of parts of Spain, Greece, Belgium and the Netherlands (I.U.C.N 2013). The pine marten is native to Britain and Ireland but is now restricted to Ireland and Scotland with Ireland being the western edge of the pine martens global geographic range (O'Mahony *et al*. 2012). Figure 1.5 shows the International Union for Conservation of Nature (IUCN) distribution of pine marten.

Figure 1.5 Global distribution of pine marten (I.U.C.N, 2013).

The density of the pine martens distribution varies both between, and within countries due to varying degrees of suitable habitat, forest fragmentation and available dietary requirements (Proulx *et al*. 2004; Kurki *et al.* 1998). Density variation has also been found in Poland with winter mean densities in Bialowieza National Park ranging from 4.44-7.5 individuals/10 km^2 (Zalewski *et al.* 1995).

1.3.2 Pine marten conservation status

The I.U.C.N lists the pine marten as a species of Least Concern due to its large global population, wide distribution, its presence in a number of protected areas and its ability to adapt to minor habitat alterations (I.U.C.N, 2013). Many continental European countries still have large forested regions and most pine marten populations are considered to be stable. Ten European countries currently have wildlife legislation in place to protect the pine marten (Albania, Britain, Bulgaria, Denmark, Hungary, Ireland, Italy, Portugal, Spain and The Netherlands). However, of 25 countries surveyed, 13 (Austria, Croatia, Czech Republic, France, Germany,Latvia, Lithuania, Poland, Romania, Switzerland, Turkey, Sweden and Yugoslavia) countries still permit the hunting of pine marten either due to pest status or as a furbearer (Proulx *et al.* 2004).

1.3.3 Stone marten range and distribution

The stone marten is widespread across much of continental Europe and Central Asia. Its range extends west to Spain and Portugal and eastwards to Mongolia and the Himalayas. It is absent from Ireland, the UK, the Scandinavian peninsula and northern European Russia (Proulx *et al.* 2004; I.U.C.N, 2013). Denmark is the northernmost limit of the stone martens range (Lachat, 1991). It is absent from most of the mediteranean islands with the exception of Crete and possibly Ibiza although the species is believed to now be extinct on the island (Amores & Delibes, 1986). Stone marten also occur in India, Afghanistan, Pakistan, Nepal and Myanmar (I.U.C.N 2013). Across most of Europe the stone marten and pine marten ranges overlap extensively and in the eastern Himalayas and Mishmi Hills it co-exists with the yellow throated marten (Choudhury, 1997). Established populations of stone marten have been reported in small pockets in the USA, presumably as a result of escapes from fur farms (Long, 1995; Hoffmeister, 1967). Figure 1.6 shows the I.U.C.N distribution of stone marten.

Figure 1.6 Global distribution of stone marten (I.U.C.N, 2013).

1.3.4 Stone marten conservation status

The IUCN list the stone marten as a species of Least Concern due to its large population, wide distribution and presence in a number of protected areas. The stone marten is common in parts of its range and populations in Central and Western Europe have been increasing.

The population that occurs in India is however protected (Tikhonov *et al.* 2008). In many countries where it occurs the stone marten is legally hunted either as a pest in response to damage to houses and cars or poultry depredation (Proulx *et al.* 2004). In some areas of the stone martens range, rabies is an issue and the species is Key listed at level 2 in China (I.U.C.N, 2011).

1.4 The diet of pine marten and stone marten

Zhou, *et al.,* (2011) describe all martens as adaptive foragers. Their study incorporated all marten species but focused mainly on stone marten, pine marten and american marten species. They found that small mammals, fruits and insects to be the primary or secondary food categories in the diet of all marten species throughout their range but also that marten diet is flexible and can also include birds and carrion. Both pine marten and stone marten are polyphagous feeders allowing them to potentially capitalise on a variety of food categories (Goszczynski, 1976). Being able to utilise the most abundant and available food source at any time allows for better adaptation and survival as the animal may exploit different trophic niches and habitats (Wierzbowska & Skalski, 2012). Supplementing live prey with fruits and berries provides opportunist feeders like the stone marten and pine marten with an improved energy balance as there is no pursuit phase as there is for animal prey (Rosellini *et al.* 2008). Pine marten will also scavenge the remains of other predators kills. In Bialowieza Forest in Poland, Selva *et al.,* (2005) showed scavenging by pine marten on the kills of grey wolves that lie within the forest increases in winter as the temperature drops significantly. Also in Poland, while investigating scavenging in foxes, Jedrzejewski & Jedrzejewski, (1990) found 10 of 38 deer carcasses in their survey site had been visited by pine marten. In Sweden, Wikenros *et al*., (2014) found pine marten scavenging on moose carcasses increased during April suggesting carrion to be an important food source during reproduction. The study also found the carcasses that were visited by pine marten had to have been killed in an area with suitable protective vegetation and cover. Sidororvich *et al*., (2005) found that carrion availablity in winter in Poland was a crucial factor in determining pine marten winter densities in some of the more ecologically poor areas of Bialowieza Forest. Stone marten will also scavenge and their scats have been shown to contain evidence they have been feeding on wild boar and deer on which they would not be predating (Branji, 1995).

During a study in Belarus, Sidorovich *et al.,* (2010), found bank voles and small birds to make up nearly half the diet of pine martens in the study area and also found other small mammals and some larger birds, fruits, seeds, honey, insects, eggs, hares, squirrels and wild deer carcasses demonstrating the breadth of the pine martens dietary niche. Pine marten diet on Sardinia was found to consist mainly of fruit (mostly *Rosaceae* fruits) small mammals (rodents) and birds (Murgia *et al*. 1995). A study in southern Poland showed that while the pine marten and fox both prey on insects, pine marten tend to prey mainly on nest building insects while foxes eat more necrophagous insects (Skalski & Wierzboska, 2008). This dietary division between fox and pine marten is also highlighted by Reig & Jedrzejewski, (1988) in eastern Poland. Both species predated small mammals, but pine marten tended to prey on forest rodents like the bank vole *Myodes glaryoles* and *Apodemus sp*. while foxes preferred *Microtus sp*. which is found in more open areas. In areas of Russia, where the pine marten is present, its diet will be adapted to include more squirrels during the long harsh winters although this may be from scavenging more than predation (Yazan, 1970). In Scotland the dietary niche of the pine marten has been shown to expand and contract seasonally with a continual preference for fruit displayed irrespective of the abundance of other available food (Caryl *et al*., 2012). This same study found the pine martens diet to consist of three principle components; *Microtus agrestis* (39%), berries (*Sorbus aucuparia* and *Vaccinium myrtillus*: 30%) and small birds (24%) prompting Caryl *et al*., (2012) to propose a facultative foraging strategy for the pine marten. In Ireland the pine martens dietary niche breadth was also described as wide by Lynch & McCann, (2007), where fruit, arthropods, small mammals, passerine birds and frogs constituted its diet in descending frequency. Woodmouse was found to be the dominant mammalian prey species in the pine martens diet in the midlands and east of Ireland where red and grey squirrels (*Sciurus vulgaris* & *Sciurus carolinensis*) were also predated. In areas where the invasive grey squirrel was available as a food source it featured significantly more in the pine martens diet than the native red squirrel (Sheehy *et al*. 2014). In Poland, Zalewski, *et al.,* (1995) showed a 2 year time lag between years with heavy seed crops and pine marten densities, attributing the increased density to the abundance of small rodents (bank vole and yellow necked mouse) feeding on the seeds. These rodents increased with a 1 year time lag.

The diet of the stone marten has been found to be very similar to that of the pine marten, but with more emphasis on fruit than that of the pine marten. In Greece a stone marten population was found to have a high dependence on fruit during the summer. This population

specialisation was contrasted with individual animal's flexibility in diet where insects and larvae were consumed with various frequencies depending on the individual animal and prey availability (Bakaloudis *et al*. 2012). In central Italy stone marten were found to consume mainly fruit in summer/autumn followed by vertebrates then invertebrates and then favour vertebrates during the winter and spring months (Posillico *et al*. 1995). In the Turew district in Poland, both marten species were found to prey heavily on common voles (*Microtus arvalis*) if present in their habitat, and Goszczynski, (1976) showed that in years where common vole abundance was low, both martens species preyed more frequently on woodmouse and bank vole, but returned to predation of common voles the following year when this species became more abundant. It was also noted during this study that marten rarely preyed on house mice, harvest mice or root voles. Rosellini, *et al*., (2008) suggest the pine marten is not an absolutely opportunistic predator and propose describing it as an intermediate between opportunistic and specialist predator. This is as a result of a study performed in North West Spain where they found that pine marten consumed the same proportion of small mammals in this area, even in seasons where small mammal abundance increased significantly. This contrasts markedly with the findings in Hungary of Lanski, *et al*., (2007) who found a positive correlation between the amount of small mammal prey consumed and the abundance of the prey in the available food resources.

As an urban adapter, some stone marten will incorporate anthropomorphic food into their diet. Higher levels of trace elements in the liver, kidney, and muscle tissue in urban stone marten than those of urban foxes in Croatia lead Bilandzic *et al*., (2010), to propose the stone marten as an important bio-indicator for the accumulation of toxic metals in urban habitats.

Both species are frugivorous and as such may play an important role in an ecosystem as seed dispersal vectors and it has been shown that seeds from the European blueberry *Vaccinium myrtillus* exhibit enhanced germination after passing through the gut of a marten (Schaumann & Heinken, 2002). Guitian & Munilla, (2010) showed that pine marten in mountain habitats in Spain tended to feed on high density patches of fallen fruit which were located under trees with the highest fruit yield, leading to seeds from these trees being preferentially dispersed. In the area above the Nietoperek bat reserve in western Poland seeds and fruit constituted the largest part of the stone martens diet with 70% frequency of occurrence and 58% biomass (Tryjanowsky, 1997). These results contrast with a study on the diet of the stone marten in south eastern Romania where birds and mammals predominated in the diet while reptiles, amphibians, insects seeds and fruits are described as supplementary foods (Romanowski &

Lesinski, 1991). A further year long study from Luxembourg found minimal difference in frequency of occurrence between mammals and fruits in the stone martens diet (Baghli *et al*, 2002). The differences in these three sets of data maybe attributed to variation in geography and habitat, namely fragmented agricultural land in western Poland, the Romanian Steppes and elevated meadows and forests in Luxembourg where habitat characteristics may contribute to defining the stone martens dietary niche.

The proportion of prey consumed will vary with availability and seasonality but will also vary considerably in areas where pine marten and stone marten are sympatric and share this region with other predators with similar trophic niches (Goszczynski, 1986). In an area where pine marten and stone marten are sympatric, Posluszny *et al*., (2007), showed that while there was considerable trophic niche overlap, pine marten preyed more frequently on small mammals and birds while fruits and insects were significantly more abundant in the diet of stone marten. Both species were found to use habitat edges to capitalise on higher small mammal abundance in fragmented landscapes during a study in the Czech Republic (Salek *et al*. 2010). In Central Spain where the stone marten are also sympatric with the common gennet (*Gennetta genetta*) the most abundant food source by percentage biomass was found to be fungi, highlighting the dietary adaptability of this species (Barrientos & Virgos, 2006).

In their paper on biogeographical variation in the diet of Holarctic marten species, Zhou, *et al*., (2011), found that while small mammals constituted a large portion of prey species for both pine marten and stone marten, insects and vegetable matter were consumed more frequently in lower elevation localities. Variations in diet with longitudinal and latitudinal position have also been noted with pine marten, along with contraction and expansion of their dietary niche on a seasonal basis (Zalewski, 2004). Marchesi *et al.*, (1989) found that the pine marten eats significantly more mammals and birds than the stone marten with both species' diet being strongly influenced by season and diet and to a lesser extent by geographic situation.

To date, bats have rarely been found in the diet of either pine marten or stone marten. In 1987, morphological analysis of stone marten scats found in underground marl pits in the Netherlands contained bone fragments from Daubenton's bats (Bekker, 1988). Of the 87 stone marten scats collected in Budapest in 1998, one was found to contain an unidentified bat species (Tothe-Apathy, 1998). The skulls from two bat species were found in scats in 1991 in Romania. One of them was identified to species level as that of a Particoloured bat

Vespertilio murinus. In reporting this study it was stated that bats are rarely found in the diet of stone marten. The scat was identified as being from a stone marten rather than pine marten due to the habitat where it was found even though the species are sympatric in the area (Romanowski & Lesinski, 1991). Obuch, (2012) found clusters of bat skeletons in caves in Slovakia which he attributed to old pine marten food reserves. In Nietoperek, remains of bats were found in only 3 of 88 scats gathered in the tunnels (Tryjanowsky, 1997). One was identified morphologically as *Plecotus auritus* while the others remained unidentified. Tryjanowski (1997), like Romanowski & Lesinski, (1991) assumed any scat found was not from pine marten due to its location alone.

1.5 Bats in and around the Nietoperek hibernation site

There are 24 (20 breeding) species of bat present in Poland with distribution patterns and species richness varying considerably across the country (Sachanowicz *et al.* 2006). There are 18 species of bat found in the Lubusz region of western Poland where the project survey site is situated. Eleven of these bat species hibernate in the tunnels or bunkers each winter. This has been determined by an annual bat census which takes place for 2 days each January to monitor the health and well being of the entire population as well as paying particular attention to any species protected by the Habitas II Directive (EU Council, 2012). The survey teams count each species of bat individually in each sub-section of the system and total their findings. Each year the survey is limited to 2 days to minimise any interference to the hibernating bat population. Each of the 11 species of bat present in the bunker system have been known to hibernate in caves in Poland (Piksa *et al.* 2013) which would have a similar microclimate to that of the bunker system. As they are seeking areas of high relative humidity Daubenton's bats tend to hibernate lower down on cave walls than the other bat species (Kokurewicz, 2004).

Table 1.1 lists all bats found in western Poland including bats found in hibernating in the tunnel system each year from 2011 to 2015 including; their abundance in the tunnels, their I.U.C.N redlist designation, population trends, and protection status in the European Union.

Table 1.1 List of bats present in Western Poland including bats counted at the Nietoperek bat hibernation site during annual censuses (2011-2015). (Kokurewicz & Schofield *Pers comm*.; EU Council, 2012; I.U.C.N, 2013; R.E.P.D Gorzow Wielkopolski, 2015).

Species	Avg no.in tunnels Jan censuses 2011-2015	I.U.C.N Redlist status	E.U Population trend	Annex II of Habitats Directive
M.myotis	25028	Least concern	Stable	Yes
M.daubentonii	4873	Least concern	Increasing	No
M. nattereri	2971	Least concern	Stable	No
B.barbestellus	884	Near threatened	Decreasing	Yes
P.auritus	535	Least concern	Stable	No
M. msytacinus	58	Least concern	Unknown	No
M.brandtii		Least concern	Stable	No
M.bechsteinii	34	Near threatened	Decreasing	Yes
M.dasycneme	29	Near threatened	Decreasing	Yes
E.serotinus	9	Least concern	Unknown	No
*E.nilssonii**	0	Least concern	Stable	No
Vespertilio murinus	0	Least concern	Stable	No
Pipistrellus pipistrellus	0	Least concern	Stable	No
Pipistrellus pygmaeus	0	Least concern	Unknown	No
Pipistrellus nathusii	0	Least concern	Unknown	No
Nyctalus noctula	0	Least concern	Unknown	No
Nyctalus leisleri	0	Least concern	Unknown	No
Plecotus austriacus	0	Least concern	Unknown	No

* *E.nilssonii* has only ever been found in bunkers that are not connected to the tunnel system.

1.6 Sampling techniques used in ecological studies of terrestrial mammals

When dealing with elusive, endangered, dangerous, or nocturnal animals non-invasive sampling is the preferred method (Taberlet *et al.* 1999). In non-invasive sampling, the animal does not have to be interfered with in any way to obtain a sample, and only methods in which the morphological remains or source of DNA is left by the animal and may be collected without having to catch the animal may be considered non-invasive. The sampling techniques described here may be divided into two groups, passive and active techniques. Passive techniques involve collecting evidence of an animal's presence without the setting of traps, bait or lures to entice the animal to leave a sample. Conversely active techniques use bait or lures in order to obtain a sample. However, a combination of both passive and active

techniques is often used. The examples of the methods described in this review will focus on their application with mustelid species.

1.6.1 Direct observation and roadkill.

With elusive animals, sightings or direct observation can be rare but may be used as grounds for further investigation as long as the sighting can be verified (Messenger & Birks, 2000). Direct observation may be suitable for presence/absence surveys but with elusive animals like the stone marten and pine marten it would be of limited use for abundance or distribution studies. Animals found dead on the road are a valuable source of material for both morphological and genetic analysis and may give some indication of a species' range and distribution. Surveys using this method may be carried out by untrained volunteers over a wide area and at relatively low costs of money and time (George *et al*. 2011). One disadvantage of this survey technique however is the inability to control the sampling effort.

1.6.2 Tracking and snow tracking

The detection of animal prints is terrain and weather dependent. However it may be used successfully in certain survey areas. An example of this is the "Mostela" box (Mos, 2012). This box contains a camera, bait, and a powdered track plate on which the animal will leave prints. Mud, wet sand, and snow are all media that may contain clear identifiable tracks. Altering the terrain to allow clear identification of tracks has been used to maximise this methods effectiveness and minimises Latency of Initial Detection (Gompper *et al.* 2006). Snow tracking is obviously dependant on snowfall but is also reliant on knowledge of the animals range, the type of ground and structure of the snow cover (Vaisfeld, 1972). It has been shown that it is possible to identify pine marten individuals, as well as the sex of these individuals by use of snow tracking alone (Zalewski, 1999). How an animal makes use of its terrain may also be determined by snow tracking. Goszczynski *et al*., (2007) used snow tracking to monitor symaptric pine marten and stone marten. The pine marten was shown to make more extensive use of the 3-dimensional forest habitat than the stone marten while the stone marten made use of the area outside the forest thereby reducing competition between these species.

1.6.3 Remote Camera Traps

Remote cameras may be used to assess the presence of a target species or to distinguish individuals of the same species, or even to give an indication of mammal biodiversity in a survey area or community (Long *et al.* 2007). The camera has an infra-red motion sensor that is triggered by the passing of an animal to give still photographs or video footage. The camera may be placed along known or potential tracks that the target species may use. The location of the camera is an important consideration, as is the potential range of the target species (Mondol *et al*. 2009), the habitat type, and the proximity to human developments (Se Quin *et al.* 2007). Remote camera surveys used alone, (Bartolommei *et al.* 2012) and in conjunction with other methods, (Rosellini *et al.* 2008), have been used successfully to survey for marten species.

1.6.4 Scent stations

Scent stations can be used to determine and monitor the density and distribution of carnivores (Wilson & Delahay, 2001). A scent station usually consists of a lure placed on a raised platform or rock with a circle of a tracking medium scattered on the ground around the lure in order to record the paw prints of any animals that are attracted to the lure. The tracking medium may consist of sand, marble dust, gypsum, flour, ashes, dust or talcum powder. The lure can be a visual lure, a sound lure, or a scent lure and can comprise of anything that will attract the animal in question to the scent station. Bait for marten scent stations may consist of chicken, eggs, rabbit, jam, peanut butter (Roche 2008; Gompper *et al*. 2006). The bait or lure should be stable and detectable from a distance (Roche, 2008).The choice of bait or lure used is dependent on the objective of the survey.

Scent stations have not been used as extensively in Europe as they have in North America. However, Mortellitit & Boitani, (2008) conducted a survey to evaluate the method for use with various European carnivores. Some bias in their distribution data was detected highlighting the need for care when selecting the sampling and survey methods for a particular species.

1.6.5 Radio tracking

Radio tracking involves trapping the animal and attaching a transmitter that will allow the tracking of the target species through space and time. As such, this is an invasive technique

but is included here as it is a technique used widely in mammal surveys allowing studies over wide ranges. It has been recommended to use as short a sample interval as realistically achievable over an extended period of time to avoid sampling bias (Rooney *et al.* 1998).

Radio tracking of marten has given important information on the social ecology, (Genovesi & Boitani, 1995) habitat selection and use (Herr *et al.* 2009), determination of home ranges and activity patterns (Pereboom *et al.* 2008), and may allow location of den or resting sites in a survey area (Birks *et al.* 2005).

1.6.6 Hair collection

Hair may be passively collected from den sites, resting sites, birds' nests, or even from scats as a result of self-grooming. However hair snares or baited hair tubes may also be used to actively obtain a hair sample.

Hair tubes have been used for many years and were first described in the 1970's in a study of small mammals in trees in Australia (Suckling, 1978). Hairs left in the tubes were identified using the morphological method of comparing the hairs to a reference hair library after examination under a dissection microscope. The use of DNA based methods to identify hair samples has increased the success rate when using hair tubes and snares (Foran *et al.* 1997).

The use of bait minimises the risk of multiple captures of different animals or species, as once the bait is removed, a second animal is less likely to enter the tube. Many modifications of this hair tube method have been made over the years and it has been found that hair trap design is an important factor when conducting a mammal survey and may influence both survey efficiency and results (Harris & Nicol 2010; Portella *et al.* 2013). Other important factors include the means by which the hair is captured and the type of bait used to attract the target species (Mills *et al*. 2000).

Baited hair tubes have been used for surveying for marten including the pine marten, fisher and American marten (Mullins *et al.* 2010; Zeilinski *et al.* 2006; Mowat & Paetkau, 2002). A desk top search of the literature shows only one example of a hair tube survey on pine marten failing to return a positive sample in an area where pine marten were found using other non invasive techniques (Bartolommei *et al.* 2012). While previous models of hair traps for marten have been bulky or heavy (Mowat & Paetkau, 2002) more recent methods have described use of much lighter traps in the form of compact and durable PVC tubes (Mullins *et*

al. 2010). The use of wire to keep the bait in position, forces the marten to struggle while in the tube increasing the likelihood that a sample will be left on the glue patch.

1.6.7 Scat collection

One potential drawback of using hair tubes or scent stations is the introduction of bias to any distribution data. This is highlighted by Powell & Proulx, (2003) who warn that as marten are naturally inquisitive animals they may temporarily stray from their natural habitat to investigate a new source of food. A method of sampling that avoids this potential bias is scat collection.

Scat is the faeces of marten. Figure 1.7 shows an image of a pine marten scat. Some mammals, including marten, use their faeces as territorial markers, throughout their territory and on range boundaries (Lenton *et al.* 1980; Balharry, 1993b). It is easier find the scat from an elusive or nocturnal species than to find the actual animal itself. As a non-invasive technique it also allows the study of rare or endangered animals without any direct contact with that animal.

Figure 1.7 Pine marten scat found on forest road, Nietoperek, Poland.

A scat survey is a relatively inexpensive method of monitoring a species over a wide area and a relatively long time period, especially if experienced volunteers are used. It may be described as a systematic search for scats over a predefined area generally along linear features like tracks or forest roads (Strachan *et al.* 1996).

Scat survey design is dependent on the objectives of the survey which are generally presence/absence, point-in- time distribution and population status on a regional or local scale (Birks *et al*. 2004). Considerations should include; the geographical area, habitat, terrain, and which sampling approach to take. Secondary objectives such as habitat selection and topographical influences may also be determined using scat surveys (Strachan *et al.* 1996; Bright & Harris, 1994). The use of specially trained scat dogs may increase the liklehood of finding scats in areas of low population density (Long *et al.* 2007).

It is not always possible to visually differentiate between the scat of a target species and that of a different sympatric species, and this problem was highlighted by Davison *et al.* (2002) where fox scat was incorrectly identified as marten scat by expert surveyors. In areas where pine marten and stone marten are sympatric this is particularly problematic as it is impossible to differentiate between the scats from these two species using morphological methods alone (Marchesi *et al.* 1989). As scats from both marten species were found within forested areas in a survey site around Rogow in central Poland, Pilot *et al*., (2007) state that it is impossible to draw any inferences on which marten species deposited the scat solely on the habitat in which the scat was found.

With the advent of molecular techniques, ambiguity associated with scat identification has been removed (Davison *et al.* 2002). It is possible to determine to species level, morphologically similar or even identical scats by a number of different methods including PCR, qPCR and RFLP (Statham *et al.* 2005; Moran *et al.* 2008; Leucentini *et al.* 2007; Pilot *et al.* 2007). Not only can the species be determined from a scat but also the sex of the animal (Lynch & Brown 2006) and the individual itself may be identified (O'Neill *et al.* 2013). This is more difficult due to the inevitably degraded nature of the DNA in some faecal samples. While the DNA extracted from a fresh hair sample will be less degraded than scat DNA, one advantage scat collection has over hair collection is the extra information on an animal's diet that can be obtained from examining the the scat either morphologically under a dissection microscope (Goszczynski, 1986), or by use of molecular techniques such as DNA analysis (Bohmann *et al.* 2011).

1.7 Genetic analysis of non-invasively collected samples

Non-invasive sampling involves only material that has been left behind by the target animal and can be collected without having to trap, interfere with, or even see the animal (Taberlet *et*

al. 1999), which is an advantage when dealing with rare, elusive, nocturnal, wide ranging or dangerous species. Non-invasive genetic sampling approaches are continuing to increase understanding in ecology, conservation genetics and other related disciplines. While faeces and hair are commonly used, bone, blood, urine, and saliva have also been used (Beja-Pereira *et al.* 2009).

Molecular techniques typically target mitochondrial DNA (mtDNA) when used to identify species from non-invasively collected samples. Mitochondrial markers are used because mtDNA is relatively easy to work with, is much smaller in size with respect to nuclear DNA and has a conserved arrangement of genes which means that many pairs of universal primers will amplify regions of the mitochondria in a wide variety of both vertebrates and invertebrates. Therefore data about a particular species can be obtained without previous knowledge of the genome. Another major advantage of using mtDNA is the number of copies present in a cell which can be anywhere from 1000 to 10 000 (Freeland, 2005). Figure 1.8 shows a map of the mitochondrial genome highlighting some of the more common target sequences used in molecular ecology.

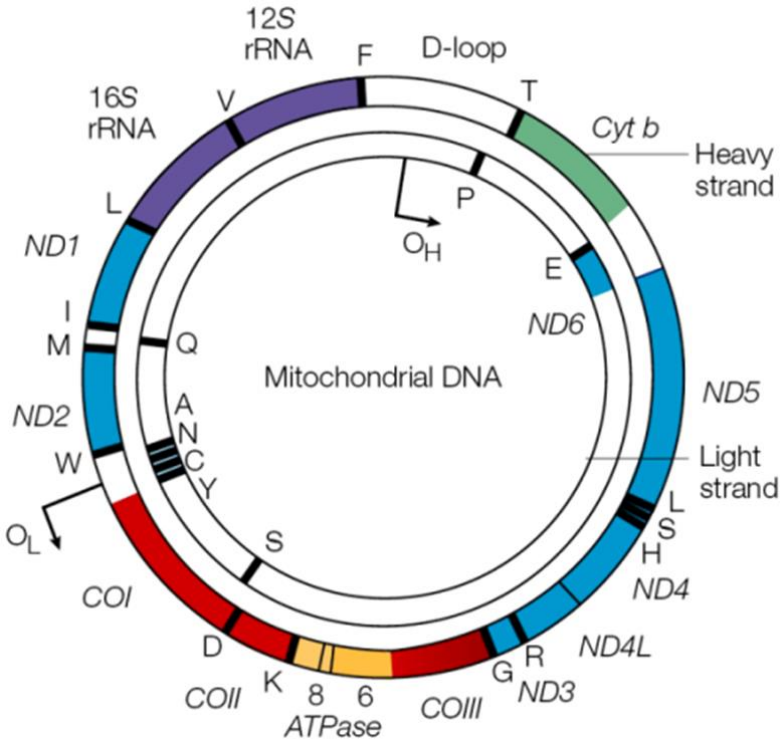


Figure 1.8 Map of mitochondrial genome (Chial & Craig, 2008); Common regions targeted in molecular ecology are ND1- NADH dehydrogenase subunit 1; CO1= cytochrome oxidase subunit 1; Cytb= cytochrome b; d-loop=displacement loop (non-coding).

Whilst the arrangement of genes is conserved the overall mutation rate is high and is as much as 10 times higher than that of nuclear DNA (Freeland, 2005). The non-coding control region which includes the displacement loop (d-loop) evolves particularly rapidly in many taxa and is due mainly to by-products of respiration and the less stringent repair mechanism that exists in mtDNA when compared to nuclear DNA. High mutation rates mean mtDNA show more polymorphisms and will often show multiple lineages in, and among populations. The more conserved mtDNA cytochrome b (Cyt b) gene is also a target in molecular ecology studies for species identification (Parson *et al.* 2000) as well as the mitochondrial cytochrome c oxidase I (COI) gene which is the suggested target for species identification from the Consortium for the Barcode of Life (CBOL) (Hebert *et al.* 2003). Another reason mtDNA is preferred over nuclear DNA in some cases is that mtDNA generally lacks recombination. As mtDNA is inherited uni-parentally, offspring have exactly the same genome as their mother barring mutation meaning individual lineages can be tracked over space and time and can be extremely useful in areas like phylogeography. As mtDNA is haploid and uni-parentally inherited it is effectively 25 % of the population size of diploid nuclear DNA so as there is less of it to begin with, it is particularly sensitive to bottlenecks or other demographic events like marked population reductions due to disease or catastrophe (Bekkevold & Hansen, 2004)..

1.7.1 Molecular analysis versus morphological analysis of non-invasively collected samples

Traditional methods of species identification using non-invasive methods are morphological and include direct observation and identification, camera traps, the use of hard-part analysis of faecal samples and microscopy. Each of these approaches has its drawbacks. For instance, visual discrimination between the scats of some species may prove difficult or impossible. This was demonstrated with pine marten and fox scats by Davison *et al.*, (2002) where a number of scats assumed to be from pine marten turned out to be from fox. Camera traps rely on the position of the camera being correct and are expensive with results varying considerably with the animal being detected and location of camera, as some animals have very large territories (Mondol *et al.* 2009). Identification of hairs using microscopy requires a degree of expertise to ensure the hairs are from the target species and not by-catch from non-target species (Kendall & McKelvey, 2008).

Hard-part dietary analysis relies on a degree of expertise in both taxonomy and morphology and also relies on the morphological integrity of the sample and as such may only be used if the diagnostic parts survive digestion (Parsons *et al*. 2003). Other animals "cough up" the hard parts of their diet leaving no hard parts in their faecal matter. Insect larvae or frog spawn would never be found in a diet using hard part analysis only, as there are no hard parts to begin with. Phenotypic plasticity is also a problem with morphological analysis with the possibility for a single genotype to develop into multiple alternative phenotypes under various environmental conditions (Freeland, 2005). While each of these methods has their drawbacks, the best approach may be to use both morphological and molecular methods together. Purcell *et al.,* (2004) used hard part analysis to firstly identify salmonid bones in scats from Pacific harbour seals (*Phoca vitulina richardsi*) to family level. As it was not possible to distinguish between bones of different species of salmonid by morphological techniques, molecular techniques were then used to tell which salmonid species were present in the seal scats. The initial use of hard part analysis saved both on time and consumables.

Molecular based tests target specific sequences of DNA. If the target DNA is present, there is no other identification of the prey necessary increasing both accuracy and precision. Some of the molecular techniques using DNA are described below along with some applications to non-invasively collected samples from mustelids.

1.7.2 Molecular methods

1.7.2.1 PCR

The Polymerase Chain Reaction (PCR) is a simple and rapid method for selectively amplifying defined sequences of either DNA or RNA from a complex nucleic acid mixture and produces a detectable amount of product from a small target sample. This is particularly advantageous with non-invasively collected samples where there may be only a small of target DNA. The number of copies generated in a PCR reaction is dependent on the number of cycles performed but the amount of starting material required is very small. The primers used should be non-identical and should anneal to opposite ends of the target DNA.

There are three phases to PCR amplification; the exponential phase where assuming 100% efficiency, the PCR products double after each cycle, the linear phase where one or more of the reagents is limiting, and the plateau phase where there is no further amplification due to

reagent depletion. Standard or end point PCR products are visualised after the plateau phase using gel electrophoresis and a UV source and therefore give no indication of starting DNA concentration (Figure 1.9).

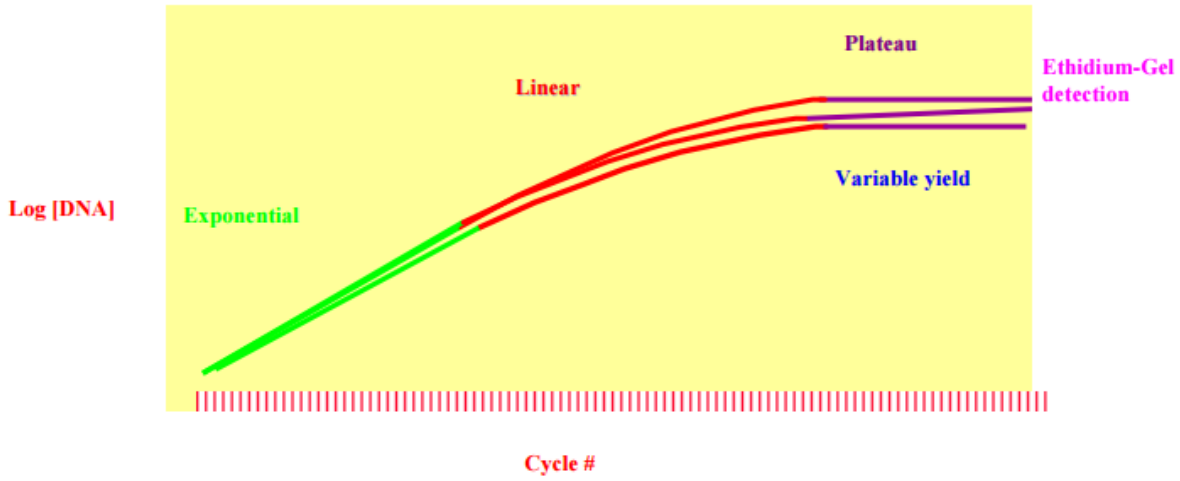


Figure 1.9 Example of PCR phases (Applied Biosystems).

1.7.2.2 Quantitative PCR (qPCR)

Quantitative PCR is similar to standard PCR in that it is also based on the selective amplification of defined sequences of DNA from a complex nucleic acid mixture and produces a detectable amount of product from a small target sample. The amplification is however monitored in real time and the method is therefore sometimes referred to as "Real-time PCR". The reactions are monitored throughout cycling using either a fluorescent DNA binding dye or fluorogenic probes, and visualised using an electronic laser-based detection system connected to a computer software system that displays amplification plots and facilitates analysis of results. As no gel electrophoresis is needed this greatly reduces sample analysis times. Another advantage qPCR has over standard PCR is the amplicon size which can be from 50-150 bp in length which is smaller than product sizes visualised in standard PCR. Given standard PCR discriminates via product size it would be difficult to discriminate between a genuine product of 50 bases and primer dimer. This is particularly advantageous when dealing with non-invasively collected samples which may contain low quality or quantities of DNA. Also qPCR facilitates the monitoring of the kinetics of PCR amplification and therefore may also be used for quantitation of template DNA. Automation is a further advantage of qPCR over end point PCR. qPCR detects amplification of product in the

exponential phase of PCR (Figure 1.10) after each cycle allowing quantitation of starting material by first constructing a standard curve.

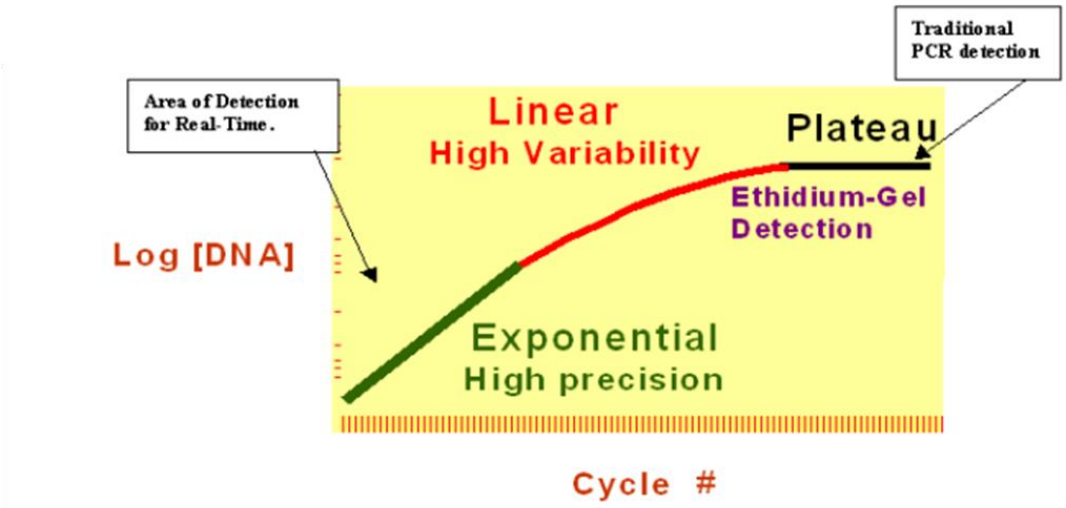


Figure 1.10 Comparison of detection areas between standard PCR and qPCR (Applied Biosystems).

As amplification proceeds the degree of fluorescence increases. The fractional cycle number at which the fluorescence from the sample reaches a pre-set threshold value is known as the Cycle threshold (C_T). Once the software receives a signal at a detectable level the result is displayed in an amplification plot (Figure 1.11). The X-axis of the amplification plot shows the cycle number and the Y-axis shows the change in fluorescence for each cycle.

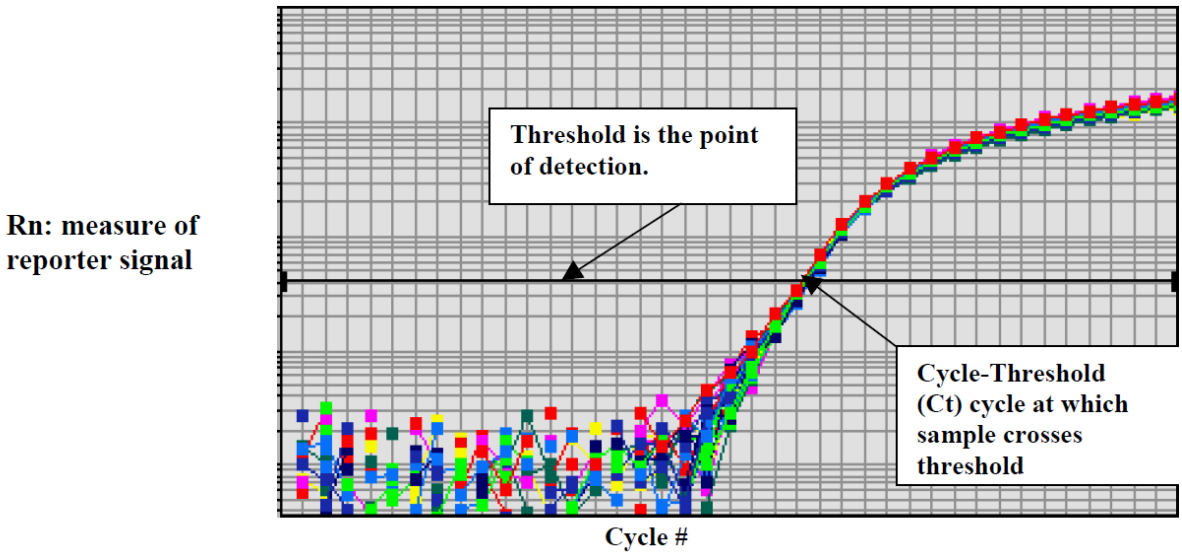


Figure 1.11 Example of amplification plot from qPCR (Applied Biosystems).

There are many different qPCR chemistries available. This project utilises two of these chemistries; intercalating dyes (SYBR® Green) and fluorogenic probes (TaqMan® MGB).

1.7.2.2.1 Intercalating dyes

There are a number of different intercalating dyes available for qPCR (reviewed in Monis *et al*. 2005) that utilise various platforms. In this project the intercalating dye used is SYBR® Green dye. Intercalating dyes bind to the minor groove of double stranded DNA and on binding, the intensity of fluorescent emission increases. Therefore as more double stranded amplicons are produced in each PCR cycle the SYBR® Green dye signal will also increase with the net increase in fluorescence being proportional to the increase in amplicon.

SYBR® Green dye enables monitoring of any double stranded DNA sequence. The disadvantage of SYBR® Green is the fact that since it binds to any double stranded DNA there is the potential for false positives especially if the reaction is not optimised as primer artefacts may also bind SYBR® Green dye and fluoresce. A built-in post-PCR confirmation method is available when using SYBR® Green dye. A melt-curve analysis step which determines the melting temperature (T_M) of the amplified DNA based on the decrease in fluorescence on the separation of the two DNA strands. Non-specific amplification may be identified as having a different, usually lower, melting temperature to the correct product.

1.7.2.2.2 Fluorogenic probes

This assay detects the amplification of a PCR product by the hybridization and cleavage of a doubly labelled fluorogenic probe during the amplification reaction**.** In the 5' assay (Holland *et al.* 1991) an oligonucleotide TaqMan® MGB probe is included in the PCR reaction which anneals to a specific sequence within the area between the forward and reverse primers so when the polymerase reaches the annealed probe its 5' nuclease activity cleaves the probe. At the 5' end of the probe there is a high energy dye attached (reporter) and at the 3' end of the probe a low energy dye is attached (quencher) (Livak *et al.* 1995). If the probe is excited by a light source while intact there will be no emission from the reporter dye due to its proximity to the quencher dye but when the probe has been cleaved by the polymerase the distance between the dyes is increased causing energy transfer to cease between dyes and the emission from the reporter dye to increase markedly. This is what is used for detection purposes and this increase is detected by the qPCR machine and displayed on screen. For any given sample the increase in reporter dye signal is proportional to the amount of product being produced.

Once the reporter signal has reached a detectable level it is displayed on an amplification plot in the same way as previously described. The major groove binding (MGB) moiety attached to the quencher molecule stabilises the hybridised probe. The use of intercalating dyes increases specificity as well as offering the ability to multiplex with additional probes as long as the labels have different excitation levels. One disadvantage however is a separate probe must be designed for each target.

1.7.2.3 Microsatellite analysis

Microsatellites are tandem repeats of 2-6 nucleotides in nuclear DNA with a microsatellite locus varying in length between 5 and 40 repeats (Selkoe & Toonen, 2006). A single locus, co-dominant nature and high allelic diversity make microsatellites very informative markers. An individual locus had two copies unless the marker is positioned on the X chromosome of a male. Otherwise, if both copies contain the same number of repeats then the individual is said to be homozygous for that marker. Likewise if both copies contain a different number of repeats the individual is said to be heterozygous for that particular marker. At any one marker, if two samples contain the same alleles, it is possible they came from the same individual. If the alleles are different then the samples are not from the same individual. Therefore, the use of multiple microsatellite markers increases the probability of distinguishing individual animals of a species. Cross species amplification of microsatellite loci within the family Mustelidae means it is not necessary to re-design a set of target primers for each species (Davis & Strobeck, 1998). Bio-informatic software is used to assess this probability and determine the number of markers required for a positive identification. Although nuclear DNA is less abundant than mtDNA in cells, microsatellite markers are PCR amplification based and therefore are also suitable for use with non-invasively collected DNA samples such as hair and scat even though they may contain poor or degraded DNA.

1.7.2.4 Sanger chain termination sequencing

The Sanger chain termination method of sequencing is also known as the dideoxy method (Sanger *et al.* 1977) as it depends on the incorporation of dideoxy nucleotides (ddNTP) into the newly synthesised strand of DNA. As the ddNTP contains no 3’ OH group chain DNA synthesis is terminated. PCR is first used to amplify the target sequence to a detectable concentration. The target is then extended in a reaction including fluorescently labelled

ddNTPs which when run capillary electrophoresed will be detected by a laser and the resultant sequence displayed by use of detection software. Direct sequencing has many applications in molecular ecology including; determination of species specific sequences to allow species identification or differentiation (Colli *et al.* 2005), the isolation of microsatellites (Domingo-Roura 2002), or in phylogenetic studies (Sato *et al.* 2012).

1.7.3 Application of molecular techniques

1.7.3.1 Species Identification

As mustelids are often nocturnal, elusive and sometimes present in low densities they can prove difficult to survey. This is compounded by the similarity of some mustelid scats to other meso-carnivores present in a survey area (Davison *et al.* 2002). In many areas the sympatric pine marten, stone marten and fox have different conservation statuses (Pilot *et al.* 2007; IUCN 2013). Misidentification of fox scats as pine marten scats, for example, could lead to an over estimation of that marten population which may affect any subsequent conservation plan. In areas where morphological misidentification of scats from these species is possible molecular techniques are important tools (Birks *et al.* 2004) to avoid incorrect abundance estimations due to incorrect identification of the species leaving the scat.

For the identification of mustelid species from non-invasively collected samples, Foran *et al*., (1997), successfully used a combination of targets on the mitochondrial cytochrome b gene and d-loop region using PCR amplification followed by Restriction Fragment Length Polymorphisms (RFLP) to distinguish between American marten, Fisher, Lynx and Wolverine. RFLP involves fragmenting amplified DNA samples by digestion with restriction enzymes which recognise and cut DNA at short recognition sequences. These restriction fragments are separated by length using gel electrophoresis. Different sequences within the target area from different samples, in this case different species, will be cut at different points and result in fragments of different length.

PCR-RFLP was also used by Ruiz-Gonzalez, et al., (2008), Balestrieri *et al*. (2008) and Ruiz-González *et al.,* (2013b) to distinguish between pine marten and stone marten while investigating the range expansion of pine marten in Italy. Figure 1.12 shows the amplified

mtDNA d-loop fragment used by Ruiz-Gonzalez, *et al*., (2008), to differentiate between pine marten and stone marten using the restriction enzymes *HaeIII* and *RsaI.*

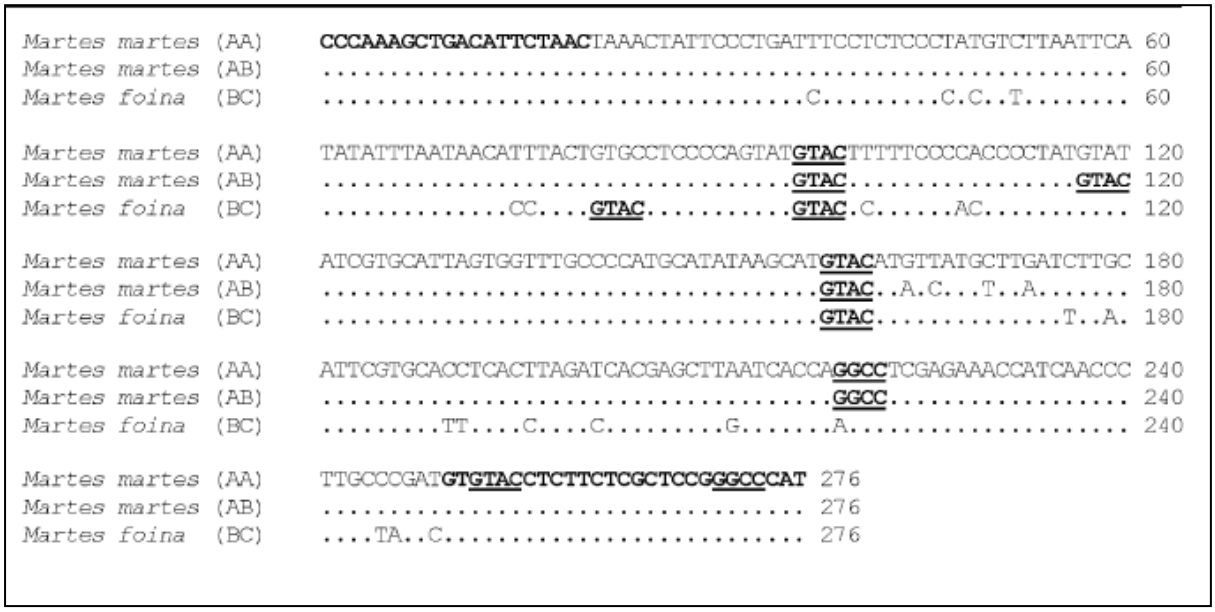


Figure 1.12 Aligned sequences of the amplified mitochondrial D-loop fragment in *Martes martes* and *Martes foina* (Ruiz-Gonzalez *et al.* 2008). Primer sequences are shown in bold. Restriction sites for HaeIII (recognition sequence GGCC) and RsaI (recognition sequence GTAC) are underlined and in bold.

Other studies using PCR-RFLP in their identification of marten species utilise a similar approach and differ mainly in the mtDNA target region or product size. For example, Vercillo *et al*., (2004) used a fragment of the cytochrome b gene as the target for their method to distinguish between stone marten and pine marten scats using PCR-RFLP. A recognition site for the restriction enzyme *AluI* (AG'CT) that produces two fragments of 78 and 140 bp in stone marten is absent in pine marten allowing successful discrimination between scats from both these species (Figure 1.13).

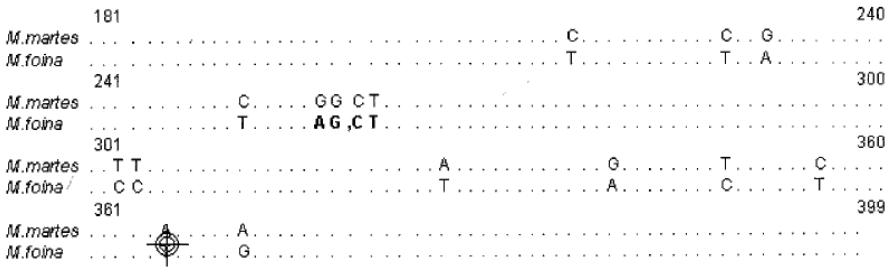


Figure 1.13 Cytochrome b partial sequence showing the AluI restriction enzyme recognition site (in bold) present in stone marten but absent in pine marten (Vercillo, *et al*. 2004).

Figure 1.14 shows the restriction patterns for polecat, fox, pine marten and stone marten when a 365 base sequence of cytochrome b sequence from each species is digested with *HinfI*, *AluI* and *TaqI* restriction enzymes (Leucentini *et al*. 2007).

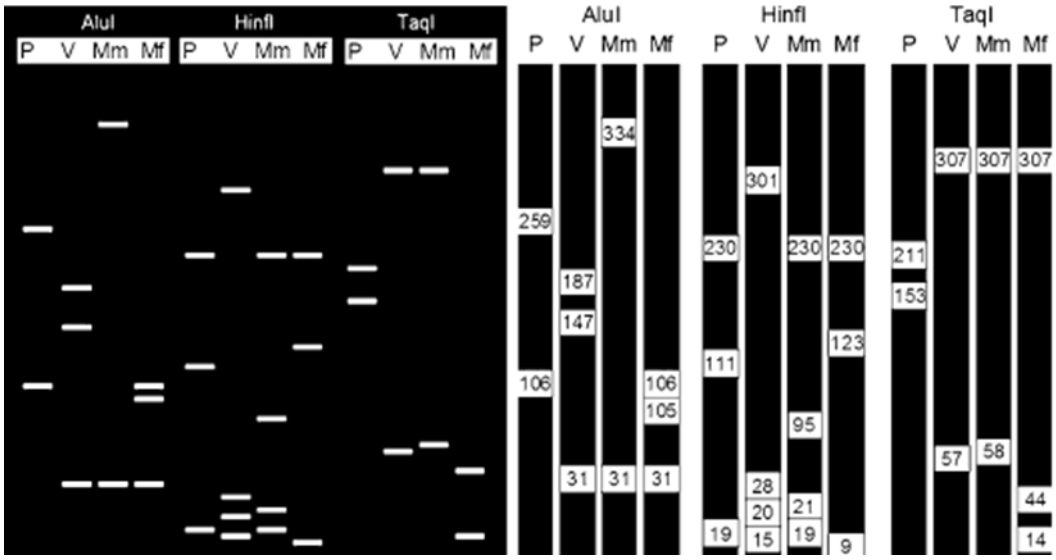


Figure 1.14 Restriction pattern of Cyt b fragment using restriction enzymes HinfI, AluI and TaqI showing a characteristic restriction pattern for polecat (P), fox (V) pine marten (Mm) and stone marten (Mf) (Leucentini *et al*. 2007).

It is sometimes possible to distinguish between faecal DNA from sympatric animals solely by using standard PCR and comparing the product sizes against a reference size standard as long as the primers used yield a different size product for each species. Kurose *et al*., (2005) distinguished between species using standard PCR amplification of a portion of the cytochrome b gene as the target. Separate sets of species specific primers were designed to target this area that yielded 4 different sized products allowing differentiation between all four small sympatric carnivores living on the Tsushima Islands of Japan: the leopard cat (*Felis bengalensis*), Japanese marten (*Martes melampus*), Siberian weasel (*Mustela sibirica*), and feral cat (*Felis catus*).

In a non-invasive genetic survey of pine marten, Mullins *et al*., (2010), used hair tubes to gather samples and a TaqMan® MGB probe based qPCR assay to amplify an 85-base pair fragment of the mitochondrial control region which allowed identification of the samples as being from pine marten. Also in Ireland, and using both SYBR® Green intercalating dye and Taqman® MGB probes qPCR protocols, O'Reilly *et al*., (2008) successfully discriminated between sympatric fox and pine marten using scats samples. The use of qPCR allowed the identification of pine marten and fox scats overcoming the problems of small quantities and

poor quality DNA associated with scat samples (O'Reilly *et al*. 2008). The primers used in this study amplified both pine marten and fox DNA samples. The addition of differently labelled species specific probes allowed identification of the offending species (Figure 1.15).

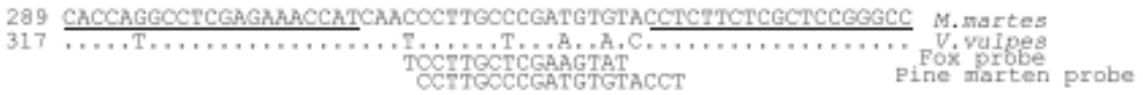


Figure 1.15 DNA alignment of the short sequence of mitochondrial D-loop sequences of fox and pine marten showing the positions of primers (underlined) and probes (below) used in qPCR 5' nuclease assay (O'Reilly *et al*. 2008).

It is also possible to distinguish between non-invasively collected samples from pine marten and stone marten using a single microsatellite marker, originally isolated from the Eurasian badger (*Mel10*). This marker is relatively short and fixed in size in stone marten (128bp) while longer and variable in size (153-165bp) in pine marten (Domingo-Roura, 2002; Posluszny *et al.* 2007). The use of microsatellite markers to differentiate between stone marten and pine marten was also used by Goszczynski *et al.,* (2007) to assess the accuracy of the snow tracking method they used to investigate the locomotion and winter foraging habits of these two sympatric species. Another example of the use of molecular methods to test the accuracy of morphological methods is found in the work of Bartolommei *et al*., (2014) who used PCR-RFLP, with DNA extracted from hair samples, to test the accuracy of their morphometric studies to distinguish between stone marten and pine marten. Morphometrics included colour variability of coat, of body sides, rhinarium, pinnae, pads, muzzle, and the colour, size and shape of the throat patch and found the morphometric measurements corresponded completely with the molecular analysis. qPCR was also used in a study to estimate the age of American martens using DNA analysis. By amplifying telomere DNA using qPCR and combining the results with biologically meaningful covariates like sex, species, and estimates of population density, in a Bayesian network model, Pauli *et al*., (2011) assigned age group to American martens with a 75-88% accuracy. A combination of molecular techniques was used in a study of the effect of tourist pressure on a wild pine marten population in northwest Spain (Barja *et al*. 2007). PCR-RFLP was used for species identification and hormone levels were measured in the scats by enzyme immunoassay to estimate the stress responses to high tourist traffic in a pine marten population in northwest Spain. The data indicated that marten show elevated stress levels during times of high tourist traffic and this physiological response was increased further during the reproductive season.

1.7.3.2 Sex determination

Determining the sex of an individual animal, or the sex ratio in a population is important for the conservation and managment plans of a species (Lynch & Brown, 2006). Zalewski, (1999) showed that it is possible to determine the sex of a pine marten simply by measuring the tracks it leaves in the snow. However, in the absence of snow it is not always possible to differentiate between the sexes of an animal by traditional methods such as sex-biased behaviour, direct observation of genitalia, or sexual dimorphism. Molecular sex determination is an obvious advantage in such instances but also when dealing with elusive, endangered, nocturnal or potentially dangerous animals. While the target for sex identification is nuclear DNA, PCR amplification allows molecular sex identification to be performed using non-invasively collected samples. Genes typically targeted for sex typing include zinc finger (ZF) genes with homologs on the X (ZFX) and Y (ZFY) chromosomes (Statham, *et al.* 2007) and the sex determining region Y (SRY) gene which is responsible for male sex determination (Lynch & Brown, 2006). Statham *et al*., (2007) determined the sex of pine marten samples as well as 4 other mustelid species targeting zinc finger gene sequences on the heteromorphic X (ZFX) and Y (ZFY) chromosomes using Restriction Fragment Length Polymorphism (RFLP). Mullins *et al*., (2010) employed a 5' nuclease TaqMan® qPCR assay for the molecular sexing of pine marten also based on amplification of zinc finger gene sequences (Figure 1.16).

Figure 1.16 Alignment of ZFX and ZFY introns (Mullins *et al*. 2010) used to sex type pine marten hair DNA extracts using a probe based qPCR assay. The binding sites of qPCR primers are underlined and the TaqMan MGB probes areas used for molecular sexing are shaded. Dots in the ZFY sequence signify identity to ZFX sequence.

O'Neill *et al.,* (2013) adopted a similar approach when sex typing Eurasian otters. TaqMan® probe based qPCR was used targeting a 79 and 75 base fragment of the final intron regions of *Lutra lutra* ZFX and ZFY genes respectively. The male specific SRY gene region has also been used to determine the sex of pine marten (Lynch & Brown, 2006). A 135bp fragment of the SRY gene was used to determine the sex of four different species of carnivore on the Tsushima Islands of Japan including the Japanese marten. As the SRY gene is male specific however lack of amplification does not necessarily indicate a female sample as a negative result may also indicate a failed PCR reaction (Statham *et al.* 2007).

1.7.3.3 Identification of individual animals

In order to estimate the density and abundance of a particular species it is important to be able to identify individual animals. The molecular approach to identifying individual animals involves the amplification and genotyping of multiple polymorphic microsatellite loci. Microsatellites are tandem repeats of 2-6 nucleotides with a microsatellite locus varying in length between 5 and 40 repeats (Selkoe & Toonen, 2006). The polymorphic nature of microsatellites makes them potentially very informative markers. For examples the microsatellite maker ma-1 was found to have 10 alleles in American marten (Davis & Strobeck, 1998). A single locus, co-dominant nature and high allelic diversity make microsatellites very informative markers. At any one marker, if two samples contain the same visible alleles, it is possible they came from the same individual. If the alleles are different then the samples are not from the same individual. Genetic software packages are used to calculate the probability of being able to distinguish a sample from an individual which is known as the probability of identity (P.I.D) and also the probability of identifying individuals in the presence of related individuals known as the probability of sibling identification error (PIsibs).

While the traditional method of pine marten censusing is scat transect surveys (Strachan *et al.* 1996) the relationship between scat density and population abundance can be unclear (Birks, *et al.* 2004).If scats can be identified to species level but scats from individual animals are not identified it is unknown whether the scat population is a product of a large population of marten or merely a few individuals depositing scats with a higher than expected frequency. As scat deposition patterns may vary with habitat characterictics (Sadlier *et al*. 2004) as well as scat deposition rates being influenced by season (Birks, *et al*. 2004) and diet (Andelt &

Andelt, 1984) it is necessary to identify individual animals in order make any inferences about the species abundance or density.

Once the individual animals in an area have been identified, further studies may then be carried out on animal density, abundance, species occupancy, distribution and population estimates on a local, regional or continental scale. Microsatellite analysis is a common approach adopted in many of these studies. The number of markers required depends on the purpose of the study with more markers with variable loci allowing a higher degree of inference.

The number of loci required in microsatellite analysis varies with the research question and with the degree of heterozygosity of each marker in a particular population. For example, the differentiation between pine marten and stone marten only requires the amplification of a single marker (Posluszny *et al*. 2007). The study of a single population of animals requires identifying individual animals and so requires more markers. In a study censusing pine marten populations in Ireland it was found that 8 microsatellite markers were sufficient to identify individual animals within the Irish population (Mullins *et al*. 2010). Eight markers were also sufficiently informative for Mergey *et al*., (2012) to study the gentic diversity of the pine marten in France. No association between genetic variability and forested habitat characteristics was found allowing them to suggest that pine martens may exhibit an increased behavioural plascticity than previously thought. Six microsatellite markers was sufficient to estimate the population size of American marten in the central Selkirk mountains, British Columbia. (Mowat & Paetkau, 2002).

While microsatellite analysis is usually sufficient to study a single population, phlogeographic studies often employ a combination of microsatellite analysis and mtDNA sequencing. This is because microsatellites provide little information about more ancient time scales due to their high mutation rates and size homoplasy which may mask phylogenetic signals (Sacks & Louie, 2008). mtDNA sequences in the d-loop region will typically vary among and within populations allowing the grouping of individual animals into haplotypes. The assignment of mitochondrial haplotypes is therefore often used, either alone (Pertoldi *et al*. 2014) , or in conjunction with microsatellite analysis for phylogeographic studies (Vergara *et al.* 2015).

In phylogeographic studies where the heterozygosity of indivdual markers may vary between mulitple populations a bigger panel of markers is again required. Williams & Scibner, (2010)

amplified 11 microsatllite loci allowing the identification of 3 geographically related genetic clusters which allowed them to investigate the effect of multiple founder populations on the genetic structure of an amercian marten population reintroduced to an area of Michigan in North America to bolster the existing population which was in decline. In comparing the two pine marten populations of Jutland and Sealand in Denmark, Pertoldi *et al*., (2008) used 11 microsatellite markers for genetic analysis and from the resultant data were able to determine there had been a large degree of genetic drift between the two populations over a short period of time. From genetic analysis of live trapped american marten using 9 microsatellite marker Broquet *et al*. (2006) were able to show a leptokurtic dispersal in this species within their study area. This long distance dispersal was also found to be a trait of some pine marten in the Netherlands, again using microsatellite analysis (de Groot, *et al.,* 2015). In their case study of the genetic structure of stone marten on the Iberian Peninsula Vergara *et al.*, (2015), used 23 microsatellite markers complemented by mitochondrial haplotype analysis. Both the microsatellite analysis and the mtDNA data revealed a lack of phylogeographic structure suggesting a recent colonisation of the Iberian Peninsula by a small number of stone marten mtDNA lineages.

When using non-invasive samples for microsatellite analysis allelic dropout and other genotyping errors may be problematic due to the degraded nature of the samples. When assigning individual genotypes the comparative multiple tube approach is often adopted (Taberlet *et al*. 1999) to protect against allelic dropout or false alleles. Three replicates have been recommended by Frantz *et al.*, (2003) wherein genotypes are assigned based on the presence of each allele at least twice for heterozygotes and three times for homozygotes. Problems with allelic dropout can also be overcome by increasing the number of loci used during analysis to minimise the effect of a single false allele. However, the introduction of a new locus to the analysis also brings the potential introduction of a new source of allelic dropout with the optimal number of loci found by trial (Mckelvey, 2004).

Non-invasive samples have, however, been used successfully for microsatellite analysis with marten species. Mullins *et al.*, (2010) used microsatellite analysis on non-invasively collected hair samples to identify individual pine marten in a study site in Ireland. On the northern Iberian Peninsula PCR RFLP on scat DNA was used to distinguish between pine marten and stone marten. Once species had been attributed to the samples, microsatellite analysis was performed, using 15 variable loci, on the pine marten scat DNA extracts to study the gene

flow throughout the study area (Ruiz-Gonzalez, *et al.* 2014) while Sheehy *et al.* (2014), used microsatellites to detect pine marten individuals from hair DNA extracts in Ireland.

1.7.3.4 Dietary analysis

Extensive work has been carried out on dietary analyses of marten scats using morphological analyses including microscopy, cuticle and hair analyses, frequency of occurrence and total biomass indices (Baghli *et al*. 2002; Rosellini *et al.* 2008; Reig & Jedrzejewski, 1988). Other studies have used molecular techniques to first attribute species to the sample scat and then used morphological methods to perform dietary analysis. In their study of the seasonal diet of British pine marten, Caryl *et al.*, (2012) firstly used a probe based qPCR assay to confirm the scats were from pine marten before then using microscopy for their dietary analysis.

Dietary analysis has also been performed using molecular techniques. qPCR has been used to detect bank vole, greater white-toothed shrew and red and grey squirrel DNA in pine marten scats (O'Meara *et al*. 2013). In their study on pine marten abundance and predation, Sheehy et al., (2014) used a combination of molecular and morphological analyses. qPCR was used to detect squirrel DNA in pine marten scats and initial results confirmed by morphological analyses.

1.8 Project outline

The aim of this project was firstly to conduct molecular analyses that identify species, sex and individual marten animals using non-invasively collected samples then secondly, a molecular based dietary study to detect bat species' DNA in scats.

DNA extracted from scat samples was used to determine the following: the distribution of both marten species; if both marten are entering the tunnels; if there was a difference in ranges above and below ground; if both marten are involved in bat predation; any preferential predation of bat species; any impact on the bat population as a result of marten predation.

Scat collection and hair tube surveys were used to gather samples for molecular analysis. DNA was extracted from these non-invasively collected samples. This project investigated distribution of pine marten and stone marten in the survey area around the tunnel system. It

also determined if either, or both, species of marten are venturing into the tunnel system. A comparison of territorial behaviour in the fragmented landscape above ground with the largely linear nature of the bat reserve was also be made. Dietary analysis was performed to determine if either, or both, marten species are involved in bat predation. Novel bat species-specific primers were designed to identify bat DNA in the scats using a qPCR assay. The project also investigates if any particular bat species is being predated and if there is any significant impact on this bat species as a result of predation by pine marten or stone marten. qPCR was used for identification of samples to species level, for sex determination. A suite of qPCR species specific primers was designed to detect the presence of bat DNA in marten scats. Microsatellite analysis was used to identify individual pine marten and stone marten.

2 THE USE OF HAIR TUBE SURVEYS TO COLLECT NON-INVASIVE SAMPLES FOR MOLECULAR ANALYSIS

2.1 Introduction

This chapter investigates the use of hair tubes to collect hair samples non-invasively from pine marten and stone marten in and around Nietoperek. A control study was also set up in Corbally Wood, Kilkenny, Ireland to trial the use of hair tubes in a site where pine marten had not seen hair tubes previously. Stone marten are not found in Ireland. Molecular techniques were used for species identification, sex determination and individual animal identification. The effectiveness of using hair tubes for sample gathering in Nietoperek and Corbally Wood is discussed as well as the use of molecular techniques for the analysis of non-invasively collected samples.

2.1.1 Explanation of Thesis Terminology

For the purpose of writing this report when the term "marten" is used without specification of stone marten or pine marten, it will refer to both species. The surveyed areas during this project consist of two sites. The first is the NATURA 2000 site in and around the bat hibernation site at Nietoperek, Poland. When referring to this site without specification to any particular area within this survey site the term "Nietoperek" will be used. The second is a forested area in Corbally, County Kilkenny, Ireland. For the duration of this document, this site will be referred to as Corbally Wood.

When referring to the underground portion of the Nietoperek survey area the term "bunker system" will refer to all areas in the survey area including the tunnels but will also incorporate bunkers that are not connected to the main tunnel system but are within the survey area. The term "tunnels" will be used to refer to the area underground including only the bunkers that are connected by these tunnels. The term "survey area" will refer to the entire underground area as well as the area directly above and to the west of the bunker system. "Sections 1-9" will refer to individual segments of the tunnels .There are two main forested areas in the above ground portion of the survey area. Two relatively small forested areas (area 3.55 km^2) in the south of the survey area in Boroszyn which will be collectively termed the "Southern Forest" and a portion of a much larger segment of forested area (10.82 km^2) directly over the

northern part of the bunker system and continues to cover an area to the west. This forest will be termed the "Northern Forest". The area in between these forests has islands of woodland present but is mostly open arable land and will be referred to as the "Middle Section" of the above ground survey area. As there are multiple bat species in and around Nietoperek when the general term "bat DNA" is used, it refers to DNA from any of the bat species involved in this study.

2.2 Survey methodologies

2.2.1 Hair tube survey sites

2.2.1.1 Nietoperek hair tube survey site, Poland

The hair tube survey conducted in Nietoperek covered the Southern Forest portion of the survey site (Figure 2.1). This area is locally known as "Petla Boryszynska" or the "Boryszyn Loop". The Southern Forest comprises two smaller woodlands with a combined area of 3.55 km^2. It is a mixed forest with dense understory almost throughout. The understory is similar to that found to be suitable for use by marten in previous studies (Birks *et al*. 2005). Only the above ground portion of the survey site was included in the hair tube survey.

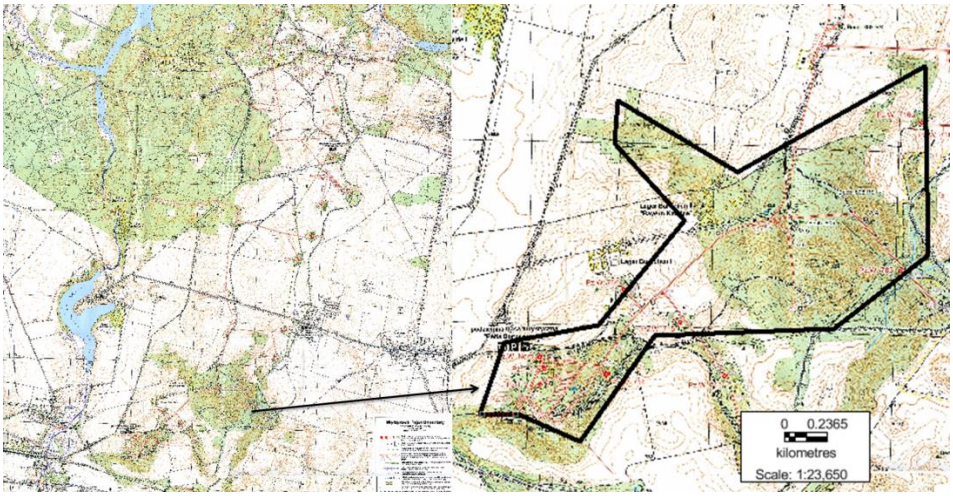


Figure 2.1 Location of Nietoperek hair tube survey, September 2012- March 2013

2.2.1.2 Corbally Wood survey site, Ireland

Corbally Wood is in County Kilkenny, Ireland. It is a managed conifer forest with a dense understory. The proposed survey area covers approximately 1.5 km^2 (Figure 2.2). Pine marten

were known to have been present in the area as previous hair tube surveys had been conducted. However it had been 7 years since any survey work had been carried out in this forest (August 2006) and as such Corbally Wood may be considered a naïve site (*Turner, Pers comm*).

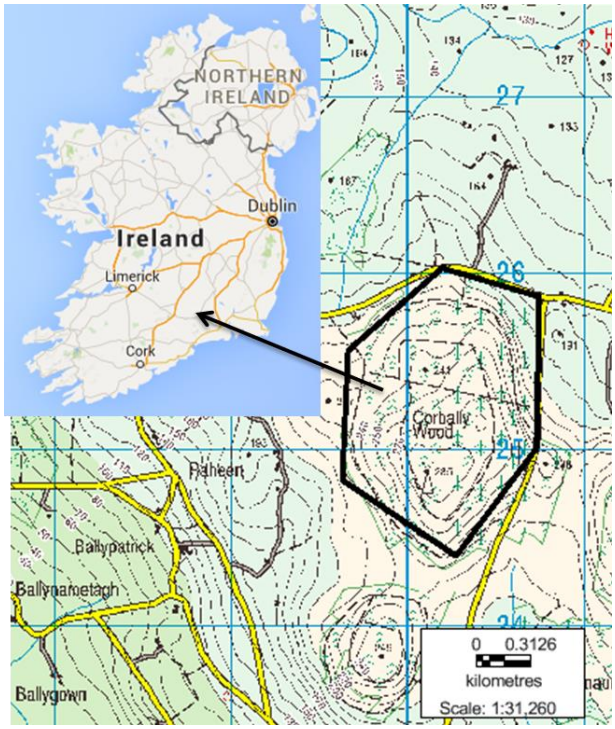


Figure 2.2 Location of Corbally Wood hair tube survey, autumn 2013.

2.2.2 Hair tube survey in Nietoperek , September 2012 to March 2013

To obtain hair samples from pine marten and stone marten, 10 baited hair tubes were erected as described by Roche (2008). The bait used was the distal part of a chicken wing with wire threaded through the bones, forcing the marten to struggle to get the bait. This will result in a hair sample being left on the glue patch on the opposite side of the tube. These tubes were erected at the start of the September 2012 scat survey.

The site chosen for the hair tube survey was Petla Boryszynska in the Southern Forest (area 3) as there had been scats from pine marten and stone marten previously found in this area. Locations were selected that were close to forest tracks but were in areas with plenty of undergrowth. Some tubes were placed on trees close to the forest boundary with the adjacent fields.

Figure 2.3 Hair tube being positioned during September 2012 survey.

The hair tubes were erected approximately 1.2-1.5 m above ground and attached to the tree with metal wire (Figure 2.3). An additional 16 hair tubes were erected by two students from Wrocław University of Environmental and Life Sciences, Poland as part of their M.Sc. program in October 2012. This extended the area covered by the hair tubes along the forest boundaries in both directions to encompass the Southern Forest (Figure 2.4). Each tube was checked for a hair sample, and rebaited, every two weeks (weather permitting) until March 2013. This task was again performed by the collaborating students. An additional experiment to determine the best bait for use with pine marten and stone marten was carried out during the scat survey in January 2013. In the Southern Forest, a board was fixed to the ground through the snow and a variety of possible baits were fixed in place. Baits used were; peanut butter, jam, chicken hearts, sardines, cheese, cherries and sausages. A trail camera was positioned to monitor any animal feeding on the bait. The camera was checked for activity two days later.

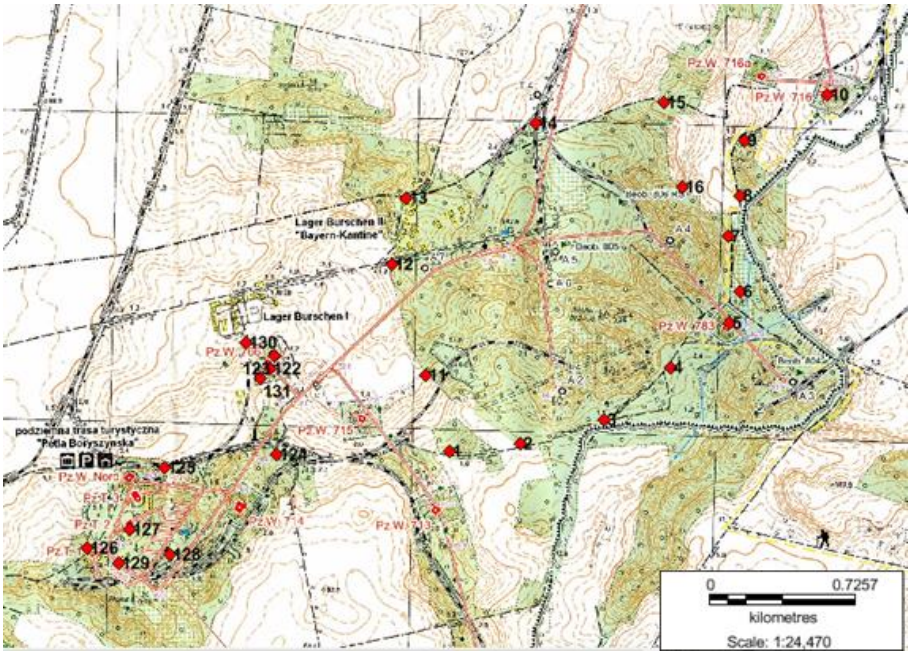


Figure 2.4 Location of hair tubes ★ erected in the Southern Forest, September/October 2012.

2.2.3 Hair tube survey in naïve site in Corbally Wood, Ireland, Autumn 2013

A hair tube study was performed in Corbally Wood, County Kilkenny to determine the effectiveness of the hair tube design and methods employed in Nietoperek and to estimate the latency period required before marten find and access the hair tubes.

2.2.3.1 Preliminary scat survey in Corbally Wood, Kilkenny, Ireland

Prior to undertaking a hair tube survey, an initial scat survey was performed to investigate the presence or absence of pine marten in the area. Pine marten scats are approximately 8-10 cm long, and 1 cm in diameter. They have a cylindrical shape with pointed ends. Sometimes the cylinder has a twist (Velander, 1986). However, diet and environmental exposure may alter scat morphology. Other mammals or invertebrates may also interfere with scats (Birks *et al.* 2004; Jedrzejewski & Jedrzejewski, 1990). Any scat that was similar in morphology to a pine marten scat was collected. If there was any doubt over what species of animal had left a scat it was collected as the species was to be verified by DNA analysis. Scats identified as possibly from pine marten were individually placed in pre-labelled zip-lock bags and given an individual identifier. This was achieved by either picking the scat up with a stick or by turning

the bag inside out and picking up the entire scat before turning the bag back around leaving the scat inside. Where a stick was used, a separate stick was used for each scat to avoid cross contamination.

The scat survey in Corbally Wood was performed in a single day where all forest roads within the survey site were surveyed for scats. The position of each possible pine marten scat was recorded using GPS. All scats were brought to the Molecular Ecology Research Laboratory in W.I.T for analysis.

2.2.3.2 Hair tube survey in Corbally Wood, Kilkenny, Ireland

Once the presence of pine marten was confirmed in the area, a hair tube survey was performed to determine how many pine marten were occupying this area of woodland. Hair tubes locations were selected approximately 200 m either side of where a known pine marten scat was picked up. Hair tubes were erected on trees in areas with dense understory, as described in section 2.2.2 of this document, and tubes were again baited with chicken wings and a sticky patch on the bottom of the inside of the tube was used to obtain a hair sample from the marten (Figure 2.5).

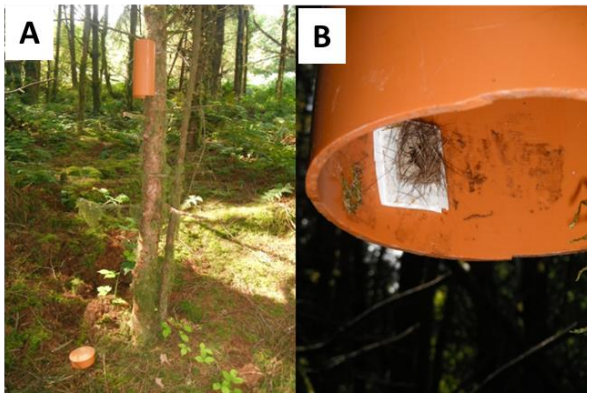


Figure 2.5 Position of hair tube on tree in Corbally wood (A) and (B) a pine marten hair sample left on a sticky patch inside a hair tube.

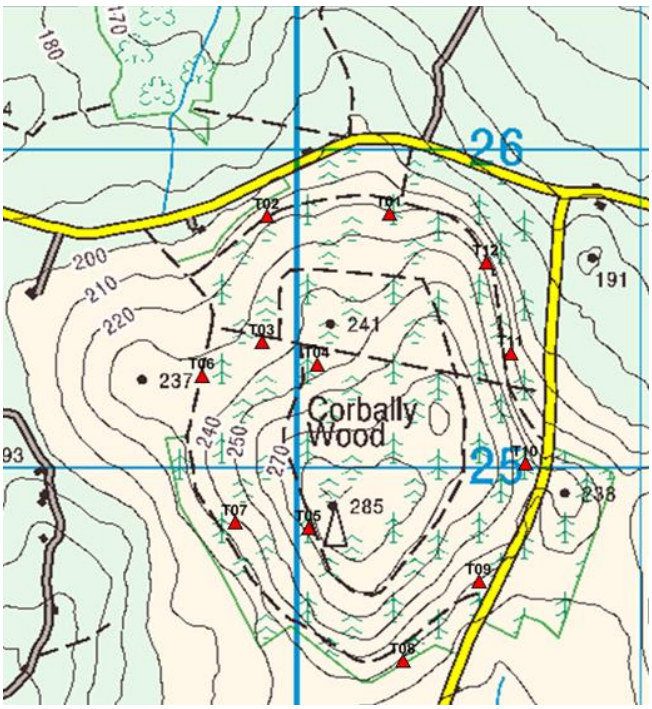


Figure 2.6 Position of hair tubes in Corbally Wood; ▲ T=tube.

The location of each hair tube was recorded (Figure 2.6). Each hair tube was visited on a weekly basis for a period of 6 weeks. Positive hair tubes were recorded on a log sheet. The quantity (HQ =Hair quantity) of hair obtained on each glue patch was broadly divided into three categories; 1-(very few hairs), 2-(10-20 hairs) and 3-(more than 20 hairs). Other information recorded on the log sheet included; the presence of a hair sample, if the marten had taken the bait and if a fresh hair patch was required. From previous studies using hair tubes to detect pine marten, a latency period of 5 – 10 days in which the pine marten find and familiarise themselves with the hair tube was expected to be sufficient (Lynch *et al.* 2006; Mullins *et al*. 2010).

2.3 Molecular analysis of non-invasively collected samples

2.3.1 Scat D.N.A extraction

Approximately 0.2 g of scat was transferred to S.T.A.R® buffer (Roche cat. no. 03 335 208 001) in a micro-centrifuge tube (Zymo Research cat. no C-2001-50) using disposable wooden spatulas. Scat samples in S.T.A.R® buffer were vortexed to mix the scat and buffer and allowed stand for 30 minutes. 150 µl of supernatant was transferred to a clean micro-

centrifuge tube. DNA was extracted using a ZR Genomic DNA II kit (Zymo Research cat. no D3007) using the following protocol; 600 µl of genomic lysis buffer (Zymo Research cat. no D3004-1-100) was added to the supernatant and the micro-centrifuge tubes vortexed and allowed to stand for 5 minutes. The 750 µl of solution was then transferred to a Zymo–Spin™ IC column (Zymo Research cat. no C1004-50) over a 2 ml collection tube (Zymo Research cat. no C1001-50) and centrifuged at 10000 x g for 1 minute in a Sigma® 1-14 micro-centrifuge. The effluent was discarded. This step was repeated first with 200 µl of pre-wash buffer (Zymo Research cat. no D3004-5-50) and then with 500 µl of g-DNA wash buffer (Zymo Research cat. no D3004-2-100). The column which now contains the scat DNA was then transferred to a clean 1.5 ml micro-centrifuge tube and 150 µl of DNA-free water added and allowed to stand for 5 minutes. Scat DNA extracts were stored at -20 °C for molecular applications.

2.3.2 Hair DNA extraction

Using a Pasteur pipette, a single drop of xylene (99%) (Lennox cat. no CLL03788.AP) was placed on each glue patch to facilitate the removal of hair samples. Using forceps, heated in a Bunsen flame and cooled to avoid cross contamination, hair samples were removed from each glue patch. Hairs were transferred to 1.5 ml micro-centrifuge tubes and DNA was extracted using the ZR Genomic DNA™-Tissue MiniPrep kit (Zymo Research cat. no D3050) using the following protocol; Hair samples were first digested by adding; hairs from a glue patch, 90 µl molecular grade water, 90 µl of 2X digestion buffer (Zymo Research cat. no D3050-1-20), 10 µl of Proteinase K (AppliChem cat. no. 39450-0-6) and 10 µl of 1M Dithiothreitol, (DTT), (Sigma Aldrich cat. no. DO632-SG) to each micro-centrifuge tube. Samples were then vortexed and incubated for 3 hours at 56 °C in a BIOSAN TS-100 Thermo shaker. 700 µl of genomic lysis buffer was then added and samples were vortexed to mix and allowed stand for 5 minutes. To remove any insoluble debris samples were centrifuged at 10000 x g for 1 minute in a Sigma® 1-14 micro-centrifuge. 700 µl of supernatant was then transferred to a Zymo–SpinTM IC column over a 2 ml collection tube and centrifuged at 10000 x g for 1 minute. The effluent was discarded. This step was repeated first with 200 µl of pre-wash buffer and then with 400 µl of g-DNA wash buffer. The spin column, which now contains the hair DNA, was then transferred to a clean micro-centrifuge tube and 100 µl of DNA free water added. Samples were allowed to stand for 5 minutes then centrifuged at 15 000 x g for 30 seconds. DNA eluates were stored at -20 °C prior to molecular analysis.

2.3.3 PCR primers and probes used to target marten DNA

The qPCR primers and TaqMan® MGB probes used for the identification of pine marten species and for sex determination, including the region being amplified and product size are listed in Table 2.1 and Table 2.2.

Table 2.1 qPCR primer pairs used to detect pine marten DNA; CR=control region, ZFX=X chromosome zinc finger gene sequence, ZFY= Y chromosome zinc finger gene sequence. Primers were supplied by Eurofins Scientific.

Primer pairs	Sequence	Target	Product size (bp)	Reference
PM3F	CTTGCCCCATGCATATAAGCA	CR	99	(Statham *et al.* 2007)
PMREV2	GCCTGGTGATTAAGCTCGTGAT			
MMXF	GGCAGAGCAACCCTGTCATAA	ZFX	76	(Mullins *et al.* 2010)
MMXR	GGGCCTGAGGTTGGTACCACCA			
Y2F	CTTGTCCAGAAACTTCATTCAATGTAA	ZFY	76	(Mullins *et al.* 2010)
MMYR3-	GGGGGCAGACAAGTAAAATCTA			

Table 2.2 TaqMan® MGB probes used for pine marten identification and sex determination using qPCR. Probes were supplied by Applied Biosystems

Probe name	Sequence	Primer pairs	Target	Reference
PM3	FAM-CGTGCACCTCACTTAG-MGB	PM3F,PMREV2	CR	(Mullins *et al.* 2010)
MMX	FAM-TGTGTCTCTCTCTGTCAA-MGB	MMXF,MMXR	ZFX	(Mullins *et al.* 2010)
MMY	VIC-CCTGGTCTGAAAACT-MGB	Y2F,MMYR3	ZFY	(Mullins *et al.* 2010)

2.3.4 Detection of pine marten DNA using TaqMan® MGB probe based qPCR

All DNA extractions were analysed using TaqMan® MGB probe based qPCR assays. All qPCR amplifications were performed in an ABI 7300 Real-time PCR system using MicroAmp® optical 96 well reaction plates (Applied Biosystems cat. no. N8010560). Applied Biosystems Sequence Detection Software (SDS1.2.3.) was used to analyse all results.

Species identification was performed using TaqMan® MGB fluorogenic probe based assay with forward PM3F and reverse PMREV2 primers (Statham *et al.* 2007) (Table 2.1) with a pine marten species specific FAM labelled probe (PM3) (Mullins *et al.* 2010).

Each 10 µl reaction contained; 5 µl FastStart Universal Probe Master (Roche cat. no 111 808 00), 0.5 µl of primer mix (PM3F+PMREV2) and pine marten probe (PM3) both at 0.25 µM final concentrations, 1 µl of molecular grade water and 3 µl of template DNA. Pine marten DNA of known origin was used as positive control and PCR blanks containing all reagents without template DNA were used to monitor for contamination. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol 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. Detection is based on the accumulated fluorescence (C_T value) of the FAM-labelled probe.

2.3.5 Sex determination of pine marten DNA extracts using TaqMan® MGB probe based qPCR.

DNA extracts that were positive for pine marten DNA using the TaqMan® MGB probe based qPCR species assay were screened for suitability to be selected for sex determination. Samples that had a high C_T value were not selected as a high C_T value in the species determination indicates a low starting concentration of template DNA. C_T values <= 30 are an indicator of good quality amplifiable DNA and so any samples below this threshold were selected for sex typing (O'Reilly *et al.* 2008). Both hair and scat DNA extracts positive for pine marten DNA were included in this screening process.

Molecular sex determination was based on the successful amplification of zinc finger gene sequences on the X (ZFX) and Y (ZFY) chromosomes using primers and probes designed by Mullins *et al.*, (2010). As this assay is based on the the co-amplification of homologous genes from both sex chromosomes there is increased reliability as the X chromosome product serves as an internal PCR control. As there is less nuclear DNA present in the sample than mtDNA, the number of cycles is increased from the 40 cycles used in species identification to 50 cycles.

Each 10 µl multiplex reaction contained 5 µl of FastStart Universal Probe Master, 0.5 µl each of; MMX primer mix ((MMXF and MMXR), MMY primer mix (Y2F and MMYR3) (Table

2.1), MMX probe (MMX) and MMY probe (MMY) (Table 2.2) all at a final concentration of 0.25 µM and 3 µl of template DNA. Male pine marten DNA of known origin was used as a positive control to confirm successful amplification using both probes and negatives controls containing all reagents without template DNA were used to monitor for contamination. All sex determination assays were performed in duplicate with a positive sample showing amplification in both. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol. Detection is based on the accumulated fluorescence (C_T value) of the FAM-labelled (MMY) and VIC-labelled (MMX) probe.

2.3.6 Microsatellite analysis of pine marten hair DNA extracts

2.3.6.1 Selection of samples for genotyping

Data generated from the sex determination assay was used to screen for samples suitable for microsatellite analysis. As the MMX target will be amplified in both sexes of pine marten these results were selected to screen for samples suitable for genotyping. Increasing C_T values in the sex determination assay reflect a reduction of nuclear DNA template (O'Neill *et al.* 2013). Samples with the lowest MMX C_T values were therefore selected for genotyping and analysis performed for samples with C_T values <= 34.0. Only hair DNA extracts were included in this screening process.

2.3.6.2 Selection of microsatellite markers for genotyping pine marten hair DNA extracts

A panel of 11 microsatellite markers currently in use in the Molecular Ecology Research Laboratory in W.I.T. (O'Reilly, *unpublished*) and known to be polymorphic in the Irish pine marten population was selected and can be seen in Table 2.3.

Table 2.3 Microsatellite primer pairs used to identify pine marten individuals from scat DNA; product = the expected product range for this study, bp = base pairs, F=forward, R=reverse, PIG-tailed (Brownstein *et al.* 1996) reverse primers are marked with an asterisk

Locus	Reporter	Product (bp)	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Reference
Mel 1	FAM	108-112	CTGGGGAAAATGGCTAAACC	*GCTCTTATAAATCTGAAAATTAGGAATTC	(Bijlsma *et al*. 2000)
Gg 7	FAM	132-142	GTTTTCAATTTTAGCCGTTCTG	GTTTATCTCCCTCTTCCTACCC	(Davis & Strobeck 1998)
Mar-43	VIC	160-178	CTTGTCACCCCAGGAGAGG	CCTAAGCCCAAATCTAAGTGC	(Natali *et al*. 2010)
Mar-53	FAM	242-244	TCTCCAGCATTTACCTTTACCC	GAACAGCCAACCCCATACC	(Natali *et al*. 2010)
Mer 041	FAM	155-161	TGTGTGATCTCTGGGAATTCTC	*GTTTCTGCTCCCCAGATAAAAGC	(Fleming *et al*. 1999)
Mvi1341	AT565	170-180	GTGGGAGACTGAGATAGGTCA	*GGCAACTTGAATGGACTAAGA	(Vincent *et al.* 2003)
Mel 105	FAM	192-196	GATATTCCCCTCCCACCACT	*CTCCAAGGGATCCTGGAACT	(Carpenter *et al*. 2003)
Ma 2	Yak	131-137	ACCCATGAATAATGTCTTAT	*ATCTTGCATCAACTAAAAAT	(Davis & Strobeck 1998)
Mvi 1354	FAM	202-208	CCAACTGGAGCAAGTAAAT	*CATCTTTGGGAAAGTATGTTT	(Vincent *et al.* 2003)
Mvis 075	FAM	151-153	GAAATTTGGGGAATGCACTC	*GGCAGGATAGGATGTGAGCT	(Fleming *et al.* 1999)
Mar 21	PET	185-195	ACATGCATACCTCCCAGACC	TTTGCTTCCTCCATCTCTCC	(Natali *et al.* 2010)

Combining loci in a single PCR (multiplexing) significantly reduces the time and cost of microsatellite analysis. Four previously optimised reactions (O'Reilly, *unpublished*) were selected for the 11 microsatellite marker panel (Table 2.4).

Table 2.4 Reactions used with panel of microsatellite primers to identify pine marten individuals from hair DNA extracts

Reaction	Marker (µM)	Marker (µM)	Marker (µM)
1	Gg7 (0.25)	Mvi1341 (0.25)	Mvi1354 (0.25)
2	Mar 43 (0.25)	Mar 21 (0.25)	Mel105 (0.25)
3	Mel1 (0.25)	Mvis075 (0.25)	Mar 53 (0.25)
4	Ma2 (0.25)	Mer041 (0.25)	

2.3.6.3 Microsatellite analysis

Amplification of DNA fragments was performed in a 2720 thermal cycler (Applied Biosystems cat. no. 4359659). Each 10 µl reaction contained 5 µl of Hot Start PCR-to-Gel Taq PCR Master Mix 2X (Amresco, cat. no IB1409-100), 1 µl of multiplex (1-4) and 4 µl of template DNA. Cycling parameters were as follows; a single cycle at 95° C for 5 minutes, 40 cycles of 95° C for 30 seconds, 57° C for 90 seconds and 72° C for 30 seconds with a final single hold at 60° C for 30 minutes. Samples were rapidly cooled to 4° C. Samples were diluted 1:20 in molecular grade water.

1 µl of each sample was denatured in 15 µl Hi-Di formamide (Applied Biosystems, cat. no. 4311320) with 0.15 µl GS500LIZ™ size standard (Applied Biosystems, cat. no. 4322682) for 5 minutes at 95 °C, followed by rapid cooling to 4 °C.

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.

Microsatellite allele sizing was performed using GeneMapper software v. 3.7 (Applied Biosystems). The 250-bp peak was excluded from the GS500LIZ^{TM} size standard as it has been found to be particularly effected by run temperature changes (Klein *et al*. 2003). Samples were assigned allele calls if the peak heights were above 100 RFU (Relative Fluorescent Units). Each sample was genotyped in triplicate with agreement in two of the three samples required for a positive heterozygous sample and all three samples in agreement required for homozygous samples. Ambiguous samples were repeated with a lower dilution factor. Samples that were found to be overloaded were repeated at a higher dilution factor.

2.4 Results

2.4.1 Hair tube survey in Nietoperek, September 2012 to March 2013

From September 2012 to March 2013 eleven of the possible thirteen visits to the hair tube survey site were reported by the collaborating students from Wrocław University of Environmental and Life Sciences. Two survey sessions were omitted in January 2013 due to heavy snow fall making it impossible for the students to travel. For the first two survey periods 10 hair tubes were checked for the presence of hair and rebaited. With the addition of 16 more hair tubes in October, the students were checking a total of 26 hair tubes for the remaining 9 survey sessions totalling 254 opportunities for a pine marten or stone marten hair sample to be collected. No hair tube was positive for a hair sample during any of these 254 visits. The bait trial showed no positive results. The chicken and sausage showed evidence that an animal had been feeding on them but when the camera trap footage was checked, the animal in question was a domestic cat.

2.4.2 Preliminary scat survey in Corbally Wood, Kilkenny, Ireland

A preliminary scat survey of Corbally Wood was performed to confirm the presence of pine marten in the area. 13 possible pine marten scats were collected and the position of each scat recorded using GPS (Figure 2.7).

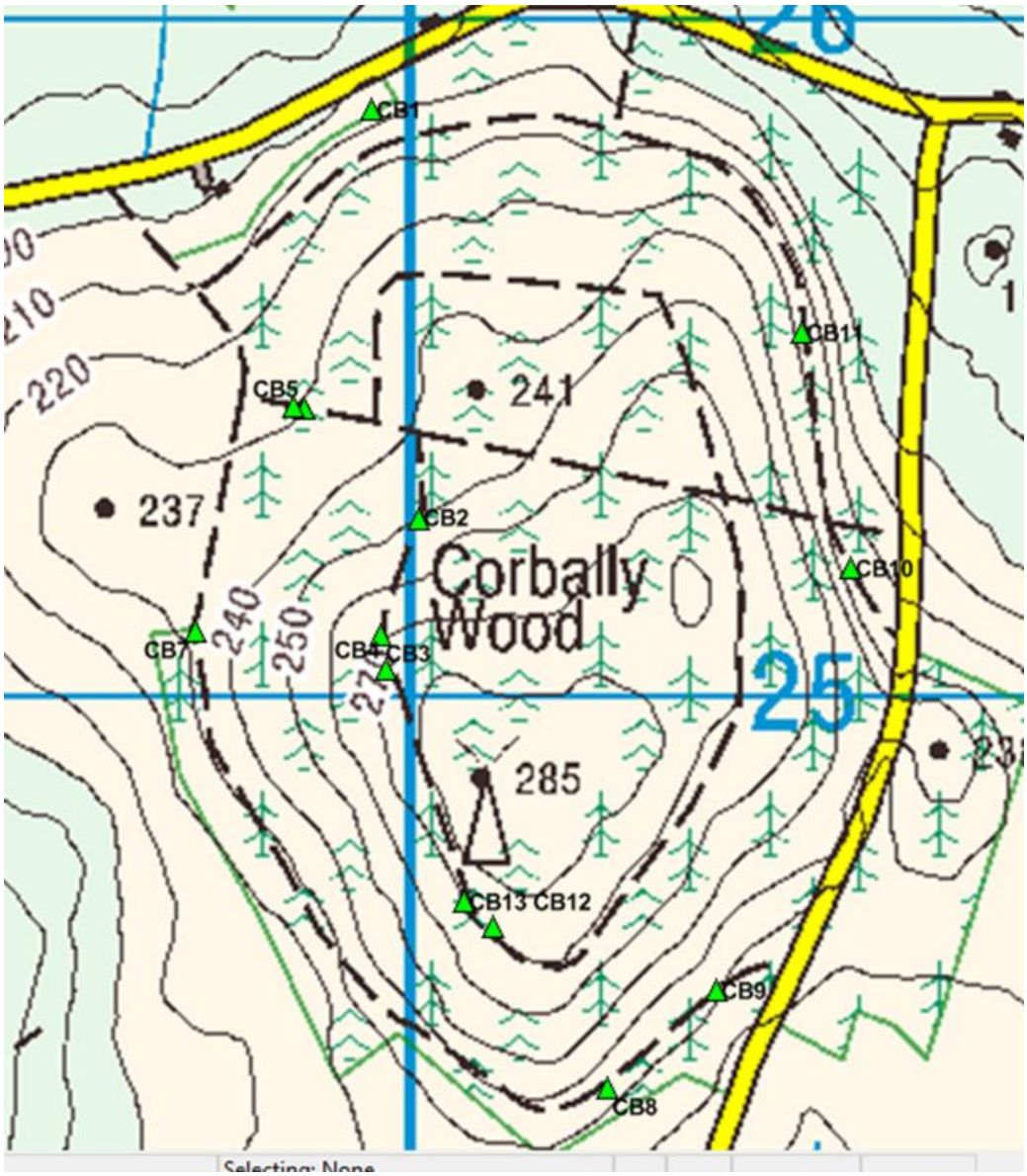


Figure 2.7 Location of scats collected ▲ during preliminary scat survey of Corbally Wood; CB = Corbally

2.4.2.1 Detection of pine marten in scat DNA extracts

Positive samples were defined as DNA extracts in which pine marten DNA was detected with a C_T value <= 35.0 using the TaqMan® MGB probe based qPCR species assay. All 13 scats

collected in Corbally Wood and analysed using a TaqMan® MGB probe based qPCR species assay were positive for pine marten DNA (Table 2.5).

Table 2.5 qPCR assay to detect pine marten DNA in scat DNA extracts

DNA no		PM3 C_T
JP300813	1	30.5
JP300813	2	28.0
JP300813	3	25.2
JP300813	4	27.3
JP300813	5	23.8
JP300813	6	32.7
JP300813	7	25.4
JP300813	8	24.3
JP300813	9	31.3
JP300813	10	33.2
JP300813	11	27.2
JP300813	12	29.3
JP300813	13	31.9

2.4.2.2 Sex determination of pine marten scat DNA extracts.

Positive samples were defined as DNA extracts in which male or female DNA was detected in both replicates with a C_T value <= 40.0 using the TaqMan® MGB probe based qPCR sex determination assay (Figure 2.8).

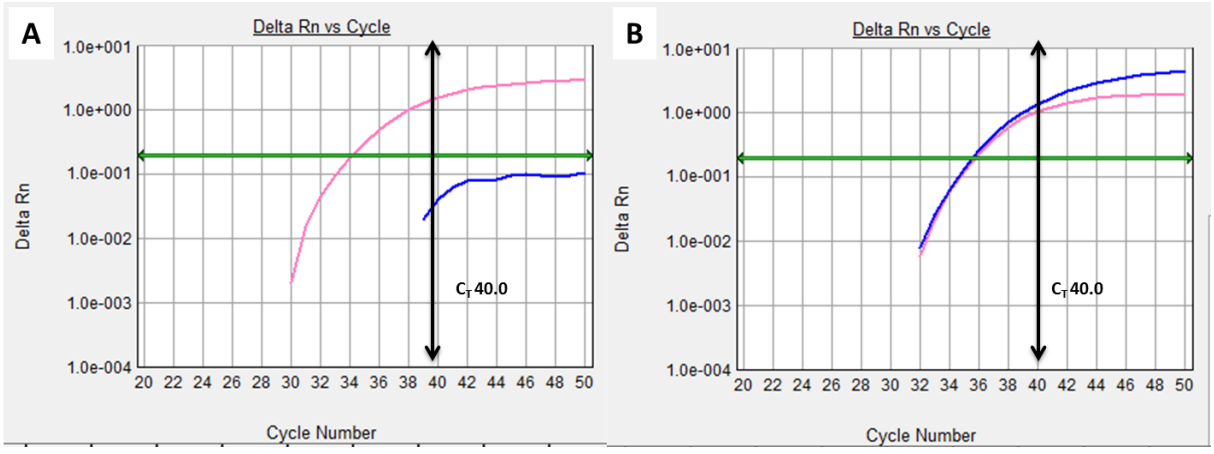


Figure 2.8 Sample qPCR plots from sex determination assay. A; amplification of the MMX probe only indicating a female B; amplification of the MMX and MMY probes indicates a male , arrow indicates cut-off for positive DNA extracts (C_T 40.0).

Eight of the 13 scat DNA extracts were selected for sex determination. All 8 were successfully sex typed consisting of 6 scat DNA extracts form male pine marten and 2 scats DNA extracts from female pine marten confirming the presence of at least two pine marten individuals in the survey area. As the purpose of the preliminary scat survey was to determine if pine marten were present in the survey site no further molecular analysis was performed on the scat DNA extracts.

2.4.3 Hair tube survey in Corbally Wood, Kilkenny, Ireland

A total of 12 hair tubes were set up throughout Corbally Wood (Figure 2.6). The hair tubes were checked for the presence of a hair sample and re-baited if necessary on a weekly basis for a period of 6 weeks. After 2 weeks all 12 of the hair tubes had been visited by an animal on at least one occasion. Fifty three hair samples were collected throughout the survey. The success rates for each hair tube can be seen in Table 2.6. The hair samples varied from one or two hairs to clumps with over 20 hairs present. Twenty two samples had 20 or more hairs present (Table 2.7).

Table 2.6 Summary of Corbally hair tube survey results; 1=hair sample found, 0= no hair sample.

	week no.						
Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**Total/tube**
1	*1*	*1*	*1*	*1*	*1*	*1*	**6**
2	*0*	*1*	*1*	*1*	*0*	*0*	**3**
3	*1*	*0*	*1*	*1*	*1*	*1*	**5**
4	*1*	*0*	*1*	*1*	*1*	*1*	**5**
5	*0*	*0*	*0*	*1*	*0*	*1*	**2**
6	*1*	*0*	*1*	*1*	*1*	*1*	**5**
7	*0*	*1*	*1*	*1*	*1*	*1*	**5**
8	*1*	*1*	*1*	*1*	*1*	*1*	**6**
9	*1*	*0*	*1*	*1*	*1*	*1*	**5**
10	*1*	*0*	*0*	*0*	*1*	*1*	**3**
11	*1*	*0*	*0*	*0*	*1*	*1*	**3**
12	*1*	*1*	*1*	*0*	*1*	*1*	**5**
Total/survey	**9**	**5**	**9**	**9**	**10**	**11**	**53**

Table 2.7 Summary of hair quantity using grading mechanism 1= (<10 hairs), 2=(10-20 hairs) and 3=(more than 20 hairs).

Hair quality	no of samples
1	13
2	18
3	22

2.4.4 Molecular analysis of hair samples from Corbally Wood

DNA was extracted from all hair samples. Hair DNA extracts were analysed for species and sex using TaqMan® MGB probe based qPCR and individual pine marten were identified from hair DNA extracts using microsatellite analysis.

2.4.4.1 Detection of pine marten DNA in hair DNA extracts

All 53 hair DNA extracts tested positive for pine marten DNA using the TaqMan® MGB probe based qPCR species assay. Screening of the results from this assay showed all hair DNA extracts gave a C_T value less than 30.0 therefore all hair DNA extracts were selected for sex typing.

2.4.4.2 Sex determination of pine marten hair DNA extracts

Table 2.8 contains a summary of the TaqMan® MGB probe based species and sex specific qPCR assay results from all hair DNA extracts analysed. 52 (98%) of the 53 hair DNA extracts were successfully sex typed. Of the 52 hair DNA extracts successfully sex typed 34 (65%) were from male pine marten 18 (35%) were from female pine marten.

Table 2.8 Species and sex typing assay results; PM3 C_T= results of species assay, MMX C_T Avg, & MMY C_T Avg are both averages of 2 replicates, F=female, M=male ND = not determined, HQ=hair quantity as in Table 2.7.

DNA no	PM3 C_T	MMX C_T Avg	MMY C_T Avg	Sex	HQ
JP181013-10	16.9	27.7		F	3
JP301013-2	18.4	33.6	33.9	M	3
JP250913-19	18.9	29.6		F	3
JP250913-14	19.2	29.9		F	3
JP141013-2	19.3	33.5		F	3
JP250913-22	19.4	31.1		F	3
JP300913-7	20.1	30.1	31.7	M	3
JP250913-7	20.6	33.7	33.4	M	3
JP250913-23	20.6	31.5	32.1	M	3
JP141013-4	20.7	33.5	33.0	M	3
JP300913-8	20.9	31.8	32.6	M	3
JP141013-9	21.5	30.9	31.4	M	3
JP141013-10	21.6	32.2	32.7	M	3
JP141013-5	21.6	32.2	33.1	M	3
JP250913-24	21.6	33.2	33.9	M	3
JP300913-5	21.7	32.5		F	3
JP181013-7	21.7	31.5	32.0	M	3
JP250913-11	21.7	32.9	33.4	M	3
JP250913-17	22.0	28.5	30.1	M	3
JP250913-2	22.0	33.5	33.9	M	3
JP181013-6	22.1	27.8	29	M	2
JP250913-4	22.4	34.0		F	3
JP250913-6	22.5	34.2	34.1	M	2
JP250913-20	22.6	34.7	36.1	M	2
JP141013-7	22.8	34.6	34.4	M	2
JP141013-1	22.9	32.6	33.2	M	3
JP141013-3	22.9	33.9	33.9	M	2
JP300913-3	22.9	34.5		F	2
JP181013-2	23.4	34.7	34.5	M	1
JP300913-6	23.4	36.8	38.9	M	1
JP250913-13	23.5	34.1	34.3	M	2
JP250913-3	23.5	34.7		F	2
JP300913-4	23.5	34.7	35.3	M	1
JP141013-6	23.6	34.3	34.7	M	2
JP250913-15	23.7	34.4	34.5	M	2
JP250913-21	23.7	34.6	34.9	M	2
JP141013-8	23.9	34.8	34.9	M	2
JP250913-10	23.9	34.2		F	2
JP250913-16	23.9	34.2		F	1
JP300913-1	24.3	34.9		F	1
JP181013-3	24.5	34.9		F	1
JP181013-8	24.5	35.2	35.9	M	1
JP181013-1	24.6	34.2		F	2
JP181013-5	24.6	35.6		F	1
JP250913-8	24.6	37.2	37.9	M	1
JP250913-1	24.6	42.7		ND	1
JP250913-12	24.7	34.4		F	2
JP250913-9	24.7	34.1	34.8	M	2
JP250913-22	24.8	34.2	34.3	M	2
JP250913-18	24.9	34.8	35.0	M	1
JP250913-5	25.1	34.9		F	1
JP181013-4	25.8	34.7		F	1
JP181013-9	26.0	34.6	34.8	M	2

2.4.4.3 Genotyping of pine marten hair DNA extracts

2.4.4.3.1 Microsatellite analysis of pine marten hair DNA extracts with panel of 11 microsatellite loci

Hair DNA extracts with MMX C_T values less than or equal to 34.0 were selected for genotyping (n=24) (Table 2.8). Of the 24 hair DNA extracts selected through screening for genotyping, 22 hair extracts (92%) were successfully genotyped across all 11 microsatellite loci. The 22 hair DNA extracts genotyped represented 5 distinct genotypes (Table 2.9). Statistical analysis was performed on the genotyping results using GenAlex (Peakall & Smouse, 2006) to assess the power of this microsatellite panel to identify individual animals in this pine marten population.

Table 2.9 Multi-locus genotypes of pine marten in Corbally Wood identified from hair DNA extracts; sizes are alleles in base pairs. n= no of alleles found in this population for each marker

DNA no	Marker	Gg7		Mvi1341		Ma2		Mer041		Mel105		Mar-43		Mar-21		Mvi1354		Mel1		Mvis075		Mar-53	
	ID/n	3		3		3		3		3		3		2		2		2		2		2	
JP300913-5	CBF1	132	132	170	170	135	137	155	155	194	196	160	162	185	195	208	208	112	112	151	153	242	244
JP250913-14	CBF1	132	132	170	170	135	137	155	155	194	196	160	162	185	195	208	208	112	112	151	153	242	244
JP181013-10	CBF2	132	138	170	180	137	137	155	161	194	194	160	162	185	195	202	208	112	112	151	153	242	242
JP141013-2	CBF2	132	138	170	180	137	137	155	161	194	194	160	162	185	195	202	208	112	112	151	153	242	242
JP250913-22	CBF2	132	138	170	180	137	137	155	161	194	194	160	162	185	195	202	208	112	112	151	153	242	242
JP250913-19	CBF2	132	138	170	180	137	137	155	161	194	194	160	162	185	195	202	208	112	112	151	153	242	242
JP301013-2	CBM1	132	138	170	170	131	135	155	161	196	196	160	178	195	195	208	208	108	112	151	151	242	242
JP141013-10	CBM1	132	138	170	170	131	135	155	161	196	196	160	178	195	195	208	208	108	112	151	151	242	242
JP250913-17	CBM1	132	138	170	170	131	135	155	161	196	196	160	178	195	195	208	208	108	112	151	151	242	242
JP181013-6	CBM1	132	138	170	170	131	135	155	161	196	196	160	178	195	195	208	208	108	112	151	151	242	242
JP141013-1	CBM1	132	138	170	170	131	135	155	161	196	196	160	178	195	195	208	208	108	112	151	151	242	242
JP141013-5	CBM2	132	142	170	176	131	135	157	161	194	194	178	178	185	195	208	208	112	112	151	153	242	244
JP300913-7	CBM2	132	142	170	176	131	135	157	161	194	194	178	178	185	195	208	208	112	112	151	153	242	244
JP181013-7	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP141013-9	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP141013-4	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP250913-23	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP250913-24	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP250913-2	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP250913-7	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP250913-11	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244
JP300913-8	CBM3	132	142	170	176	137	137	155	157	192	192	160	178	195	195	208	208	112	112	151	153	242	244

2.4.4.3.2 Descriptive statistics

Once individual genotypes had been identified the probability of identity (PI) statistics were calculated using the GenAlEx 6.5 to assess the power of the panel of microsatellite loci to identify individual animals (PI), including related animals (PIsibs), from DNA extracted from hair samples collected in Corbally Wood. PI (Figure 2.9) was obtained for increasing numbers of loci. It is recommended that a PIsibs value should be below 0.01 to ensure discrimination between individual animals even if some of the animals are related (Waits *et al.* 2001). Five distinct genotypes were detected in Corbally Wood. And each distinct genotype can be attributed to an individual animal ($P < 0.001$) (Figure 2.9), with this panel of 11 loci yielding a cumulative PID of 6.0E-06 and a cumulative PIsibs of 6.5E-02 (GenAlEx 6.5).

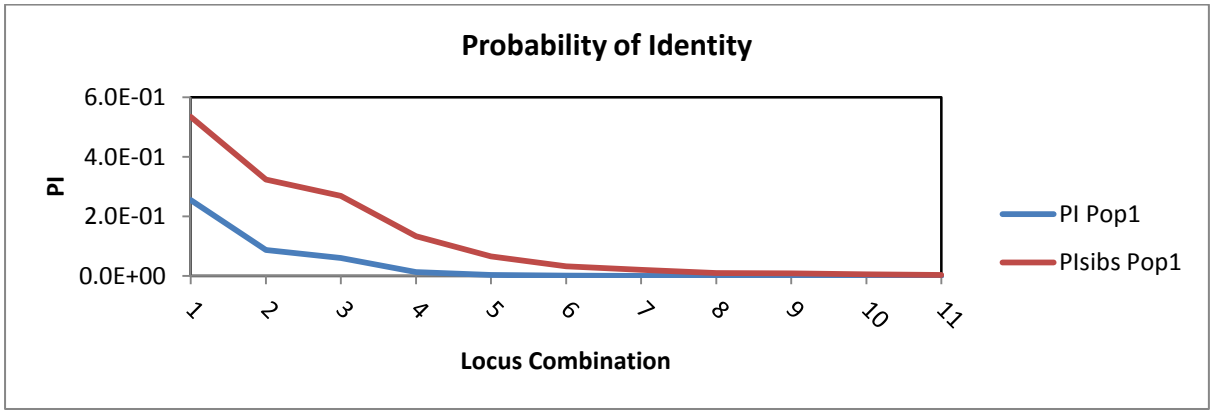


Figure 2.9 Probability of Identity (PI) for each locus and for increasing combinations of the 11 loci used to identify pine marten individuals.

There are two copies of each locus in the mammalian genome. If both copies are the same the individual animal is referred to as being homozygous at that locus. If the copies differ then the individual is heterozygous. Observed heterozygosity (H_O) refers to the observed proportion of heterozygotes at each marker in a population. Expected heterozygosity (H_E) is the expected proportion of heterozygotes in a randomly mating population and is considered a good measure of genetic variation (Allendorf & Luikart, 2007). Testing for significant differences between H_O and H_E in a population is important to identify if factors like mutation, migration, or fluctuations in population size may be affecting the distribution of

allelic variation in a population. If mating in a population is random and no factors like those above are acting on the population then allele and genotype frequencies will remian constant which is known as Hardy-Weinberg Equilibrium (HWE). Heterozygosity was calculated for each locus. The loci were entered into GenAlex in order of decreasing number of alleles present (Table 2.10). The Corbally Wood population did not deviate significantly from HWE. H_O is comparable to H_E at all markers except Mel105 which had a much lower observed heterozygosity than expected. It must be noted however, that this is a very small sample size (n=5).

Table 2.10 Descriptive statistics of panel of microsatellite loci in order of decreasing number of alleles. N=number of individuals, Na= number of alleles, H_O= observed heterozygosity, H_E= expected heterozygosity. Chi-square test (P = 0.957)

Locus	N	Na	H_O	H_E
Mar-43	5	3	0.8	0.64
Ma2	5	3	0.6	0.62
Mer041	5	3	0.8	0.62
Mel105	5	3	0.2	0.62
Gg7	5	3	0.8	0.56
Mvi1341	5	3	0.6	0.46
Mvis075	5	2	0.8	0.48
Mar-21	5	2	0.6	0.42
Mar-53	5	2	0.6	0.42
Mvi1354	5	2	0.2	0.18
Mel1	5	2	0.2	0.18

2.4.5 Distribution of DNA extracts positive for pine marten DNA in Corbally Wood, Kilkenny, Ireland.

2.4.5.1 Distribution of scat and hair DNA extracts positive for pine marten DNA

All scats collected in the preliminary scat survey of Corbally Wood were positive for pine marten DNA. All hair samples collected throughout the survey period were also positive for pine marten DNA. Therefore the distribution of pine marten scats and hair samples during

this survey is identical to the distribution of all scats and hair samples collected during the survey as presented in Figure 2.7 and Figure 2.6 respectively.

2.4.5.2 Distribution of individual pine marten hair samples collected throughout the survey period in Corbally Wood

The distribution of pine marten individuals from microsatellite analysis of hair DNA extracts can be seen in Figure 2.10. Three male and two female pine marten were identified. Of the 12 hair tubes, 3 were visited by more than one individual (Table 2.11). No hair tube was visited by two individuals of the same sex.

Table 2.11 Summary of hair tube visits by individual pine marten to each hair tube during the survey period; number in brackets denotes the total number of each animal to each tube

Tube	Animal (no of visits)
T01	CBM1(2) CBF2(1)
T02	CBM1(2)
T03	CBF1(1) CBM1(1)
T04	CBM3(2)
T05	CBM3(1)
T06	CBM3(1)
T07	CBM3(1)
T08	CBF1(1) CBM3(2)
T09	CBM3(2)
T10	CBM2(1)
T11	CBM2(1)
T12	CBF2(3)

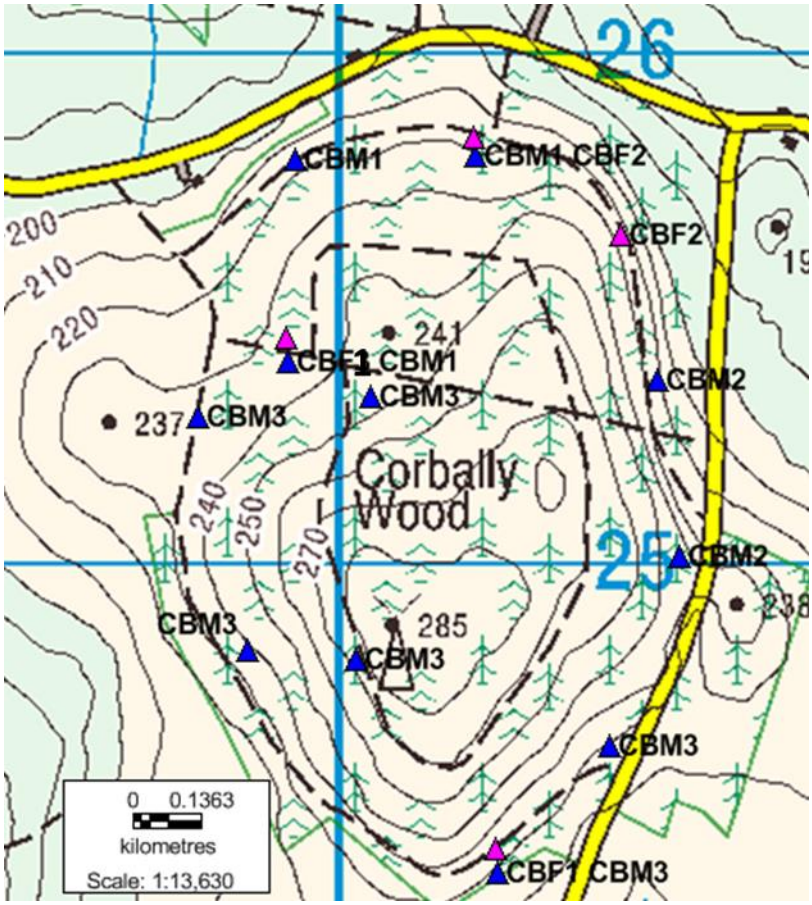


Figure 2.10 Location of hair samples captured from individual pine marten in Corbally Wood; males (CBM) ▲, females (CBF) ▲.

2.5 Discussion

2.5.1 Hair tube survey in Nietoperek , September 2012 to March 2013

The hair tubes erected in Nietoperek, and checked regularly for six months, failed to yield a positive hair sample. The bait trial also gave no positive results with none of the baits attracting marten. The methods employed with both hair tubes and selected bait have been used successfully in the past for obtaining hair samples from pine marten in other studies (Roche, 2008; Mullins, *et al.* 2010; Sheehy *et al.* 2014). Only one study has reported the failure of hair tubes to successfully detect pine marten (Bartolommei *et al*. 2012) as part of a comparative study also using scat collection and remote camera trapping which were both successful in that study.

Tourist pressure has been shown to elicit a physiological response in marten whose scats have been found to contain elevated levels of stress hormone in areas of high tourist activity (Barja *et al.* 2007). This increase in stress may have a negative effect on marten investigating any human constructs including hair tubes. The museum at Pniewo hosts touring parties that walk along the paths through the Southern Forest portion of the survey site close to where some of the tubes were situated but the volumes of tourists are low (*Pers obs*.) and it is unlikely that this was a factor as the marten ignored the hair tubes close to the tourists trails as well as those tubes which were removed from such trails.

Neither pine marten nor stone marten are protected species in Poland and both are hunted, although hunting bags show that lately, hunting of marten is only sporadic with only two individual marten recorded in the hunting bag of 2008-2009 in over 100 km^2 around Rogow in central Poland (Gryz *et al*. 2011). However while hunting of marten may not be extensive at present, historical hunting of marten may have led to an increased learned vigilance and possible fear of humans in marten on mainland Europe in comparison to the pine marten in Ireland which are protected (Wildlife Act 1976). Given the stone marten's status as an "Urban adapter" however, increased vigilance in the presence of humans may not be the entire reason for these animals not entering the baited hair tubes.

Primary and secondary food resources for opportunistic feeders are virtually unlimited in an agricultural landscape in mainland Europe (Lanski & Heltai, 2011). This increased availablity of prey compared with the small mammal abundance in Ireland where previous studies have been conducted may be a reason for marten not entering the tubes; they simply do not need to take the risk. However, hair tubes have been previously found to be a successful sampling method for marten detection in areas of high small mammal abundance (Foran *et al*. 1997).

Some hair tubes were erected along the forest edge in order to maximise the chances of getting hair samples from both pine marten and stone marten. It has been shown that small mammal abundance may be highest at habitat edges (Salek *et al.* 2010). This may contribute to marten not entering hair tubes in these areas but as the hair tubes that were not situated at the habitat edges were also not visited then the level of small mammal abundance was probably not a factor.

One possible reason for the failure of the hair tubes may be an increased vigilance from both pine marten and stone marten in areas where large predators are found. Pine marten do not

display increased vigilance in areas where foxes are present (Wikenros *et al.* 2014) but there may be other larger predators present in the area around Nietoperek which may cause marten to exhibit increased vigilance making them wary of entering a tube to get at the chicken. Wolves, lynx or raccoon dogs may be repsonsible for this increased vigilance, animals not present in areas where this hair tube method has previously been successfully employed like Ireland and Scotland. Any future efforts to obtain hair samples in Nietoperek should take all these factors into consideration with the use of a longer latency of initial detection period possibly advised to compensate for increased vigilance as a result of hunting or predation pressures. In studies with longer latency periods peanut butter may be used instead of chicken as it will not decay as rapidly as chicken and will remain a viable feeding opportunity for the marten for a longer period.

Hair tubes were not trialled in the tunnels as they did not work above ground. However, in future studies that may successfully use hair tubes in Nietoperek, the tubes erected in the tunnels will need to be modified as the glue patch may pose a hazard to the resident bats as they may become stuck to the tube. Tubes that are closed at the bottom and have a lid that can be open by the marten may be a viable alternative.

2.5.2 Hair tube survey in naïve site in Corbally woods, Ireland, autumn 2013

The original research methodology for this project relied on the use of baited hair tubes to non-invasively collect hair samples from pine marten and stone marten in the above ground portion of the survey area in Nietoperek. The capture of hair samples from both marten species and from individuals from both species at a predefined location in space and time would have provided increased resolution into the density and distribution of both species above ground and also in the tunnels. For monitoring populations using molecular analysis of non-invasively collected samples, hair samples are preferred over scat samples as they provide a more reliable and cost effective DNA source (Mullins *et al.* 2010).

As the hair tube survey in Nietoperek failed to yield any positive hair samples a similar hair tube study was performed in Corbally Wood in Ireland using the same methodology to investigate its effectiveness in an area where marten were known to be present. A scat survey was initially performed to confirm this. As the scats collected were relatively evenly distributed (Figure 2.7) their locations were used as a guide when selecting hair tube locations with approximately 400 m between tubes (Figure 2.6).

After two weeks, all 12 hair tubes had yielded at least one hair sample confirming the assumed latency period of 1-2 weeks was sufficient (Table 2.5). This is consistent with latency of detection periods found in other pine marten studies which also used hair tubes to obtain samples (Lynch *et al*. 2006; Mullins *et al.* 2010). Of the 53 hair samples retrieved 40 contained 10 or more hairs (Table 2.6) indicating the method of obtaining the hair was effective in this instance.

2.5.3 Molecular analysis of DNA extracts from Corbally Wood

2.5.3.1 Detection of pine marten in scat DNA extracts

All scats collected were from pine marten and were relatively evenly distributed throughout the survey site (Figure 2.7). The use of the TaqMan® MGB probe based qPCR pine marten species assay proved a successful tool in identifying all DNA extracts from scats as being from pine marten.

2.5.3.2 Sex determination of pine marten scat DNA extracts

Scats from male and female pine marten were identified using a TaqMan® MGB probe based qPCR sex typing assay. Male and female scat DNA extracts confirm the presence of at least two pine marten in Corbally Wood during the survey period. The scat survey was performed to confirm the presence of pine marten in the survey site. As this had been achieved from the use of qPCR assays, no further molecular analysis of pine marten scat DNA extracts was required. The results also confirm the effectiveness of using the TaqMan® MGB probe based qPCR sex typing assay with DNA extracted from scats.

2.5.3.3 Detection of pine marten DNA in hair DNA extracts

All 53 hair DNA extracts were positive for pine marten DNA with an average C_T value during the TaqMan® MGB probe based qPCR species assay of 22.6 (Table 2.8). As discussed earlier DNA from hair samples will be less degraded than scat DNA. The average C_T value obtained for the same assay using scat DNA extracts was considerably higher at 28.5 (Table 2.7). qPCR is quantitative even without the use of a standard curve and a difference in C_T values of 3.33 equates to a 10-fold difference in starting template (O'Connor & Runquist,

2008). The results obtained here are in agreement with previous findings that hair samples are a better source of DNA than scats for non-invasive population studies using molecular techniques (Mullins *et al*. 2010).

Table 2.8 also includes the average C_T values obtained during the TaqMan® MGB probe based qPCR sex typing assay. The average MMX C_T value obtained using this assay was 33.35. This equated approximately to a 1000-fold difference in starting template DNA between the species assay and the sex typing assay and is indicative of the difference between the starting template concentrations of mtDNA when compared to nuclear DNA.

Of the 53 hair DNA extracts 52 (98%) were successfully identified to sex using the TaqMan® MGB probe based qPCR sex typing assay suggesting the use of this assay as an effective method for use with non-invasively collected hair samples.

2.5.3.4 Microsatellite analysis of pine marten hair DNA extracts

Through the screening of MMX C_T values 24 hair DNA extracts were selected for microsatellite analysis. Of these 24 hair DNA extracts, 22 were successfully amplified across all loci giving a genotyping success rate of 92% of samples selected for genotyping or 41.5% of all hair samples collected during the survey. This success rate is comparable with other studies that used microsatellite analysis of pine marten hair DNA extracts (Mullins *et al.* 2010).

The 22 successfully genotyped hair DNA extracts represent 5 individual pine marten comprising 3 males and 2 females (Table 2.10) with a PID of 6.0E-06 and PIsibs of 6.5E-02 using this panel of 11 microsatellite loci. These results suggest that hair samples are an effective source of DNA for population studies using microsatellite analysis.

In some other studies which identified individuals by genotyping from hair DNA extracts the number of hairs required for successful identification of individual animals has varied between a single guard hair (Frantz *et al.* 2004) to between 10 and 30 hairs (Mullins *et al.* 2010). In this study the number of hairs collected on each glue patch (Table 2.6) had a strong bearing on the quality of DNA in each hair DNA extract. The only hair sample that did not yield a sex type, for example, had less than 10 hairs present while 21 of the 22 samples that resulted in complete genotypes had more than 20 hairs present on the glue patch with the remaining 1 sample having between 10 and 20 hairs present (Table 2.8).

2.5.4 Distribution of individual pine marten hair samples collected throughout the survey period in Corbally Wood

The distribution of pine marten individual captures and recaptures in each hair tube along with the number of visits to each tube can be seen in Figure 2.10 and Table 2.11 respectively. The total number of individuals detected over the 6 week survey period (n=5) may initially appear high for such a small area but given time of year, there may have been kits present. Given the relatively short survey period it is also impossible to rule out the capture of transient individuals. CBF1, a female pine marten was only ever detected once throughout the 6 weeks. CBM3, a male pine marten was detected throughout the survey period a total of 9 times at 6 different tubes suggesting this individual's territory may have made up a large portion of the survey site. A longitudinal hair tube survey would give more insight into these issues but this was not necessary as it was not part of the goal of the study in Corbally Wood. The hair tube survey in Corbally Wood was sufficient to demonstrate intra-sexual territorial exclusion over the 6 week survey period. No two animals of the same sex used the same hair-tube (Figure 2.10). Male and female pine marten visited tubes 1, 3 and 8. This is consistent with the literature that states pine marten will only share territories with individuals of the opposite sex (Balharry, 1993b).

The methods employed in this study; the use of hair tubes, hair tube positioning, bait, latency period used and molecular techniques all proved suitable for use in estimating the population density and size in this small wooded area. These findings suggest the hair tube survey in Nietoperek was conducted in an appropriate manner. At the time of writing, there are two parallel hair tube surveys taking place as part of a collaboration between researchers in the Molecular Ecology Research Group in W.I.T and the Mammal Research Institute, Białowieża, Poland. Both studies are trialling hair tube positioning, various baits and re-check timing. The results of these surveys at this time are pending.

3 PINE MARTEN AND STONE MARTEN DISTRIBUTION IN AND AROUND THE NIETOPEREK BAT HIBERNATION SITE

3.1 Introduction

This chapter investigates the distribution of pine marten and stone marten throughout the survey site, above and below ground, in Nietoperek. Scat surveys were used to gather samples for analysis. Molecular techniques were used for species identification, sex determination and individual animal identification. Above and below ground distribution is discussed and compared for species, sex and for individuals. Below ground distribution is discussed with focus on separate sections, scat abundance, and variations in both scat abundance and density.

3.2 Methods

3.2.1 S.T.A.R® Buffer trials

Assays were performed to test the stability of S.T.A.R® buffer over a longer period of time than recommended by manufacturer's guidelines and the effectiveness of transporting samples transferred directly into S.T.A.R® buffer on site in Nietoperek.

3.2.1.1 S.T.A.R® Buffer stability trial

As part of the scat DNA extraction process scats are initially placed in S.T.A.R® buffer, as described in section 2.3.1. This is usually performed in the laboratory. However, as surveys in Nietoperek were to take place over several days, scat DNA may have continued to degrade while awaiting extraction. It was proposed therefore to transfer a portion of each scat into S.T.A.R® buffer on-site in Nietoperek to minimise any further DNA degradation. S.T.A.R® buffer kills any microbes in the scat samples making them safer to work with as well as minimising microbial production of DNAses which may break down the target DNA in the scat sample. The transport of samples was also a consideration. Transfer of scats into S.T.A.R® buffer on-site would allow samples to be packed compactly into an appropriately labelled cardboard box (Sarstedt cat. no 95.064.982).These boxes can hold 81 tubes. This approach makes the samples more portable which was desirable given the high volume of samples expected while covering a large survey area.

It was first necessary to see if the S.T.A.R® buffer would maintain its effectiveness while the samples were in-transit. Manufacturer's guidelines state that the sample should remain stable in S.T.A.R® buffer for up to 5 days. It was estimated from sample gathering to DNA extraction could take up to 2 weeks so a trial was undertaken to determine the stability of samples in this medium over at least a 2 week period using 5 pine marten scats that had been collected during other studies in the Molecular Ecology Research Laboratory in W.I.T. A portion of each scat was added to S.T.A.R® buffer and DNA extracted on days 0, 4, 8 and 23. Each DNA sample was tested in triplicate for pine marten DNA using the TaqMan® MGB probe based qPCR assay described in section 2.3.4.

3.2.1.2 Comparison of same-day transfer and after-survey transfer of scats into S.T.A.R® buffer

During the first scat survey in September 2012, some of the scats (n=36) were sampled into S.T.A.R® buffer on-site in Nietoperek with the remaining portion of each scat brought back to W.I.T in labelled zip-lock bags. To compare the two methods of transport for DNA degradation, DNA was extracted from both sets of samples and tested for pine marten and stone marten DNA using the TaqMan® MGB probe based qPCR assay described in section 3.3.4.

3.2.2 Scat survey area

3.2.2.1 Tunnel system below ground survey area

Figure 3.1 shows a map of the portion of the bunker system still connected by tunnels. This constitutes the main area for underground survey.

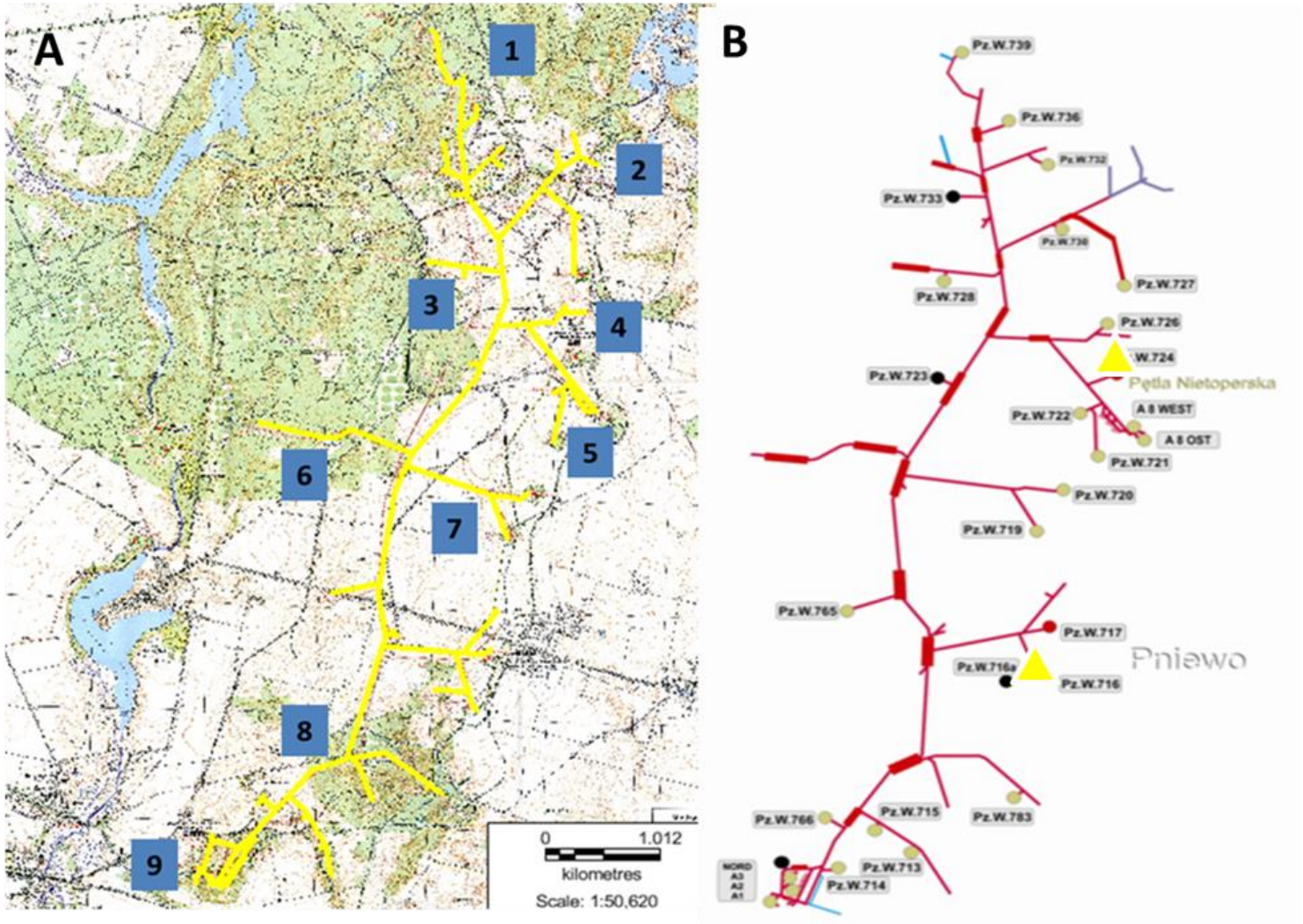


Figure 3.1 Map of system with section numbers (A) and bunker locations (B), ▲ Access points.

Originally part of a system that stretched for over 80 km there remains approximately 30 km of tunnels connecting the above ground fortifications. The majority of the system lies more than 50 m below the surface. For the purpose of counting hibernating bats during the January censuses the system is divided into 9 sections which are further divided into sub-sections. The section and sub-sections lengths including the locations of any bunkers in each section can be viewed in Appendix I. Some sub-sections are prone to flooding. Bunkers are labelled "PzW" which refers to the term "Panzerwerk" which the German army gave to these fortifications.

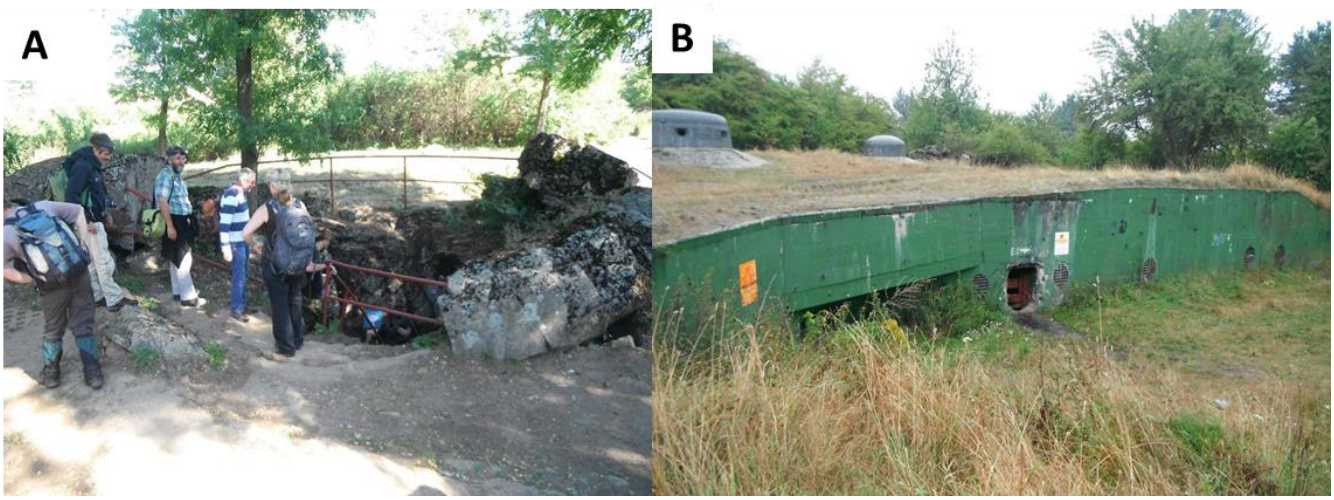


Figure 3.2 Access points to the tunnel system PzW 716 (A) PzW 724 (B).

There are two human access points to the system; one in the south of the survey area (PzW 716) and one in the centre of the survey area (PzW 724) (Figure 3.2A & B). The location of these two access points can be seen in Figure 3.1.

3.2.2.2 Above ground survey area

The above ground portion of the survey site is divided into three areas which were surveyed in an attempt to cover as much as the above ground area as possible with the time and volunteer surveyors available. These are; 1, the Northern Forest, 2, the Middle Section, and 3, the Southern Forest (Figure 3.3).

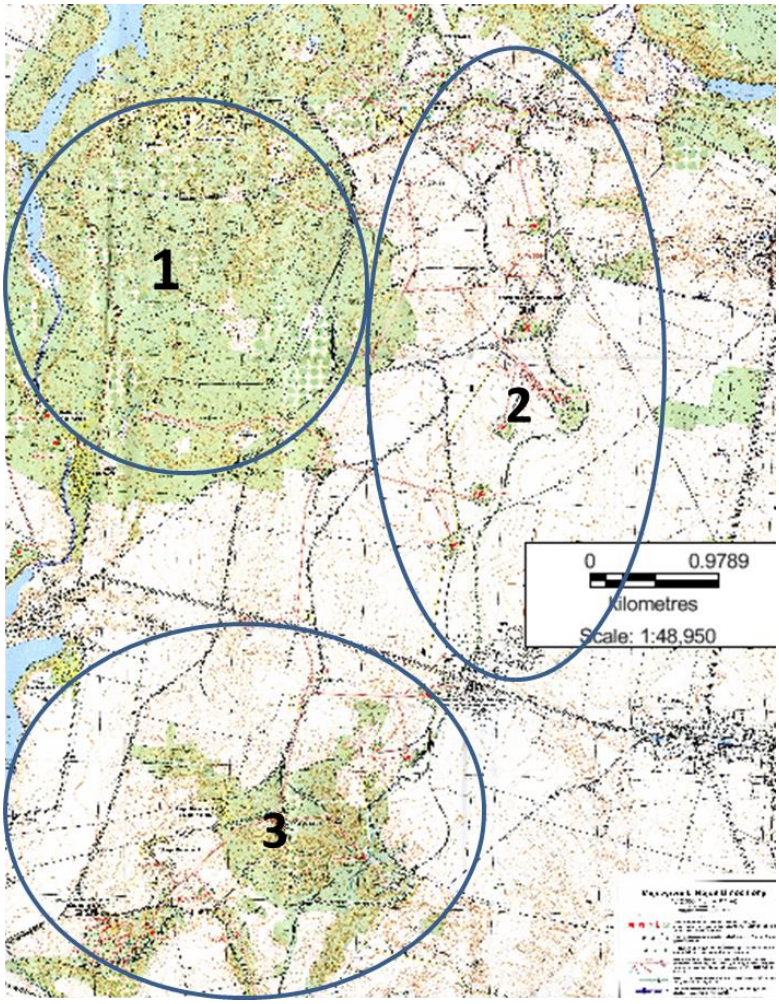


Figure 3.3 Breakdown of above ground survey area; 1= Northern Forest, 2= Middle section, 3= Southern Forest.

The Northern Forest portion of the survey area covers 7.78 km^2. It is a mixed forest and appears extensively managed. There are areas with plenty of undergrowth coupled with clear fells and open areas under the canopy where there is little understory. The Northern Forest also contains the entrance to section 6 of the system which leads to a road up to the surface from the tunnels.

The Middle Section of the survey area is mostly arable land with open fields. While the bunkers themselves have been reclaimed by nature (Figure 3.4 A & B) and are mostly surrounded by forest, gun turrets can be seen dotted around the open farmland (Figure 3.4 C).

Figure 3.4 (A) 716A (B) PzW 730 (C) Gun Turret above section 7.

The farmland consists of fields of maize and sugar beet with meadowlands in other areas. Throughout the middle section there are woodland islands which in most cases are connected to the larger forested areas by rows of small trees and brush potentially providing access corridors for wildlife. The Middle Section also incorporates the area north of the museum at Pniewo. The Middle Section of the survey area covers 7.1 km^2.

The Southern Forest portion of the survey area is as described in Chapter 2. The Southern Forest experiences far more footfall than the Northern Forest from tourist activities and dog walkers. Through the Southern Forest and continuing north along a portion of the Middle Section is a row of concrete anti-tank defences. These rows consist of 4-5 waist high concrete pyramid shapes resembling teeth and are known locally as “dragon’s teeth”. These may resemble rocks to pine marten and stone marten and as such, scats may be found lying on top of them. This linear feature is surveyed wherever it is present in the survey area. Figure 3.5 shows a portion of the dragon’s teeth in the Southern Forest portion of the survey area.

Figure 3.5 Section of "Dragon's teeth" near Pniewo.

3.2.3 Scat surveys

It is not possible to differentiate between pine marten and stone marten scats without the use of molecular techniques so the morphology of scats from both species is as described in section 2.2.3. Any scat that was similar in morphology to a marten scat was collected. If there was any doubt over what species of animal had left a scat it was collected as the species was verified by DNA analysis. Scats identified as possibly from pine marten or stone marten were individually placed in pre-labelled zip-lock bags and given an individual identifier (NP number). Scats were collected as described in section 2.2.3.

For above ground scats the location was recorded using GPS. During the above ground surveys, a minimum of three volunteers surveyed the route; one looking at each side of the track and a third volunteer recording the location of collected scats as well as the date the scat was collected and a description of the scats possible age (old versus fresh looking), morphology and immediate location. For example; "on a rock" "at the side of the track" or any other potentially relevant information was recorded in the comments section of a log sheet (Figure 3.6).

Logging sheet for Nietoperek May survey 2014(A)

Survey area:_____ Name: _____Date_____

NP no	L1	L2	Notes / Observations Scats

Figure 3.6 Log sheet used by volunteers during May 2014 above ground survey.

Surveying the tunnels involved using a head torch and a hand held torch to look for scats with emphasis on the sides of the tunnels where scats had been previously found. Care had to be taken as lying water sometimes covered holes and uneven ground so a single file approach was adopted where possible with the first volunteer calling out the location of any potential hazards. Stairwells were surveyed from the bottom up checking in any corridors and rooms at the top of each stairwell if possible. In some areas this is not possible due to large amounts of suspended debris. When surveying in the tunnels, two approaches were adopted. The survey in January 2013 was performed as part of a collaboration between this project and the annual bat reserve survey. Numbers must be kept to a minimum for this survey to avoid any unnecessary disruption to the hibernating bats. This meant that one individual was added to each of the nine bat survey teams that would survey exclusively for scats while the other members counted bats. The January 2013 survey served to clear the entire tunnel system of scats allowing a maximum age of any scat found in subsequent surveys to be determined. During May 2013, September 2013, January 2014 and May 2014, the entire system was again surveyed. During these surveys groups of at least 3 volunteers surveyed a section of the tunnel system each. Scats were collected as in the above ground surveys. For scats found in

the tunnels, the label number from the zip-lock bag was placed on a map at the point where the scat was found. This point was then given a GPS coordinate using MapInfo® GIS.

3.2.4 Survey teams

Each survey team consisted of volunteers with previous expertise in scat collection and where possible each team had at least one member that was familiar with the area above ground and had previously been inside the tunnels. Also where possible, at least one person on each team was a native Polish speaker.

3.2.5 Processing of scats

The same day a scat survey was completed, a portion (~0.2 g) of each scat was taken using a clean disposable wooden spatula (coffee stirrer) and placed in a labelled micro-centrifuge tube containing 1 ml S.T.A.R® buffer. Tubes were inverted several times to mix. Each tube was labelled with a unique identifier and placed upright in a labelled cardboard storage box and brought back to Ireland on completion of each survey. This was performed on the same evening the scats were collected to avoid keeping the scats in the anaerobic environment of the plastic bags for too long and to limit DNA degradation and potential action of microbial DNAses. On return to Ireland, the samples were either extracted immediately or placed in a freezer at -20° C until extracted.

3.2.6 Individual surveys

A total of 7 scat surveys were performed over the project period. The initial survey in September 2012 covered areas 1 and 3 of the above ground survey site (Figure 3.3) and sections 8 and 9 of the tunnels system (Figure 3.1 A). The October 2012 survey was incorporated as part of a trip to the survey site to set up hair tubes to non-invasively collect hair samples from pine marten and stone marten. This survey only covered area 3 above ground and there was no survey of the tunnels. All subsequent surveys covered the 3 areas above ground as well as the entire tunnels system below ground. During the January surveys, access to the tunnels is limited to one day to limit any disturbance to hibernating bats. Table 3.1 contains a summary of all survey conducted throughout this project. Details of each individual survey including maps showing areas surveyed can be seen in Appendix II of this document.

Table 3.1 Summary of scat surveys above and below ground in Nietoperek.

Survey period	Above ground survey days	Total survey distance (km)	Tunnels survey days	Sections surveyed (1-9)
Sep-12	4	29.2	1	8,9
Oct-12	2	8.7	0	0
Jan-13	2	3.2	1	1-9
May-13	3	24.55	3	1-9
Sep-13	3	38.39	2	1-9
Jan-14	2	16.5	1	1-9
May-14	7	63.18	2	1-9

3.2.7 Molecular analysis of non-invasively collected samples

3.2.7.1 Scat D.N.A extraction

Scat samples in S.T.A.R® buffer were vortexed to mix the scat and buffer. 150 µl of supernatant was transferred to a clean micro-centrifuge tube. DNA was extracted using a ZR Genomic DNA II kit (Zymo Research cat. no D3007) following the protocol described in section 2.3.1; DNA was eluted into 150 µl of molecular grade water. Scat DNA extracts were stored at -20 °C for molecular applications.

3.2.7.2 PCR primers and TaqMan® MGB probes used to target marten DNA in scat DNA extracts

All the primers and TaqMan® MGB probes used for the identification of pine marten and stone marten species and sex determination including the region being amplified are listed in Table 3.2 and Table 3.3.

Table 3.2 qPCR primer pairs used to detect marten DNA; CR=control region, ZFX=X chromosome zinc finger gene sequence, ZFY= Y chromosome zinc finger gene sequence. Primers were supplied by Eurofins Scientific.

Primer pairs	Sequence	Target	Product size (bp)	Reference
PM-FOR	CACCAGGCCTCGAGAAACCAT	CR	60	(Statham *et al.* 2007)
PM-REV	CTTCTCGCTCCGGGCCC			
MMXF	GGCAGAGCAACCCTGTCATAA	ZFX	76	(Mullins *et al.* 2010)
MMXR	GGGCCTGAGGTTGGTACCACCA			
Y2F	CTTGTCCAGAAACTTCATTCAATGTAA	ZFY	76	(Mullins *et al.* 2010)
MMYR3	GGGGGCAGACAAGTAAAATCTA			
LMS-3-25	TCCCTAAGACTCAAGGAAGAAGCA	CR	300	(Statham *et al.* 2007)
PM-REV	CTTCTCGCTCCGGGCCC			
DL575R	CATGGGCTGATTAGTCATTAGTCC	CR	452	*(This study)* (Statham *et al.* 2007)
LMS-3-25	TCCCTAAGACTCAAGGAAGAAGCA			

Table 3.3 TaqMan® MGB probes used for species identification and sex determination using qPCR. Probes were supplied by Applied Biosystems.

Probe name	Sequence	Primer pair	Target	Reference
PM2	FAM-CCTTGCCCGATGTGTACCT-MGB	PM-FOR, PM-REV	CR	(O'Reilly *et al.* 2008)
SM	VIC-CCTTGCTAGACGTGTACC-MGB	PM-FOR, PM-REV	CR	(O'Reilly unpublished)
FOX1	VIC-TCCTGGCTCGAAGTAT-MGB	PM-FOR, PM-REV	CR	(O'Reilly *et al.* 2008)
FOX2	VIC- TCCTTGCTCGAAGTAT-MGB	PM-FOR, PM-REV	CR	(This study)
MMX	FAM-TGTGTCTCTCTCTGTCAA-MGB	MMXF, MMXR	ZFX	(Mullins *et al.* 2010)
MMY	VIC-CCTGGTCTGAAAACT-MGB	Y2F,MMYR3	ZFY	(Mullins *et al.* 2010)

3.2.7.3 Detection of pine marten and stone marten scat DNA using TaqMan® MGB probe based qPCR

All qPCR amplifications were performed in an ABI 7300 Real-time PCR system using MicroAmp® optical 96 well reaction plates. Applied Biosystems Sequence Detection Software (SDS1.2.3.) was used to analyse all results.

Species identification was performed using TaqMan® MGB fluorogenic probe based assays with forward PM-FOR and reverse PM-REV primers (O'Reilly *et al.* 2008) (Table 3.2) with a pine marten species specific FAM labelled probe (PM2) (O'Reilly *et al.* 2008) and a VIC labelled stone marten specific probe (SM) (O'Reilly unpublished) (Table 3.3). The assay may be performed in a duplex as the primers and differently labelled probes target the same region

in the d-loop mitochondrial DNA sequence of both species with 3 sequence mismatches between the pine marten and stone marten DNA in the probes target region.

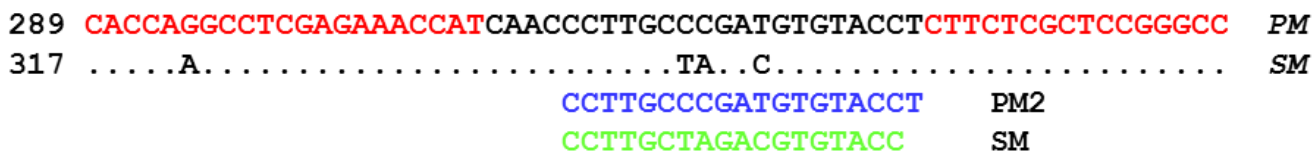


Figure 3.7 Alignment of pine marten and stone marten CR sequences highlighting position of SNP's between species; primers FAM labelled probe VIC labelled probe.

Each 10 µl reaction contained; 5 µl FastStart Universal Probe Master, 0.5 µl of primer mix (PM-FOR + PM-REV), pine marten TaqMan® MGB probe (PM2) and stone marten TaqMan® MGB probe (SM), all at 0.25 µM final concentrations with 3.5 µl of template DNA. Pine marten and stone marten DNA of known origin were used as positive controls and PCR blanks containing all reagents without template DNA were used to monitor for contamination. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol as described in section 2.3.4. Detection is based on the accumulated fluorescence (C_T value) of either the FAM-labelled (pine marten) or VIC-labelled (stone marten) probe.

3.2.7.4 Analysis of scats negative for marten DNA

3.2.7.4.1 Detection of fox DNA in non-marten scats using TaqMan® MGB probe based qPCR

While it is impossible to morphologically distinguish between pine marten and stone marten scats it is also sometimes difficult to distinguish these scats from fox scats (Davison *et al.* 2002). Therefore any scat samples that were negative for pine marten and stone marten DNA were tested for fox DNA. This assay was initially performed on non-marten scats from the September 2012 survey (n=60). Fox identification was performed using TaqMan® MGB fluorogenic probe based assays with PM-FOR and PM-REV primers (O'Reilly *et al.* 2008) (Table 3.2) with a fox species specific VIC labelled probe (FOX1) (O'Reilly *et al.* 2008) (Table 3.3).

Each 10 µl reaction contained; 5 µl FastStart Universal Probe Master, 0.5 µl each of primer mix (PM-FOR + PM-REV), and fox probe, both at 0.25 µM final concentrations, 1 µl of

molecular grade water and 3 µl of template DNA. Fox DNA of known origin was used as positive control and PCR blanks containing all reagents without template DNA were used to monitor for contamination. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol as described in section 2.3.4. Detection is based on the accumulated fluorescence (C_T value) of the VIC-labelled fox probe.

3.2.7.4.2 SYBR® Green Dye qPCR assay

A SYBR® Green Dye qPCR assay was used to assay any samples that were negative for marten DNA using TaqMan® MGB probe based qPCR. SYBR® Green is an intercalating dye and will bind to any double stranded DNA. The primers used are the same as those used in the species TaqMan® probe based assays (PM-FOR and PM-REV). As well as pine marten, stone marten and fox, these primers will amplify DNA from a broader range of mammals, if present. Initially this assay was performed on scat DNA extracts from the September 2012 survey and was later used to assay for non-marten DNA in all scat DNA extracts collected throughout the project.

Each reaction contained; 5 µl of FastStart Universal SYBR Green Master (Roche cat. no.112 262 00), 0.5 µl of primer mix (PM-FOR + PM-REV) at 0.25 µM final concentration, 2 µl of molecular grade water and 3 µl of template DNA. Pine marten, stone marten and fox DNA of known origin were used as a positive controls and PCR blanks containing all reagents without template DNA were used to monitor for contamination. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol 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. A single cycle dissociation stage was added with the following thermal profile; 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. The melting temperature (T_M) of an amplicon varies between amplicon length and sequence composition and can be used to distinguish between specific and non-specific amplifications when used with the SYBR® Green Dye qPCR assay with non specific amplification due to primer dimer usually having a lower melting temperature than target amplicons (Ririe *et al.* 1997). Any samples that showed amplification with a C_T <28.0 and a T_M not less than 2°C lower than the positive controls were selected for sequencing using ABI BigDye Sequencing kit v1.1. Samples were analysed using an ABI PRISM 310 Genetic Analyser.

3.2.7.4.3 Standard PCR of DNA extract positives from SYBR® Green Dye qPCR assay

Standard PCR was first performed using the primer pair LMS-3-25 and PM-REV (Table 3.2) targeting a 300 base pair product in a 2720 thermal cycler.

Each 10 µl reaction contained; 5 µl GoTaq Hot Start Green Master Mix (Promega cat. no. M5122), 0.5 µl of primer mix (LMS-3-25 + PM-REV) at 0.25 µM final concentration, 1.5 µl of molecular grade water and 3 µl of template DNA. DNA of known origin was used as a positive control and PCR blanks containing all reagents without template DNA were used to monitor for contamination. Reaction conditions were as follows; 95°C for 2 minutes, followed by 50 cycles with three steps; 95°C x 30s, 50°C x 30s, 72°C x 30s then a single cycle of 72°C for 10 minutes. Samples were then cooled to 4°C. Samples were then run on a 1.5 % agarose gel

3.2.7.4.4 Gel Electrophoresis

100 ml of 1.5% agarose (1.5 g of agarose, Amresco cat. no. N605-500G) gel was prepared in 1 X Tris-acetate-EDTA (TAE) buffer and allowed to cool to approximately 50 °C. 1 µl of ethidium bromide solution (Sigma cat. no. 129k8708) was added to allow visualisation of the products and the solution was swirled to mix thoroughly. The gel was poured into the tray and the comb added to form the wells. The gel was then allowed to set. When the gel was set it was placed into HYBAID gel tank which was filled with 1 X TAE buffer. 3 µl of sample was added to each well and its position recorded. 3 µl of 100 bp size standard was added to the outer wells of each row to allow determination of product size. The gel tank was connected to an Amersham Pharmacia Biotech electrophoresis power unit and ran at 180 V, 120 mA for 35 minutes. Samples were visualised using Gene Genius Bio-imaging System™ using Gene Snap™ software. Any sample that showed a clear single 300 base product was selected for sequencing following post-PCR clean-up.

3.2.7.4.5 Post-PCR clean-up

The remainder of each sample (7 µl) and 100 µl of DNA binding buffer was added to a 1.5 ml micro-centrifuge tube. Each tube was vortexed briefly to mix. The mixtures were then transferred to Zymo-spin Columns, placed into collection tubes. All tubes were then placed in a Sigma 1-14 micro-centrifuge and spun at 16000 x g for 30 seconds and the effluent was discarded. 200 µl of wash buffer (Zymo Research cat. no. D3004-2-48) was added to each

column and centrifuged again at 16000 x g for 30 seconds. This wash step was repeated. 10 µl of molecular grade water was added directly to each column matrix. Each column was then transferred to a clean 1.5 ml micro-centrifuge tube and centrifuged at 16000 x g for 30 seconds to elute the DNA.

3.2.7.4.6 Sequencing of DNA extract positives from SYBR® Green Dye qPCR assay

Sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems cat. no. 4337450). Samples were sequenced in one direction using LMS-3-25 forward primer. In a 96-well reaction plate containing MicroAmp optical tubes (Applied Biosystems cat. no. 4316567) each 20 µl reaction contained; 1 µl of 2.5X Ready Reaction Premix (BigDye 3.1) (Applied Biosystems cat. no. 4305605), 3.5 µl of 5X BigDye Sequencing Buffer (Applied Biosystems cat. no. 4336697), 0.6 µl LMS-3-25 primer, 13.9 µl of molecular grade water and 1 µl of template DNA. The reaction plate was placed in a 2720 thermal cycler under the following conditions; a single cycle of 96 °C for 1 minute followed by 25 cycles of; 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 minutes. Samples were then cooled to 4 °C.

The reaction plate was removed from the thermal cycler. 5 µl of EDTA (125 mM) was added to each well. 60 µl of 100 % ethanol was added to each tube to aid precipitation. The plate was sealed and inverted 4 times to allow mixing. The plate was then incubated at room temperature for 15 minutes. Using a plate adapter the plate was centrifuged at 3000 x g for 30 minutes in a Sigma 4K15 centrifuge maintained at 4 °C. The reaction plate was removed from the centrifuge. The plate was inverted and returned to the centrifuge to be spun at 185 x g to remove excess liquid. The plate was removed from the centrifuge and 60 µl of 70 % ethanol was added to each tube. 70 % ethanol was made up fresh as this must not be below 70 % to avoid pellet returning into solution. The plate was returned to the centrifuge and spun at 3000 x g for 15 minutes. The plate was inverted again and spun up to 185 x g then removed from the centrifuge and air dried for approximately 30 minutes. 20 µl of Hi-Di formamide (Applied Biosystems cat. no. 4311320) was added to each well. Samples were denatured in a 2720 thermal cycler for 3 minutes at 95 °C and cooled slowly to 4 °C. Samples were electrophoresed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems) with the following sequencing parameters; polymer used was POP-4, BDT (Big Dye Terminator) BigDye 3.1, the capillary length was 36 cm and the run time selected was 25 minutes. Sequences were analysed using Sequence Scanner v1.0 software (Applied Biosystems).

3.2.7.4.7 Design of TaqMan® MGB probe for qPCR detection of fox in scat DNA extracts from Nietoperek

A fox species specific TaqMan® MGB probe (FOX2) was designed to detect fox in scat DNA extracts from Nietoperek. Fox sequences from Nietoperek, that included the target region for the fox specific TaqMan® MGB probe (FOX1) were aligned using the multiple sequence algnment editor BioEdit (Hall, 1999) to highlight single nucleotide polymorphisms (SNP's) between the FOX1 target sequence and the complementary sequence in the Nietoperek fox DNA extracts. The sequence of FOX2 TaqMan® MGB probe was designed to target the same region as FOX1 but to incorporate any SNP's in the Nietoperek fox sequences into it's target region.

3.2.7.5 Sex determination of pine marten and stone marten scat DNA samples using TaqMan® MGB probe based qPCR.

Samples that were positive for either pine marten or stone marten DNA were screened for suitability to be selected for sex determination as described in section 2.3.5. Reaction volumes and conditions were also as described in section 2.3.5 of this document.

3.2.7.6 Microsatellite analysis of pine marten and stone marten scat DNA extracts

3.2.7.6.1 Selection of samples for genotyping

Data generated from the sex determination assay was used to screen for samples suitable for microsatellite analysis as described in section 2.3.6.

3.2.7.6.2 Screening for polymorphic loci

Microsatellite loci were selected that would amplify a product less than 250 bp due to the degraded nature of scat DNA. Table 3.4 shows the microsatellite primers screened for polymorphic loci in the Nietoperek populations of pine marten and stone marten. Initial screening was performed with 23 DNA extracts (14 stone marten, 9 pine marten) as this process was performed in parallel with sample gathering.

Table 3.4 Microsatellite primers screened for polymorphic loci with pine marten and stone marten DNA extracts from Nietoperek. A GTTTCTT "PIGtail" sequence has been added to the 5' end of reverse primers marked with an asterisk to promote full non-templated nucleotide addition.

Locus	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Reference
Mel 1	CTGGGGAAAATGGCTAAACC	*GCTCTTATAAATCTGAAAATTAGGAATTC	*(Bijlsma et al. 2000)*
Gg 7	GTTTTCAATTTTAGCCGTTCTG	GTTTATCTCCCTCTTCCTACCC	*(Davis & Strobeck 1998)*
Mar-08	CCCTTTAGTTGGCACAGTCC	CTTTGGCATGAGTCATTTGG	*(Natali et al. 2010)*
Mar-43	CTTGTCACCCCAGGAGAGG	CCTAAGCCCAAATCTAAGTGC	*(Natali et al. 2010)*
Mar-53	TCTCCAGCATTTACCTTTACCC	GAACAGCCAACCCCATACC	*(Natali et al. 2010)*
Mer 041	TGTGTGATCTCTGGGAATTCTC	*GTTTCTGCTCCCCAGATAAAAGC	*(Fleming et al. 1999)*
Mvi1341	GTGGGAGACTGAGATAGGTCA	*GGCAACTTGAATGGACTAAGA	*(Vincent et al. 2003)*
Mf 4.17	GAGGCGACAAATTCCGGT	CCAAGCTTCAGAATCTAG	*(Basto et al. 2010)*
Mel 105	GATATTCCCCTCCCACCACT	*CTCCAAGGGATCCTGGAACT	*(Carpenter et al. 2003)*
Gg 454	CTTCTTACATAGTCAATGTTTTG	TGCCATTTTCTCCAGAA	*(Walker et al. 2001)*
Gg 234	TTACTTAGAGGATGATAACTTG	*GAACTCATAGGACTGATAGC	*(Walker et al. 2001)*
Ma 2	ACCCATGAATAATGTCTTAT	*ATCTTGCATCAACTAAAAAT	*(Davis & Strobeck 1998)*
Mvi 1354	CCAACTGGAGCAAGTAAAT	*CATCTTTGGGAAAGTATGTTT	*(Vincent et al. 2003)*
Mvis 075	GAAATTTGGGGAATGCACTC	*GGCAGGATAGGATGTGAGCT	*(Fleming et al 1999)*
Mer 022	CCATGCTTTGGGTAGGAGAA	CCTTGTTCTCAGGTGGTTGG	*(Fleming et al 1999)*
Mf 1.1	CTGTGTCAGAAAATGTGC	AGGGCTGGTAATACCATG	*(Basto et al. 2010)*
Mf 3.7	AAGATTTTAACCACCATG	GCCTGTTATCAGCCAGCT	*(Basto et al. 2010)*
Mf 8.10	ATCTGTATTATCTGCATA	CCAAAGTGTGATGTTTGC	*(Basto et al. 2010)*

Any marker with inconsistent amplification was discarded. Similarly, any marker which amplified consistently but showed no variation in the sample set was also discarded as these markers would not be informative for identification of individual animals.

A total of 10 microsatellite markers were selected for use in two separate panels of 8 markers to select for pine marten and stone marten individuals (Table 3.5).

Table 3.5 Microsatellite primer pairs used to identify pine marten and stone marten individuals from scat DNA; product=the expected product range for this study, bp = base pairs, F=forward, R=reverse, PIG-tailed reverse primers are marked with an asterisk, primer pairs were supplied by Eurofins and Applied Biosystems.

Locus	Reporter this study	Product (bp)	Primer pairs used with pine marten DNA extracts	Reference
Mel 1	FAM	106-116	F-CTGGGGAAAATGGCTAAACC R-*GCTCTTATAAATCTGAAAATTAGGAATTC	*(Bijlsma et al. 2000)*
Gg 7	PET	132-142	F-GTTTTCAATTTTAGCCGTTCTG R-GTTTATCTCCCTCTTCCTACCC	*(Davis & Strobeck 1998)*
Mar-53	FAM	236-242	F-TCTCCAGCATTTACCTTTACCC R-GAACAGCCAACCCCATACC	*(Natali et al. 2010)*
Mer 041	ATTO550	159-165	F-TGTGTGATCTCTGGGAATTCTC R-*GTTTCTGCTCCCCAGATAAAAGC	*(Fleming et al. 1999)*
Mvi1341	PET	168-172	F-GTGGGAGACTGAGATAGGTCA R-*GGCAACTTGAATGGACTAAGA	*(Vincent et al. 2003)*
Mel 105	FAM	191-201	F-GATATTCCCCTCCCACCACT R-*CTCCAAGGGATCCTGGAACT	*(Carpenter et al. 2003)*
Mar-43	VIC	150-164	F-CTTGTCACCCCAGGAGAGG R-*CCTAAGCCCAAATCTAAGTGC	*(Natali et al. 2010)*
Gg 454	HEX	126-134	F-CTTCTTACATAGTCAATGTTTTG R-TGCCATTTTCTCCAGAA	*(Walker et al. 2001)*
Locus	**Reporter this study**	**Product (bp)**	**Primer pairs used with stone marten DNA extracts**	**Reference**
Mel 1	FAM	106-116	F-CTGGGGAAAATGGCTAAACC R-*GCTCTTATAAATCTGAAAATTAGGAATTC	(Bijlsma *et al.* 2000)
Gg 7	PET	132-142	F-GTTTTCAATTTTAGCCGTTCTG R-GTTTATCTCCCTCTTCCTACCC	(Davis & Strobeck 1998)
Mar-08	FAM	146-154	F-CCCTTTAGTTGGCACAGTCC R-CTTTGGCATGAGTCATTTGG	(Natali *et al.* 2010)
Mar-53	FAM	236-242	F-TCTCCAGCATTTACCTTTACCC R-GAACAGCCAACCCCATACC	(Natali *et al.* 2010)
Mer 041	ATTO550	159-165	F-TGTGTGATCTCTGGGAATTCTC R-*GTTTCTGCTCCCCAGATAAAAGC	(Fleming *et al.* 1999)
Mvi1341	PET	168-172	F-GTGGGAGACTGAGATAGGTCA R-*GGCAACTTGAATGGACTAAGA	(Vincent *et al.* 2003)
Mf 4.17	FAM	205-217	F-GAGGCGACAAATTCCGGT R-CCAAGCTTCAGAATCTAG	(Basto *et al.* 2010)
Mel 105	FAM	191-201	F-GATATTCCCCTCCCACCACT R-*CTCCAAGGGATCCTGGAACT	(Carpenter *et al.* 2003)

3.2.7.6.3 Microsatellite PCR optimisation

Initial screening for polymorphic loci was performed using GoTaq® Hot Start Polymerase (Promega, cat. no. M5001). To optimise the PCR protocol for use with scat DNA which may be degraded, three alternative enzymes were trialled with a pine marten DNA extract which had shown consistent amplification when used to screen for polymorphic loci with the GoTaq® Hot Start Polymerase. The three alternative enzymes trialled were; HotStart PCR-to-Gel Taq PCR Master Mix (Amresco cat. no. IB1409), Kapa2GHS PCR Mix (Kapa Biosystems cat.no KK5609) and HotStart Plus (Qiagen, cat. no. 203643). The markers used were Mel1, Mer041 and Mel105 which represent a relatively short, medium and long product respectively. Trial criteria were; consistent amplification, peak height, clear resolution and minimal background noise. Each enzyme was trialled in triplicate.

Combining loci in a single PCR (multiplexing) significantly reduces the time and cost of microsatellite analysis. Once the pine marten and stone marten microsatellite panels had been selected using single marker assays, multiplexing was trialled and results compared with single marker assays. It was found that some markers, when multiplexed, either interfered with the amplification of other markers or they themselves fail to amplify in the presence of any other markers. A variety of multiplexes were therefore attempted to optimise the assay. Optimisation involved adjusting primer set concentrations to allow for successful and consistent amplification.

3.2.7.6.4 Microsatellite analysis

Amplification of DNA fragments was performed in a 2720 thermal cycler. Each 10 µl reaction contained 5 µl Amresco HotStart PCR-to-Gel Taq PCR Master Mix, 1 µl of multiplex or primer pair and 4 µl of template DNA. Cycling parameters were as follows; a single cycle at 95° C for 5 minutes, 40 cycles of 95° C for 30 seconds, 57° C for 90 seconds and 72° C for 30 seconds with a final single hold at 60° C for 30 minutes. Samples were rapidly cooled to 4° C. Samples were diluted 1:20 in molecular grade water.

1 µl of each sample was denatured in 15 µl Hi-Di formamide (Applied Biosystems, cat. no. 4311320) with 0.15 µl GS500LIZ™ size standard (Applied Biosystems, cat. no. 4322682) for 5 minutes at 95 °C, followed by rapid cooling to 4 °C.

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.

Microsatellite allele sizing was performed using GeneMapper software v. 3.7 (Applied biosystems). The 250-bp peak was excluded from the GS500LIZ size standard. Samples were assigned allele calls if the peak heights were above 100 RFU (Relative Fluorescent Units). Each sample was genotyped in triplicate with agreement in two of the three samples required for a positive heterozygous sample and all three samples in agreement required for homozygous samples. Ambiguous samples were repeated with a lower dilution factor. Samples that were found to be overloaded were repeated at a higher dilution factor.

3.2.7.7 Haplotype analysis of scat DNA extracts from individual pine marten and stone marten

Mitochondrial DNA (mtDNA) is maternally inherited and can be categorized into several types and groups termed haplotypes and haplogroups. The variations in the genetic code of mitochondria fit into clusters. These clusters can trace lineage far back into time. The steady rate of mutation permits a reasonably accurate estimate of exactly when a particular group of animals migrated from their primary group. mtDNA haplotype analysis is widely used in phylogeographic studies with the intra-specifically variable d-loop region of mtDNA an informative region for such studies (Davison *et al*. 2001; Ruiz-Gonzalez *et al*. 2013a).

Once individual pine marten and stone marten had been identified using microsatellite analysis mitochondrial d-loop haplotype analysis was performed on one scat DNA extract from each individual to attribute a haplotype to each individual animal. Standard PCR was used to amplify products which were then sequenced.

Sequences were compared to existing marten sequences (Davison, *et al.* 2001; Nagai *et al.* 2012; Ruiz-Gonzalez *et al.* 2013a; Vergera *et al*. 2015) in the GenBank (NCBI) database to determine the haplotype of each animal. Stone marten sequences were also compared with sequences from stone marten DNA extracts which were obtained in the Molecular Ecology Research Laboratory in W.I.T.

3.2.7.7.1 Standard PCR and sequencing of mtDNA d-loop target DNA

Standard PCR was performed using the primer pair LMS-3-25 and DL575R (Table 3.2) to amplify a 451 base pair region of d-loop mtDNA.

Each 10 µl reaction contained 5 µl GoTaq Hot Start Green Master Mix, 0.5 µl of primer mix (LMS-3-25 + DL575R) at 0.25 µM final concentrations, 1.5 µl of molecular grade water and 3 µl of template DNA. DNA of known origin was used as a positive control and PCR blanks containing all reagents without template DNA were used to monitor for contamination. Reaction conditions were as described in section 3.2.7.5.3. Samples were run on a 1.5% agarose gel against a 100 base pair size standard and visualised using Gene Genius Bio-imaging System™ using Gene Snap™ software as described in section 3.1.7.5.4. Samples were then sequenced in one direction using the DL575R primer following the protocol described in section 3.2.7.5.6.

3.2.7.7.2 Phylogenetic analysis of mtDNA d-loop sequences

Sequences of d-loop mtDNA from pine marten and stone marten were retrieved from GenBank (NCBI). Sequences for each species were aligned using BioEdit and pairwise alignment was performed in ClustalW (Larkin *et al.* 2007). Additional stone marten sequences from DNA extracts in the Laboratory in W.I.T were also aligned. Aligned sequences were transferred to MEGA version 6 (Tamura *et al.* 2013) where sequences were cut to the largest region where all sequences overlapped and all insertions were removed. Arlequin v3.5 (Excoffier & Lischer, 2010) was used to generate a resource description framework (rdf) file compatible with the phylogenetic software package Network 4.6 (Fluxus engineering; Bandelt *et al*. 1999) which was used to construct a median joining network from the mtDNA d-loop sequences

3.3 Results

3.3.1 S.T.A.R® Buffer stability trial

The 5 pine marten DNA samples were originally extracted as part of a pine marten survey of Portlaw Wood, Waterford, Ireland and on original analysis had C_T results representative of good, average and poor quality DNA extracts (Table 3.6). Samples with good or average quality starting DNA concentrations prior to re-analysis with qPCR showed little or no evidence of a decrease in quality throughout the trial as evidenced by samples 1-3 (Table 3.6). Samples 4 and 5 only showed a slight increase in C_T values over 23 days. Samples were therefore transferred directly into S.T.A.R® buffer in Poland on the evening of each survey.

Table 3.6 Comparison of qPCR C_T values from 5 samples held in S.T.A.R® buffer from 0-23 days, Original $C_{T=}$ the C_T results obtained during the original analysis of pine marten scat DNA extracts as part of the Portlaw Wood pine marten survey. P<0.01 for all samples indicating no significant degradation in DNA at the 99% confidence interval.

Sample no	Original C_T	Day 0 C_T	Day 4 C_T	Day 8 C_T	Day 23 C_T
1	24.17	24.61	24.11	24.17	25.01
2	21.88	22.06	22.29	21.89	21.16
3	18.99	19.34	18.93	19.20	18.97
4	26.42	26.01	26.55	26.65	28.72
5	27.44	27.01	27.74	27.33	28.34

3.3.2 Comparison of same-day transfer and after-survey transfer of scats into S.T.A.R® buffer

Of the 36 scats collected and transported to W.I.T both in S.T.A.R® buffer and in zip-lock bags, 19 were found to be marten scats using TaqMan® MGB probe based qPCR species assays (Table 3.7). The average C_T value for samples added directly to S.T.A.R® buffer while still in Nietopetek did not show any decrease in quality. As this approach is safer and renders the samples more portable, it was employed throughout the remainder of the project.

Table 3.7 qPCR species assay using DNA extracts from marten scats added to S.T.A.R® buffer in Nietoperek and W.I.T. PM= pine marten, SM= stone marten, NP=Nietoperek, WIT=Waterford Institute of Technology, C_T Average= the average C_T obtained for all scat DNA extracts from a species transported in S.T.A.R® buffer and in plastic zip-lock bags.

Sample no	PM NP C_T	PM WIT C_T	Sample no	SM NP C_T	SM WIT C_T
1	20.63	20.99	**1**	23.33	23.65
2	20.94	21.04	**2**	23.46	23.44
3	21.26	21.15	**3**	24.63	24.74
4	21.38	21.33	**4**	25.20	25.01
5	21.56	21.73	**5**	25.51	25.76
6	21.67	21.27	**6**	25.91	25.41
7	21.87	21.99	**7**	26.05	25.87
8	21.90	21.43	**8**	26.65	26.22
9	22.39	22.05	**9**	27.29	26.93
10	22.80	22.99			
C_T Average	**21.64**	**21.59**		**25.34**	**25.22**

3.3.3 Scat surveys

Seven main scat surveys were performed during this project from September 2012 to May 2014. Some scats had been previously collected by volunteers from the Vincent Wildlife Trust (VWT) during bat censuses. Scats were also collected during the hair tube surveys by the collaborating students from Wrocław University between October 2012 and March 2013. All the scats were combined for a total sample size of 1815 samples. On visual examination prior to processing, it was revealed that 30 samples were not scats. The dark conditions in the tunnels may have been responsible for this misidentification. Therefore the total number of scats to be analysed during this project was 1785. 659 scats were collected above ground while 1126 scats were collected throughout the tunnel system (Table 3.8).

Table 3.8 Summary of scats collected during each survey period; A=above ground scats, B=below ground scats, * scats collected prior to commencement of this project, ** main scat surveys.

Survey	Total	A	B
Jul-11*	32	16	16
Dec-11*	117	48	69
Apr-12*	89	22	67
Sep-12**	130	81	49
Oct-12**	80	51	29
Nov-12	30	29	1
Jan-13**	220	24	196
Feb-13	26	17	9
May-13**	276	88	188
Sep-13**	347	129	218
Jan-14**	249	111	138
May-14**	189	43	146
Total	1785	659	1126

3.3.4 Detection of pine marten and stone marten in scat DNA extracts

Positive samples were defined as DNA extracts in which pine marten or stone marten DNA was detected with a C_T value <= 35.0 using the TaqMan® MGB probe based qPCR species assay (Figure 3.8). Negative samples include all samples that contained non-marten DNA as well as samples which contained no, or insufficient, DNA. The total number of scat DNA extracts analysed was 1785 with 414 DNA extracts containing pine marten DNA and 802 extracts containing stone marten DNA. Table 3.9 shows a summary of the species assay results including the success rate of species identification throughout the study.

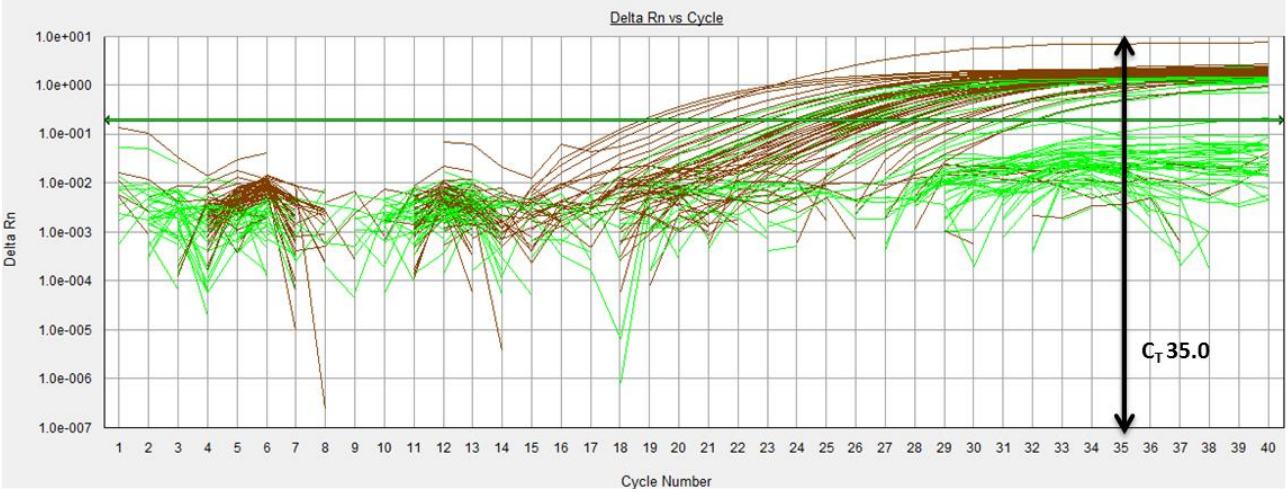


Figure 3.8 Sample plot from TaqMan® MGB probe based qPCR assay to detect pine marten and stone marten. Arrow indicates cut-off for positive DNA extracts (C_T 35.0).

Table 3.9 Species results summary; A=above ground, B=below ground, Tot=total, +ve=positive, -ve=negative PM=pine marten, SM=stone marten, S=success rate; *= survey periods covering the entire survey area

Survey	A -ve	B-ve	Tot-ve	PM A	PM B	SM A	SM B	Tot +ve	S A %	S B %	S Tot %
Jul-11	3	0	3	12	8	1	8	29	81.25	100	90.63
Dec-11	13	12	25	4	7	31	50	92	72.92	82.61	78.63
Apr-12	12	13	25	7	26	3	28	64	45.45	80.6	71.91
Sep-12	38	22	60	28	8	15	19	70	53.09	55.1	53.85
Oct-12	16	15	31	8	1	27	13	49	68.63	48.28	61.25
Nov-12	1	1	2	3	0	25	0	28	96.55	0	93.33
Jan-13*	10	59	69	6	85	8	52	151	58.33	69.9	68.64
Feb-13	15	8	23	0	0	2	1	3	11.76	11.11	11.54
May-13*	42	67	109	36	24	10	97	167	52.27	64.36	60.51
Sep-13*	47	64	111	32	46	50	108	236	63.57	70.64	68.01
Jan-14*	19	42	61	40	13	52	83	188	82.88	69.57	75.5
May-14*	17	33	50	17	9	9	104	139	60.47	77.4	73.54
Total	**233**	**336**	**569**	**193**	**227**	**233**	**563**	**1216**	**64.64**	**70.16**	**68.12**

3.3.5 Analysis of scats negative for marten DNA

3.3.5.1 Detection of fox DNA in non-marten scats using TaqMan® MGB probe based qPCR

DNA extracts that were negative for pine marten and stone marten DNA were tested for the presence of fox DNA using a TaqMan® MGB probe based fox specific qPCR assay. Of the initial 60 non-marten scat DNA extracts assayed for the presence of fox DNA using this assay with the TaqMan® MGB probe FOX1 (Table 3.3), no DNA amplification was detected in any sample.

3.3.5.2 SYBR® Green Dye qPCR assay

A SYBR® Green Dye qPCR assay was used to assay any samples that were negative for marten DNA using TaqMan® MGB probe based qPCR. The same primers were used as in the TaqMan® MGB probe based qPCR species assay but without the added specificity of a species specific probe. This assay will amplify DNA from a wider range of mammals, if present.

Positive samples were defined as DNA extracts in which amplification was detected with a C_T value <= 28.0. Of the 60 scat DNA extracts tested from the September 2012 survey, 29 were found to contain insufficient amplifiable DNA and 31 amplified successfully with a C_T less than 28.0.

For all surveys, 495 samples were tested for the presence of DNA from other mammals using the SYBR® Green Dye qPCR assay. Scat DNA extracts that amplified with a C_T value <= 28.0 (n=91) were selected for standard PCR and sequencing.

3.3.5.3 Standard PCR of DNA extract positives from SYBR® Green Dye qPCR assay

From the September 2012 survey, scat DNA extracts which were positive using the SYBR® Green Dye qPCR assay were amplified using standard PCR. Products were run on a 1.5% agarose Gel and products visualised using Gene Genius Bio-imaging System™ (Figure 3.9). From these results, 24 scat DNA extracts were selected for sequencing using the LMS-3-25 primer.

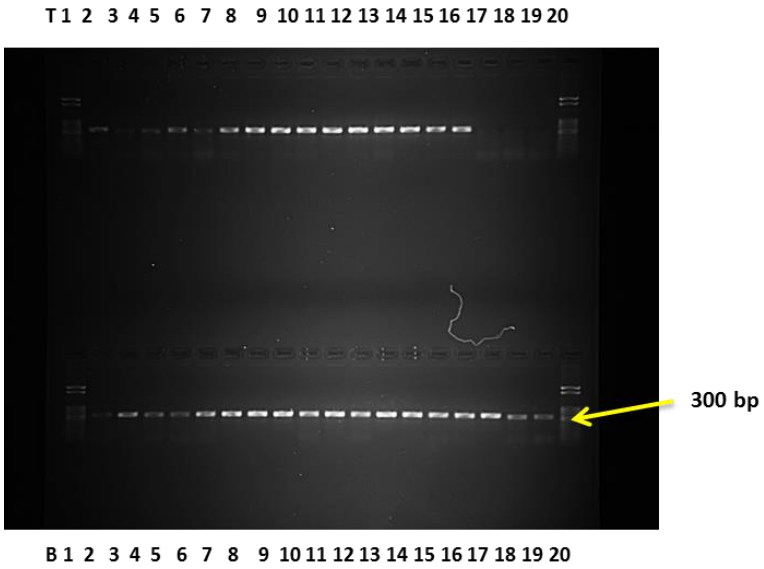


Figure 3. 9 Gel image of LMS-3-25, PMREV amplification of DNA extracts. T=top row, B=bottom row, T1, T20, B1, B20= 100 base pair ladder (product 300 bases); T2-T15, B2-B17= scat DNA extracts, B18, B19=positive controls T18, T19=negative controls, T17=empty.

For all other surveys 91 scat DNA extracts were selected for standard PCR and sequencing. Of the 91 samples that amplified using qPCR, 14 were successfully amplified using standard PCR.

3.3.5.4 Sequencing of DNA extract positives from SYBR® Green Dye qPCR assay

A total of 13 of the 24 non marten scat DNA extracts were successfully sequenced from the September 2012 survey. When these sequences were inserted to B.L.A.S.T, all 13 were 99% identical to existing fox sequences in the database over 100% of the sequence (Figure 3.10).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Vulpes vulpes haplotype 138 tRNA-Pro gene and D-loop, partial sequence; mitochondrial	412	412	100%	8e-117	99%	KJ846557.1
Vulpes vulpes mitochondrial partial D-loop, isolate POLI71	412	412	100%	8e-117	99%	HF677245.1
Vulpes vulpes mitochondrial partial D-loop, isolate D408	412	412	100%	8e-117	99%	HF677224.1

Figure 3.10 Sample B.L.A.S.T results of a 300 base CR product amplified using LMS-3-25, PM REV with non-marten DNA extracts from Nietoperek.

All 13 fox sequences obtained from scat DNA extracts from Nietoperek were aligned using BioEdit and found to have the same sequence over the TaqMan® MGB probe target region (Figure 3.11 A). There is a SNP present in all sequences when compared to the FOX1 probe target sequence (Figure 3.11 B). A second TaqMan® MGB probe was therefore designed to account for this SNP (Figure 3.12). All non-marten scat DNA extracts were then re-assayed using TaqMan® MGB probe based qPCR with the fox specific FOX2 probe.

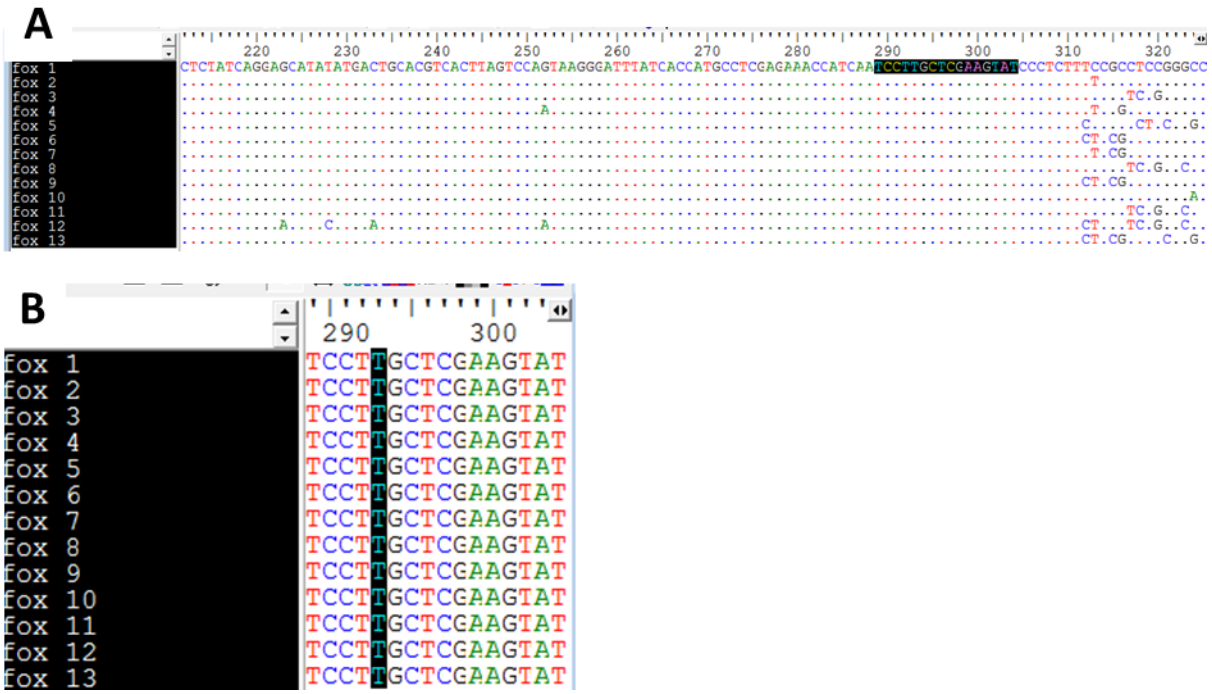


Figure 3.11 (A) Alignment of Nietoperek fox mtDNA CR sequences in BioEdit with the FOX1 probe target-region highlighted. (B) FOX1 probe target-region with SNP highlighted.

Figure 3.12 Sequence of new TaqMan® MGB FOX2 probe compared to FOX1 probe sequence. SNP highlighted in red.

From all other surveys 14 further scat DNA extracts were selected for sequencing and these 14 samples yielded 5 successfully sequencing reactions. On insertion of the resultant

sequences into B.L.A.S.T., 3 DNA extracts were found to contain badger DNA and 2 DNA extracts contained European polecat DNA. The remaining scat DNA extracts (n=490) did not contain sufficient analysable DNA for successful identification.

3.3.5.5 Re-assay of non-marten scats using TaqMan® MGB probe based qPCR with the fox specific FOX2 probe

Once the new FOX2 probe had been designed, all non-marten scat DNA extracts were tested for the presence of fox DNA using TaqMan® MGB probe based qPCR. Positive samples were defined as in section 3.3.4. Of the 569 scat DNA extracts assayed using this method, 74 were positive fox DNA.

3.3.6 Distribution of scats positive for marten DNA.

Prior to collection, the location of each scat had been recorded both above and below ground. The positions of all scats positive for pine marten and stone marten DNA were plotted. Figure 3.13 shows the locations of all marten positive scats collected above ground while Figure 3.14 shows the location of all marten positive scats collected in the tunnels. Scats from both marten species were collected throughout the tunnel system with only pine marten scats collected in the north of the system. Pine marten and stone marten scats were again collected throughout the above ground survey area with only pine marten scats collected in the Northern Forest portion of the survey site.

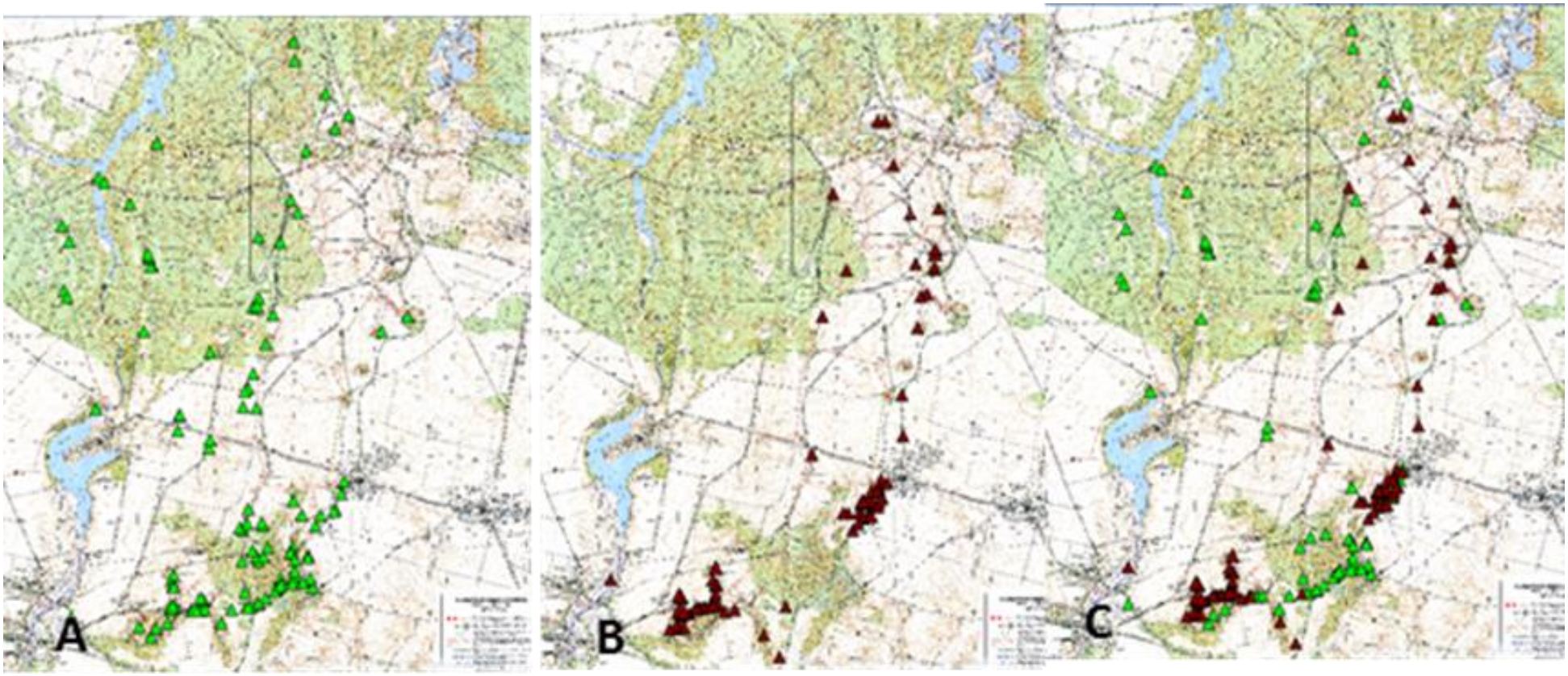


Figure 3.13 Above ground scat distribution (A) pine marten (B) stone marten (C) both species, ▲ pine marten ▲ stone marten.

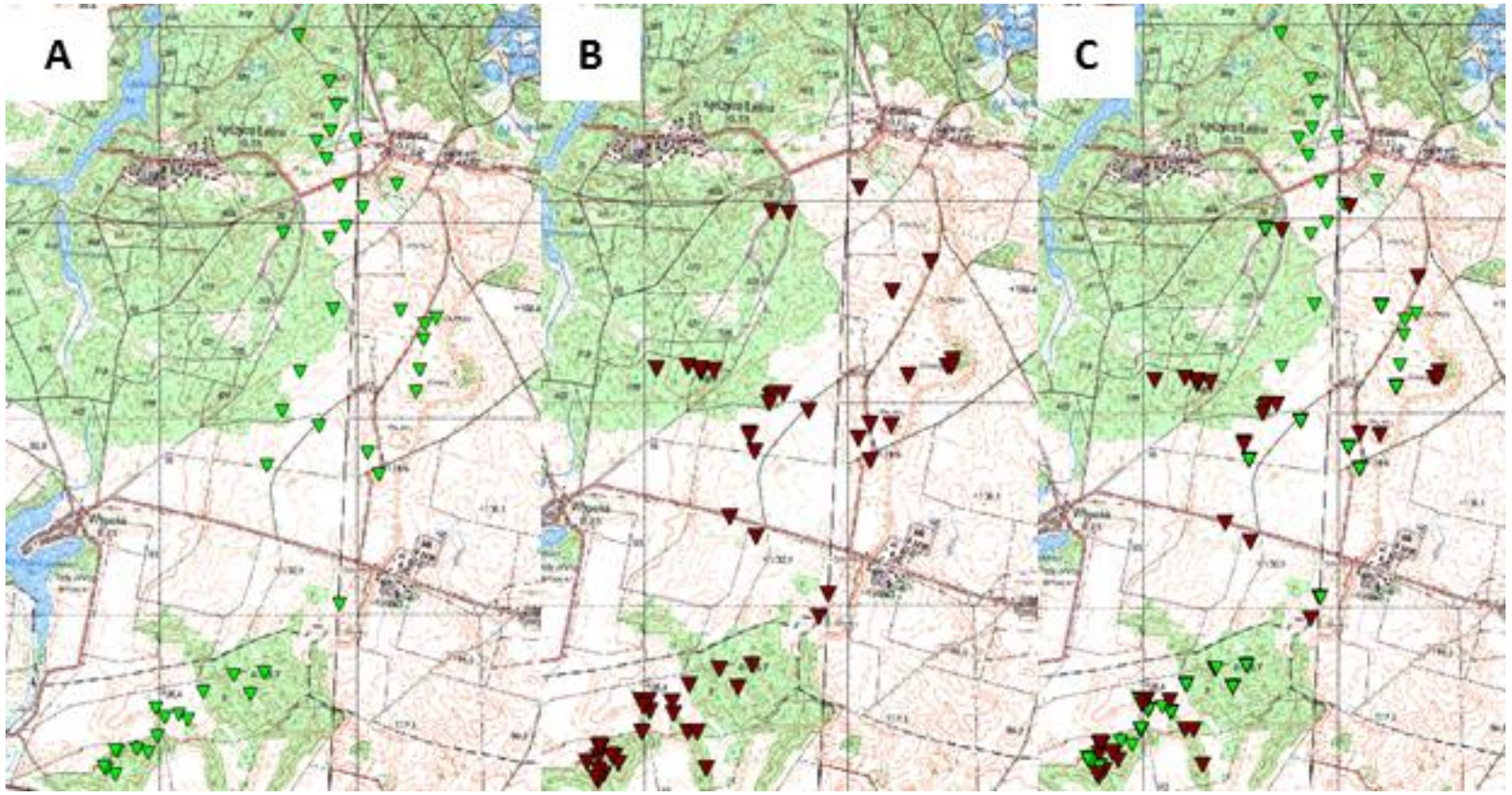


Figure 3.14 Below ground scat distribution. (A) pine marten (B) stone marten (C) both species, ▼ pine marten ▼ stone marten.

3.3.7 Distribution of scats identifiable as non-marten scats.

The locations of all scats collected that were not from pine marten or stone marten but that could be identified as being from other species have been plotted in Figure 3.15. Fox scats were collected in all 3 above ground survey areas. All scats positive for fox DNA were collected above ground. The 3 scats containing badger DNA were collected above ground and all three were collected in area 3. Both scats containing European polecat DNA were collected during the May 2014 survey in section 8 of the tunnel system.

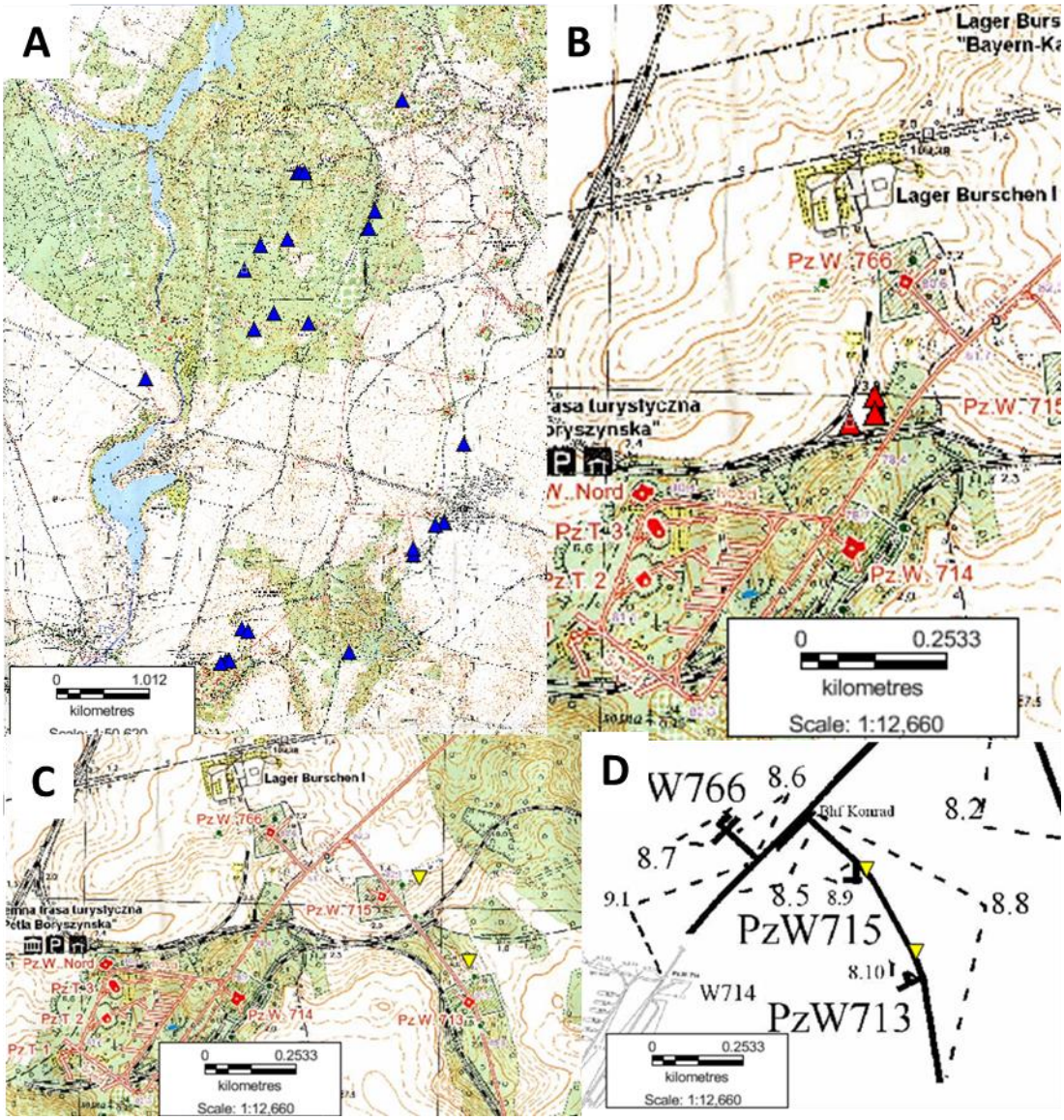


Figure 3.15 Location of non-marten scats. A=fox ▲ B=badger ▲ C=European polecat ▼ D=European polecat scat positions on a map of the tunnel system.

3.3.8 Male and female pine marten and stone marten scat DNA samples

3.3.8.1 Sex determination of marten DNA extracts

From screening of the C_T values (C_T <= 30.0) when using the TaqMan® MGB species probe based qPCR assay, 501 DNA extracts were selected for sex determination, again using TaqMan® MGB probe based qPCR. These consisted of 144 pine marten and 357 stone marten DNA extracts which accounted for 34.5% and 44.5% of the extracts identified to each species level respectively. Positive samples were defined as DNA extracts in which male or female DNA was detected with a C_T value <= 40.0 using the TaqMan® MGB probe based qPCR sex determination assay (Figure 3.16). Of the 501 samples assayed, 490 (97.8%) extracts were successfully identified to sex level (Table 3.10). This is an overall success rate of 40.3% of the initial 1216 extracts identified to species level (Table 3.11).

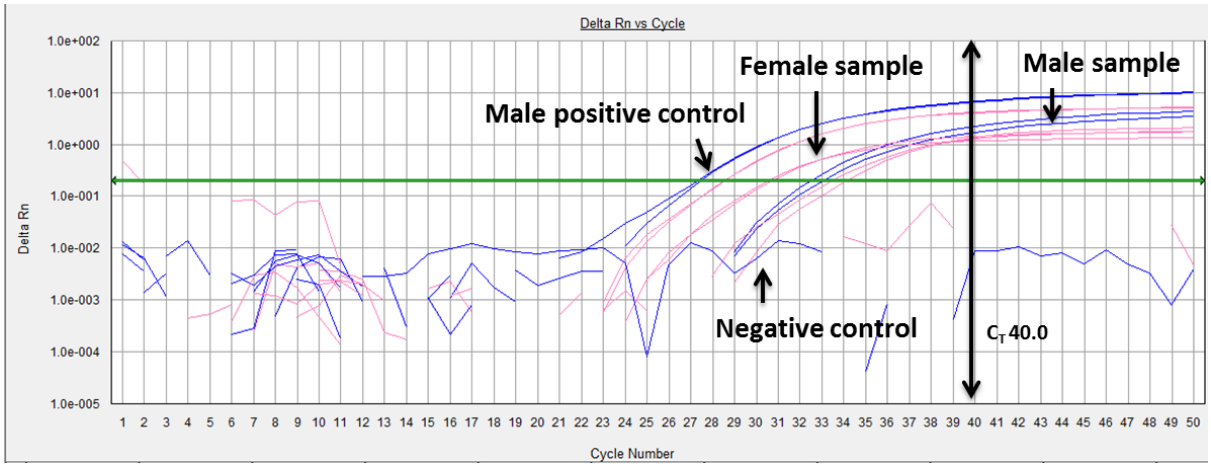


Figure 3.16 Sample plot from TaqMan® MGB probe based qPCR assay to determine sex from DNA extracts. Amplification of the MMX probe only, indicates a female while amplification of both MMX and MMY indicates a male. Double-headed arrow indicates cut-off for positive DNA extracts (C_T 40.0).

Table 3.10 Success rates of qPCR TaqMan® MGB probe based assay for sex determination of marten DNA extracts, PM=pine marten, SM=stone marten.

	PM (%)	SM (%)	Total (%)
Total	139/143 (97.2)	351/357 (98.3)	490/501 (97.8)
Above	88/89(98.8)	111/113 (98.2)	199/202 (98.5)
Below	51/54(94.4)	240/244 (98.3)	291/298 (97.6)

Overall success rates for individual species were 139/414 (33.5%) for pine marten and 351/802 (43.7%) for stone marten extracts. The range of C_T values obtained was 25.8-40.0 and 27.1-40.0 for pine marten and stone marten respectively. Overall success rates for each species and between scats gathered above and below ground varied from 51/223 (22.9%) for pine marten scats found in the tunnels to 111/229 (48.4%) for stone marten scats collected above ground. Successful determination to sex level was higher at 199/420 (47.3%) with scats collected above ground than in the tunnels at 291/796 (36%) (Table 3.11).

Table 3.11 Overall success rates of determining sex of marten species expressed as a percentage of the number of DNA extracts identified in total, by species, and by location (above or below ground). PM=pine marten, SM=stone marten.

	PM (%)	SM (%)	Total (%)
Total	139/414 (33.5)	351/802 (43.7)	490/1216 (40.29)
Above	88/191(46)	111/229(48.4)	199/420 (47.3)
Below	51/223(22.9)	240/573(41.8)	291/796 (36%)

3.3.8.2 Distribution of male and female marten scats

The location of each pine marten and stone marten scat collected that was successfully identified to sex level was plotted (Figure 3.17 and Figure 3.18). Scats from male and female pine marten and stone marten were successfully identified from above ground and in the tunnel system.

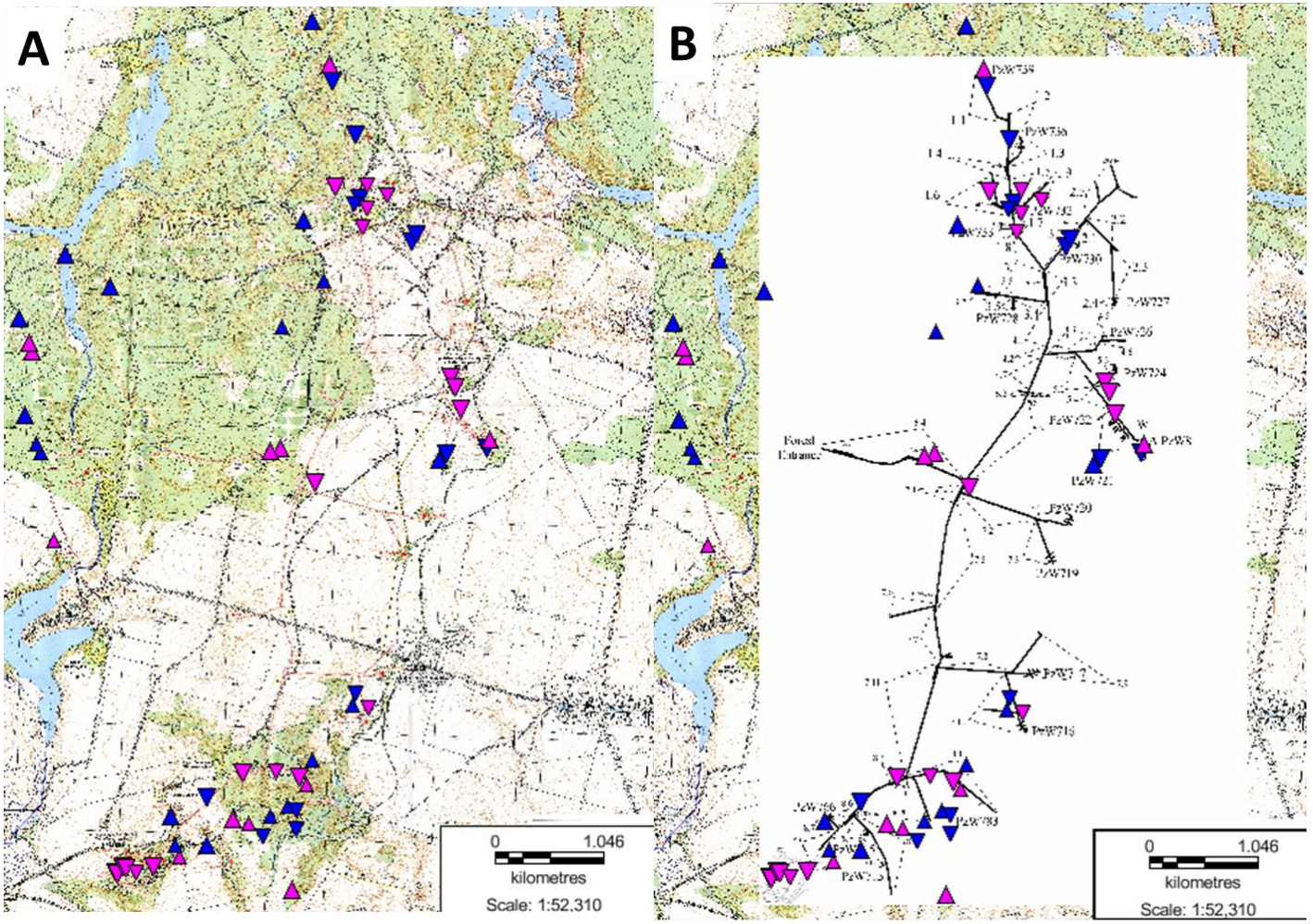


Figure 3.17 A=Location of all pine marten scats identified to sex and B= the same points with respect to the tunnel system. male ▲ female ▲ . Triangles are inverted to denote scats collected in the tunnels.

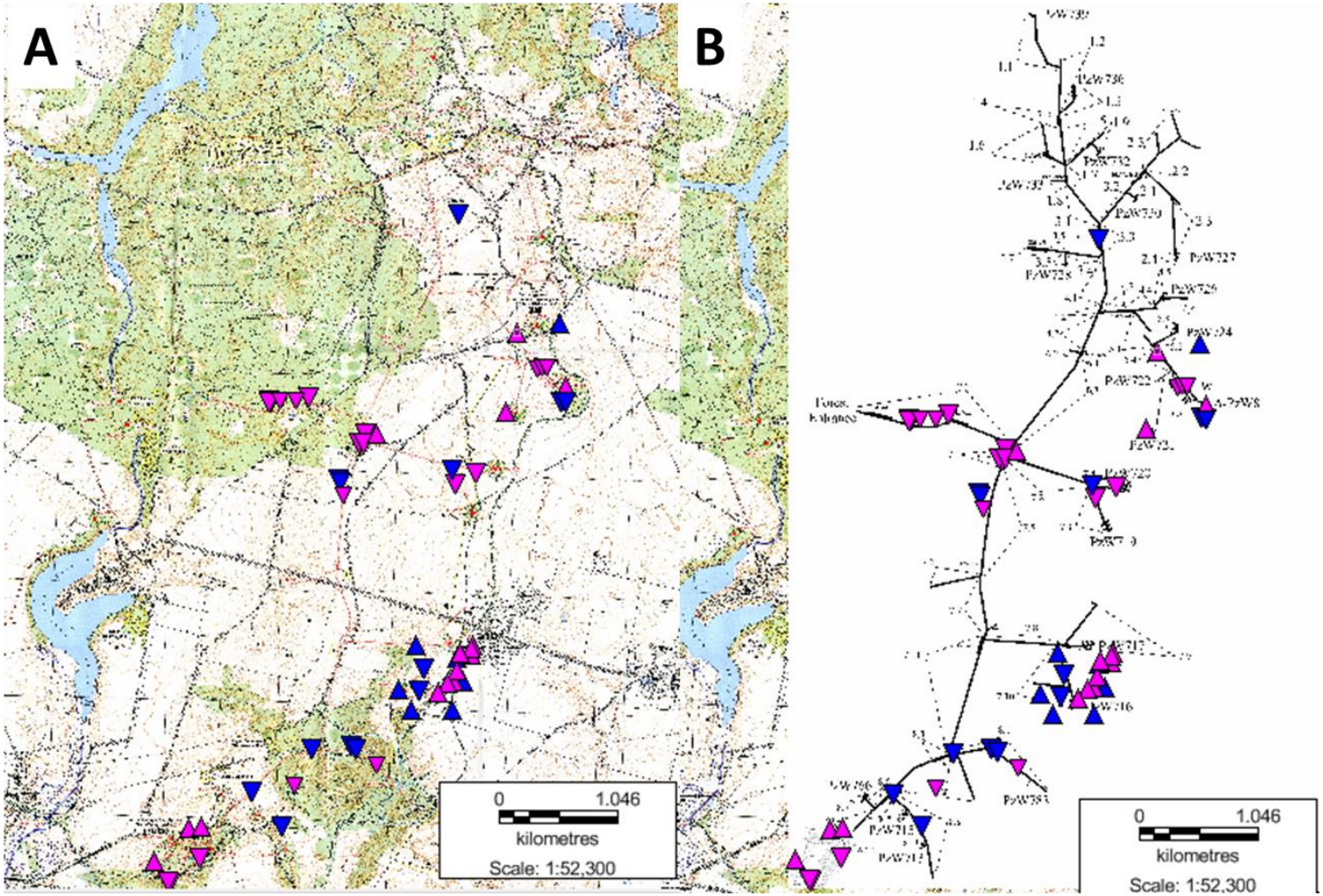


Figure 3.18 A=Location of all stone marten scats identified to sex and B=the same points with respect to the tunnel system. male ▲ female ▲ , Triangles are inverted to denote scats collected in the tunnels.

3.3.9 Microsatellite analysis of pine marten and stone marten scat DNA extracts

3.3.9.1 Selection of samples for genotyping

DNA extracts with MMX C_T values ranging from 25.8-34.6 (n=199) in the sex determination assay were selected for genotyping. Samples with C_T values above this were not analysed as it was found that amplification above this level was no longer reliable.

3.3.9.2 Screening for polymorphic loci

Any marker with inconsistent amplification was discarded. Unreliable or inconsistent amplification was defined as any DNA extract that failed to amplify at least 4 microsatellite markers. Similarly any marker which amplified consistently but showed no variation in the sample set was also discarded as these markers would not be informative for identification of individual animals. Table 3.12 contains a summary of the screening results for markers trialled during this project. Ten markers amplified consistently and with sufficient variability to be included in the final microsatellite panels. The remaining 8 markers were not selected for use either due to a lack of heterozygosity or a failure to amplify consistently.

Table 3.12 Summary of microsatellite screening results for markers trialled including markers not used for genotyping marten in this project

Result	n	primers
Consistent amplification of variable product of expected size	10	MEL1,Gg7,Mar-8,Mer041, Mvi1341,Mel105, Mf4.17, Mar-53, Gg454, Mar-43
Inconsistent amplification or amplification of product with no variation	8	Gg234,Ma2,Mvi1354,Mvis075, Mer022,Mf1.1, Mf3.7,Mf8.10

3.3.9.3 Microsatellite PCR optimisation

3.3.9.3.1 Multiplexing of microsatellite markers

Since combining loci in a single PCR significantly reduces the time and cost of microsatellite analysis, markers were trialled in multiplexes and amplifications compared with the results of single marker assays. Various combinations of markers were tried. Some markers amplified consistently in a multiplex while others did not. It was not practical to trial every possible combination of 8 markers for each marten specie. Four reactions were selected for each microsatellite panel. Two multiplexes and two single marker reactions were required for both panels (Table 3.13 and Table 3.14).

Table 3.13 Reactions used with panel of microsatellite primers to identify pine marten individuals from scat DNA.

Reaction	Marker(µM)	Marker(µM)	Marker(µM)	Marker(µM)	H_2O up to 200 µl (µl)
1	Mar43(0.25)	Mel105(0.25)	Mvi1341(0.3)	Mar-53 (0.3)	(156)
2	Mel1 (0.19)	Mer041(0.3)			(180)
3	Gg7(0.5)				(180)
4	Gg454(0.3)				(188)

Table 3.14 Reactions used with panel of microsatellite primers to identify stone marten individuals from scat DNA.

Reaction	Marker (µM)	Marker (µM)	Marker (µM)	H_2O up to 200 µl (µl)
1	Mar-53 (0.3)	Mvi1341 (0.3)	Mar-8 (0.3)	(161)
2	Mer041 (0.3)	Mel105(0.3)	Mel1(0.19)	(161)
3	Mf4.17 (0.25)			(190)
4	Gg7(0.5)			(180)

3.3.9.3.2 HotStart PCR enzyme trial

Of the four enzymes used to amplify three loci, HotStart PCR-to-Gel Taq PCR Master Mix (Amresco) gave the cleanest peaks consistently. The KAPPA2G (Kapa Biosystems) enzyme resulted in larger peaks which would be an advantage when using degraded DNA extracted from scat samples but the peaks were often staggered with only a single base between two peaks. As all three markers used are dinucleotide markers the minimum distance between two peaks of a heterozygous sample is two bases. The marker Mel1 amplified well with all four enzymes. This marker product is the shortest of the three. The markers with bigger products however did not amplify as successfully with the HotStart Plus (Qiagen) or the GoTaq HotStart (Promega). Background noise was evident when using all four enzymes. Given the consistent amplification, peak height, and clarity of peaks Amresco HotStart PCR-to-Gel Taq PCR Master Mix was selected for use in all further microsatellite analysis (Figure 3.19).

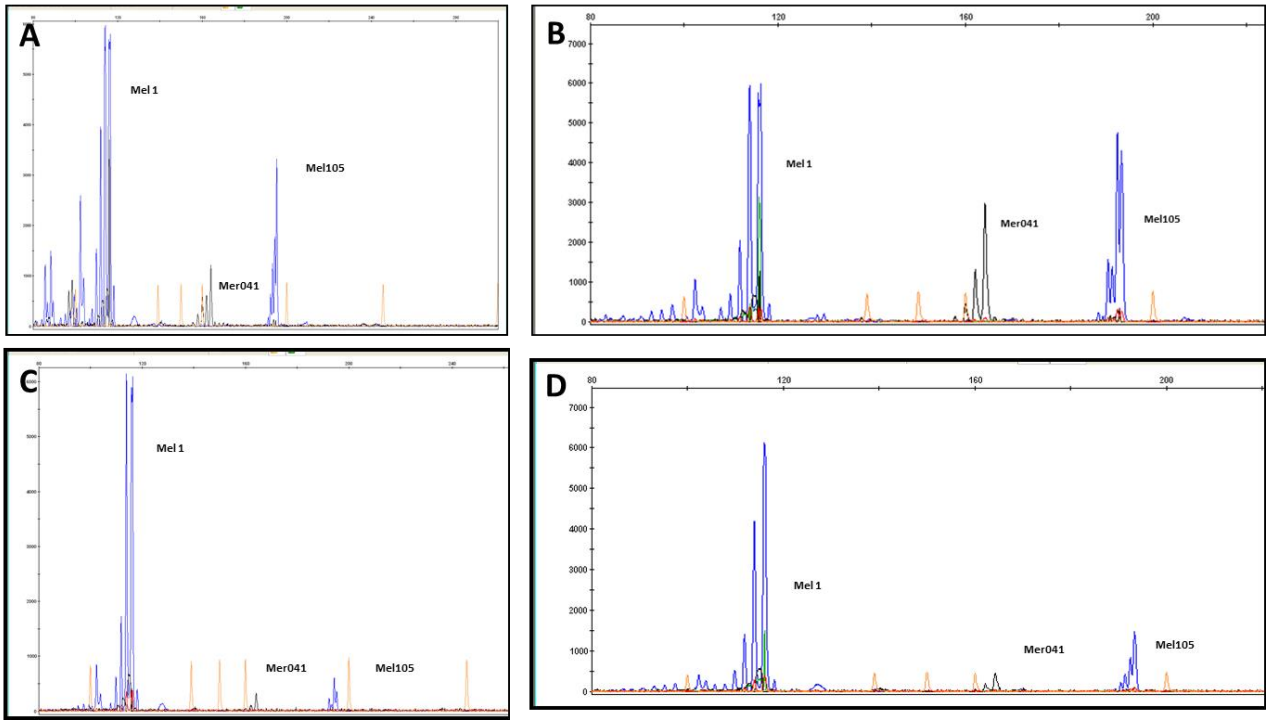


Figure 3.19 Sample plot of fragment analysis of a pine marten individual DNA sample using the loci; Mel1(FAM) Mer041(ATTO550) and Mel105 (FAM) amplified using four enzymes. A=Amresco HotStart PCR-to-Gel Taq PCR Master Mix, B= Kapa2GHS, C= Hotstart Taq Plus, D= GoTaq HotStart.

3.3.9.4 Genotyping of marten scat DNA extracts

3.3.9.4.1 Microsatellite analysis

Hair DNA extracts with MMX C_T values less than or equal to 34.0 were selected for genotyping (n=199). Of these 199 DNA extracts, 85 were from pine marten scats and 114 were from stone marten scats. Successful amplification of all 8 loci from either panel of microsatellite markers was achieved for 95 of the 199 DNA extracts. The 85 pine marten DNA extracts resulted in 45 (52.9%) successful amplifications of all 8 loci with 20 distinct genotypes (Table 3.15). From the 114 stone marten DNA extracts assayed 50 (43.8%) were successfully amplified across all 8 loci representing 21 distinct genotypes (Table 3.16) The overall success rate for scat DNA extracts selected for genotyping was 47.7%. Statistical analysis was performed on the genotyping results using GenAlex to assess the power of both these microsatellite panels to identify individual animals in the pine marten and stone marten populations in Nietoperek.

Table 3.15 Multi-locus genotypes of pine marten in Nietoperek identified from scat DNA extracts; sizes are alleles in base pairs. n= no of alleles found in this population for each marker.

DNA no		Marker	Sex	MEL1		Gg454		Gg7		Mar-43		Mer041		Mvi1341		Mel105l		Mar-53	
		ID / n		4		5		7		6		5		6		6		7	
JP210912	110	P1	F	110	116	128	128	132	144	156	160	161	165	172	172	191	195	246	246
JP170513	20	P2	F	112	116	128	130	136	146	154	160	161	165	168	176	193	197	234	244
JP170513	10	P2	F	112	116	128	130	136	146	154	160	161	165	168	176	193	197	234	244
JP220513	19	P3	F	116	116	128	134	134	142	160	162	161	165	168	176	199	201	242	244
JP210912	23	P4	F	110	116	132	134	134	142	150	160	159	163	168	170	195	195	242	244
JP061112	5	P5	F	110	116	128	130	142	144	160	162	159	165	170	172	197	197	234	236
JP160113	15	P5	F	110	116	128	130	142	144	160	162	159	165	170	172	197	197	234	236
JP270514	56	P5	F	110	116	128	130	142	144	160	162	159	165	170	172	197	197	234	236
JP210912	30	P6	F	112	116	126	126	134	144	150	164	159	163	172	172	195	201	240	246
JP061112	19	P6	F	112	116	126	126	134	144	150	164	159	163	172	172	195	201	240	246
JP200513	25	P6	F	112	116	126	126	134	144	150	164	159	163	172	172	195	201	240	246
JP200513	28	P6	F	112	116	126	126	134	144	150	164	159	163	172	172	195	201	240	246
JP210513	33	P6	F	112	116	126	126	134	144	150	164	159	163	172	172	195	201	240	246
JP180113	97	P7	F	110	116	128	128	132	144	160	162	161	165	172	172	191	197	246	246
JP210912	40	P8	F	112	116	128	134	138	144	154	160	159	167	176	176	195	197	246	246
JP210912	19	P9	F	110	116	128	134	132	136	160	162	161	163	168	170	193	193	236	242
JP030811	6	P10	F	110	116	128	134	138	144	154	160	159	163	176	176	195	195	246	246
JP160113	13	P10	F	110	116	128	134	138	144	154	160	159	163	176	176	195	195	246	246
JP160113	21	P10	F	110	116	128	134	138	144	154	160	159	163	176	176	195	195	246	246
JP030811	30	P10	F	110	116	128	134	138	144	154	160	159	163	176	176	195	195	246	246
JP210513	69	P11	F	114	116	126	126	134	142	150	164	159	165	172	172	195	201	240	246
JP030811	5	P12	M	116	116	128	134	134	142	150	154	161	165	168	170	195	199	242	244
CP030112	15	P12	M	116	116	128	134	134	142	150	154	161	165	168	170	195	199	242	244
CP030112	29	P12	M	116	116	128	134	134	142	150	154	161	165	168	170	195	199	242	244
JP220513	21	P12	M	116	116	128	134	134	142	150	154	161	165	168	170	195	199	242	244
JP210912	26	P13	M	116	116	128	134	136	142	160	162	161	165	166	174	199	201	242	242
JP210912	22	P13	M	116	116	128	134	136	142	160	162	161	165	166	174	199	201	242	242
JP210912	88	P14	M	116	116	130	130	134	144	150	150	165	165	168	176	193	195	238	240
JP210912	21	P14	M	116	116	130	130	134	144	150	150	165	165	168	176	193	195	238	240
JP030811	36	P15	M	114	116	132	132	138	142	150	154	163	165	172	172	191	193	242	242
JP210513	18	P15	M	114	116	132	132	138	142	150	154	163	165	172	172	191	193	242	242
JP130913	9	P15	M	114	116	132	132	138	142	150	154	163	165	172	172	191	193	242	242
JP130913	6	P16	F	114	116	128	132	142	142	150	154	159	159	168	172	191	201	240	242
JP130913	13	P16	F	114	116	128	132	142	142	150	154	159	159	168	172	191	201	240	242
JP130913	1	P16	F	114	116	128	132	142	142	150	154	159	159	168	172	191	201	240	242
JP270514	21	P16	F	114	116	128	132	142	142	150	154	159	159	168	172	191	201	240	242
JP210513	23	P17	M	112	116	128	134	132	136	150	160	163	165	172	172	193	195	234	236
JP120913	41	P18	M	110	116	128	130	132	144	156	160	161	165	172	172	191	195	244	246
JP130913	4	P18	M	110	116	128	130	132	144	156	160	161	165	172	172	191	195	244	246
JP130913	7	P18	M	110	116	128	130	132	144	156	160	161	165	172	172	191	195	244	246
JP270514	33	P18	M	110	116	128	130	132	144	156	160	161	165	172	172	191	195	244	246
JP280514	41	P19	M	116	116	128	130	136	142	160	162	161	165	166	174	199	201	242	242
JP160113	7	P20	M	114	116	132	132	134	142	150	154	163	165	168	172	191	193	240	242
JP170113	2	P20	M	114	116	132	132	134	142	150	154	163	165	168	172	191	193	240	242
JP280514	40	P20	M	114	116	132	132	134	142	150	154	163	165	168	172	191	193	240	242

Table 3.16 Multi-locus genotypes of stone marten in Nietoperek identified from scat DNA extracts; sizes are alleles in base pairs. n= no of alleles found in this population for each marker.

DNA no		Marker	Sex	MEL1		Gg7		Mar08		Mer041		Mvi1341		Mel105l		Mf4.17		Mar53	
		ID / n		4		4		5		4		4		5		4		4	
JP170113	55	S1	F	112	116	136	140	146	148	165	165	168	168	193	195	217	217	236	238
JP160513	24	S2	F	106	116	136	140	146	152	165	165	168	170	191	195	205	213	238	238
JP160513	13	S2	F	106	116	136	140	146	152	165	165	168	170	191	195	205	213	238	238
JP160513	8	S3	F	116	116	136	140	148	148	165	165	168	170	193	195	213	217	238	240
JP160513	10	S3	F	116	116	136	140	148	148	165	165	168	170	193	195	213	217	238	240
JP160513	12	S3	F	116	116	136	140	148	148	165	165	168	170	193	195	213	217	238	240
JP160513	3	S4	M	112	116	132	136	146	152	159	161	170	170	195	197	209	217	238	238
JP170513	28	S4	M	112	116	132	136	146	152	159	161	170	170	195	197	209	217	238	238
JP170913	67	S4	M	112	116	132	136	146	152	159	161	170	170	195	197	209	217	238	238
JP290514	43	S5	F	116	116	136	140	146	148	161	165	170	170	193	195	209	217	240	242
JP170113	44	S6	F	112	116	136	140	148	148	161	165	170	170	193	195	209	217	240	242
CP030112	37	S7	M	114	116	136	136	152	154	161	165	170	170	193	193	213	217	242	242
JP170913	73	S7	M	114	116	136	136	152	154	161	165	170	170	193	193	213	217	242	242
JP170913	56	S7	M	114	116	136	136	152	154	161	165	170	170	193	193	213	217	242	242
JP170113	17	S8	M	112	116	136	136	146	152	163	165	170	172	197	197	213	217	238	240
JP170113	50	S8	M	112	116	136	136	146	152	163	165	170	172	197	197	213	217	238	240
JP170913	63	S8	M	112	116	136	136	146	152	163	165	170	172	197	197	213	217	238	240
JP170913	37	S8	M	112	116	136	136	146	152	163	165	170	172	197	197	213	217	238	240
JP170513	46	S9	F	116	116	136	140	152	152	165	165	170	170	193	195	209	217	238	240
JP170513	37	S9	F	116	116	136	140	152	152	165	165	170	170	193	195	209	217	238	240
JP170513	40	S9	F	116	116	136	140	152	152	165	165	170	170	193	195	209	217	238	240
JP030811	22	S10	F	116	116	136	136	152	152	165	165	170	172	193	195	209	217	238	240
JP180113	92	S11	M	112	116	136	136	146	152	161	163	168	170	193	195	213	213	242	242
JP180913	45	S12	M	116	116	136	136	146	148	161	165	168	170	193	195	217	217	240	240
JP260514	23	S12	M	116	116	136	136	146	148	161	165	168	170	193	195	217	217	240	240
JP260514	2	S12	M	116	116	136	136	146	148	161	165	168	170	193	195	217	217	240	240
JP300514	13	S12	M	116	116	136	136	146	148	161	165	168	170	193	195	217	217	240	240
CP030112	17	S13	F	112	116	136	140	148	148	165	165	168	170	193	195	217	217	238	240
JP210912	82	S13	F	112	116	136	140	148	148	165	165	168	170	193	195	217	217	238	240
JP210912	57	S13	F	112	116	136	140	148	148	165	165	168	170	193	195	217	217	238	240
JP180913	8	S14	M	112	116	136	142	148	148	165	165	168	168	193	195	209	217	238	242
JP200513	24	S15	M	112	116	136	142	146	152	165	165	170	172	193	201	209	213	236	236
CP040112	11	S16	F	116	116	136	136	146	150	161	165	172	178	193	195	213	213	238	238
JP030811	35	S16	F	116	116	136	136	146	150	161	165	172	178	193	195	213	213	238	238
JP170113	43	S16	F	116	116	136	136	146	150	161	165	172	178	193	195	213	213	238	238
CP040112	22	S17	M	116	116	136	136	152	152	161	165	170	170	195	201	209	213	238	240
JP210912	87	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP210912	10	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP170513	68	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP220513	51	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP220513	20	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP270514	1	S18	M	112	116	136	136	146	146	159	165	170	170	193	193	213	213	238	242
JP290514	34	S19	M	112	116	136	136	148	148	161	165	170	172	193	195	213	213	240	240
JP270514	30	S19	M	112	116	136	136	148	148	161	165	170	172	193	195	213	213	240	240
JP270514	7	S19	M	112	116	136	136	148	148	161	165	170	172	193	195	213	213	240	240
JP270514	10	S19	M	112	116	136	136	148	148	161	165	170	172	193	195	213	213	240	240
JP290514	41	S20	F	114	116	136	140	152	154	161	165	170	170	193	195	213	217	242	242
JP270514	66	S20	F	114	116	136	140	152	154	161	165	170	170	193	195	213	217	242	242
JP170113	47	S20	F	114	116	136	140	152	154	161	165	170	170	193	195	213	217	242	242
JP180913	88	S21	M	106	116	136	140	148	148	165	165	168	170	193	195	205	213	238	238

3.3.9.4.2 Descriptive statistics

Once individual genotypes had been identified, the probability of identity (PI) statistics were calculated using the GenAlEx 6.5 to assess the power of each panel of microsatellite loci to

identify individual animals (PI), including related animals (PIsibs), from DNA extracted from hair samples collected in Nietoperek. PI was obtained for increasing numbers of loci. Twenty one distinct stone marten genotypes were detected. Each distinct genotype can be attributed to an individual animal (P<0.001) (Figure 3.20) with this panel of 8 loci yielding a cumulative PID of 7.5E-06 and a cumulative PIsibs of 4.9E-03. Twenty distinct pine marten genotypes were detected. Each distinct genotype can be attributed to an individual animal (P< 0.001) (Figure 3.21), with this panel of 8 loci yielding a cumulative PID of 6.6E-09 and a cumulative PIsibs of 6.6E-04 (GenAlEx 6.5).

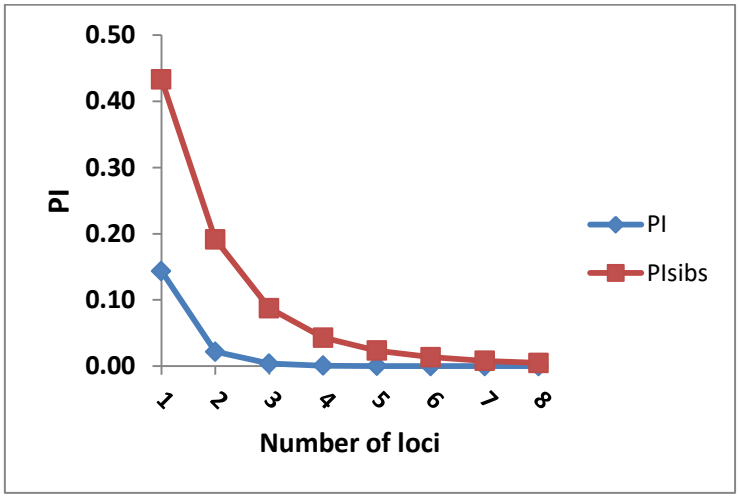


Figure 3.20 Probability of Identity (PI) for each locus and for increasing combinations of the 8 loci used to identify stone marten individuals.

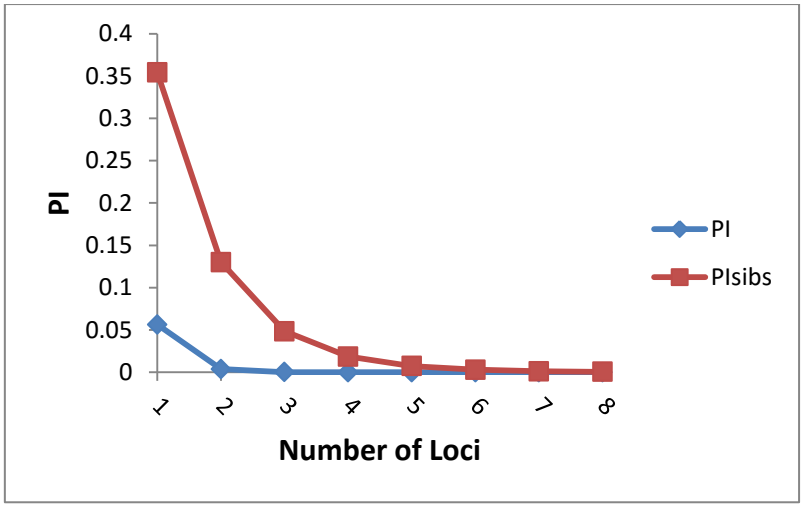


Figure 3.21 Probability of Identity (PI) for each locus and for increasing combinations of the 8 loci used to identify pine marten individuals.

For each panel of markers H_E was calculated for each locus. Loci were entered into GenAlex in order of decreasing number of alleles present (Table 3.17 & Table 3.18). H_O is comparable to H_E at all markers for both panels of markers. The stone marten population did not deviate from HWE at 6 markers. There was deviation from HWE at Mel105 ($P<0.05$) and Mf4.17 ($P<0.05$). The pine marten population in Nietoperek did not deviate from HWE at 5 markers. However there was deviation from HWE at Mar53($P<0.05$), Mar43($P<0.05$) and Gg454 ($P<0.05$). While departure from Hardy-Weinberg equilibrium (DHW) in a population can be an indication of inbreeding or genotyping errors in analysis, DHW due to a small sample size can be expected (Wittke-Thompson *et al*. 2005).

Table 3.17 Descriptive statistics for stone marten panel of microsatellite loci in order of decreasing number of alleles. N=number of individuals, Na= number of alleles, Ho= observed heterozygosity, He= expected heterozygosity. Chi-square test (P = 0.965)

Marker	N	Na	Ho	He
Mar-08	21	5	0.524	0.705
Mel105	21	5	0.857	0.624
Mar-53	21	4	0.524	0.693
Mf4.17	21	4	0.667	0.671
Mvi1341	21	4	0.524	0.545
Mel1	21	4	0.667	0.494
Mer041	21	4	0.571	0.494
Gg7	21	4	0.571	0.441

Table 3.18 Descriptive statistics of pine marten panel of microsatellite loci in order of decreasing number of alleles. N=number of individuals, Na= number of alleles, Ho= observed heterozygosity, He= expected heterozygosity. Chi-square test (P = 0.984)

Marker	N	Na	Ho	He
Gg7	20	7	0.95	0.82
Mar-53	20	7	0.65	0.791
Mel105l	20	6	0.8	0.799
Mar-43	20	6	0.95	0.778
Mvi1341	20	6	0.55	0.734
Gg454	20	5	0.65	0.745
Mer041	20	5	0.9	0.738
Mel1	20	4	0.75	0.559

3.3.10 Distribution of scats from individual pine marten and stone marten throughout Nietoperek

Figure 3.22 shows the distribution of scats collected which were successfully identified as being from individual stone marten. Of the 21 stone marten individuals detected throughout the survey site across all surveys, 12 animals were detected at least twice. Three stone marten individuals were detected both above ground and in the tunnels. The remaining animals that were detected at least twice comprise of 2 animals detected only above ground and 7 animals only detected in scats collected in the tunnels.

Figure 3.23 shows the distribution of scats collected which were successfully identified as being from individual pine marten. Of the 20 pine marten individuals detected throughout the survey site and across all surveys, 11 animals were detected at least twice. Eight individuals were detected both above and below ground and in the tunnels. Two individual pine marten were only ever detected above ground and DNA from one individual was only found in scats collected in the tunnels.

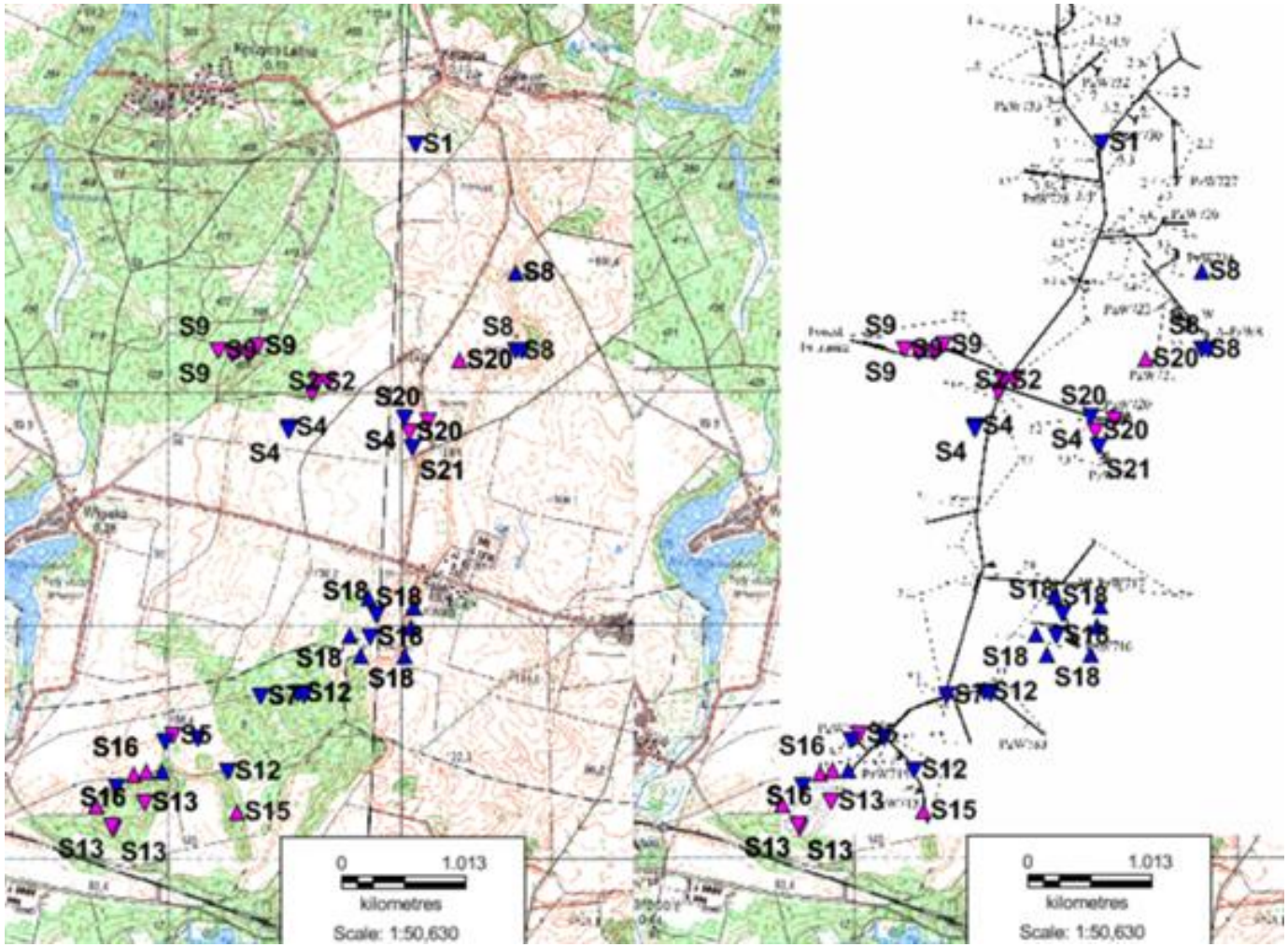


Figure 3.22 Distribution of scats collected from individual stone marten as identified by microsatellite analysis. S=stone marten male ▲ female ▲ ;inverted triangle denotes a scat collected in the tunnels.

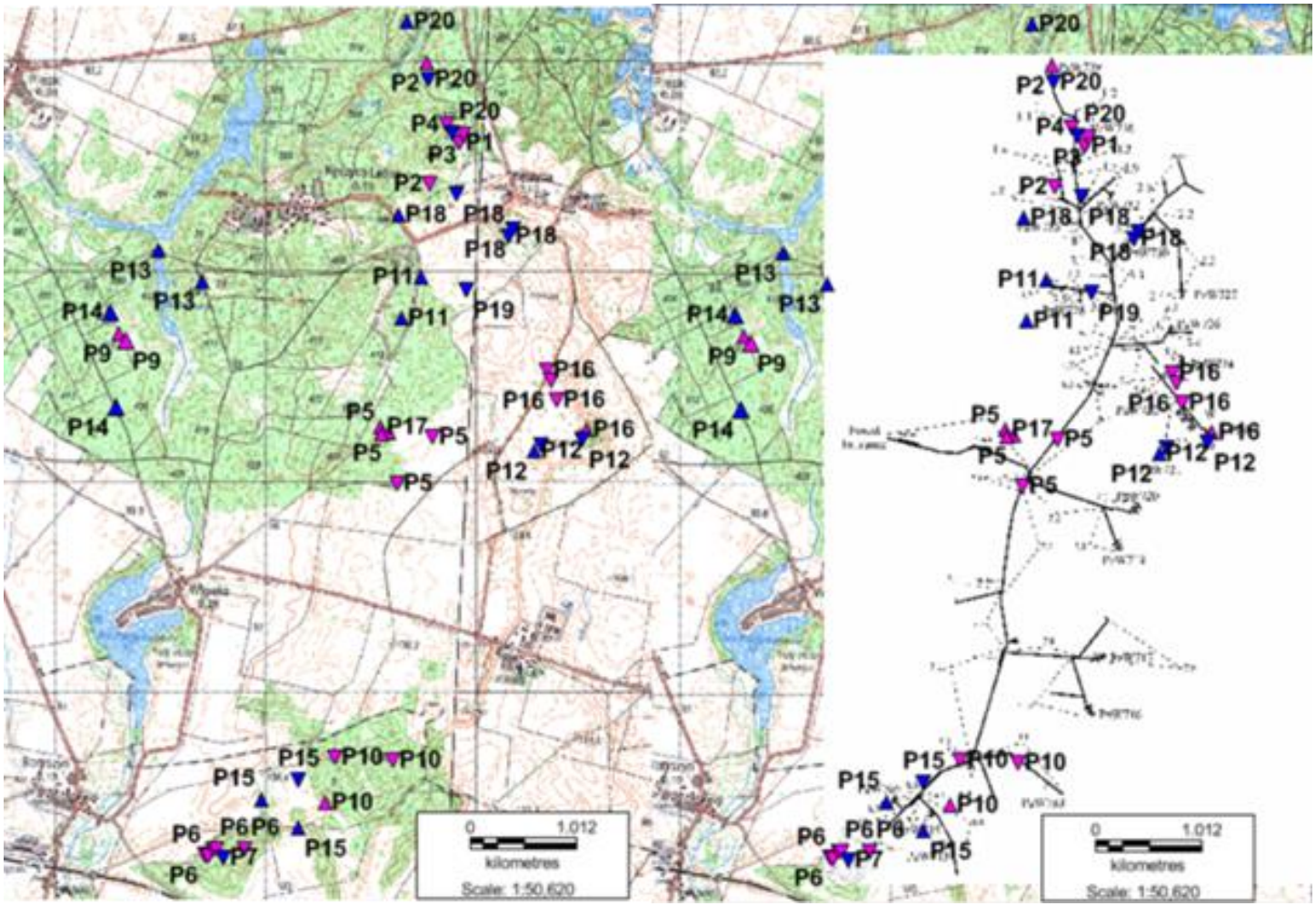


Figure 3.23 Distribution of scats collected from individual pine marten as identified by microsatellite analysis; P=pine marten, male ▲ female, ▲ ;inverted triangle denotes a scat collected in the tunnels.

3.3.11 Haplotype analysis

Scat DNA extracts identified as being from individual pine marten and stone marten were amplified targeting a 451 base region of d-loop mtDNA. From the gel image in Figure 3.24, all pine marten and stone marten DNA extracts trialled were amplified successfully. One Scat DNA extract from each identified individual was therefore selected for sequencing over this region.

Figure 3.24 Gel of PM-FOR, DL575R amplification of DNA extracts. Wells 1 and 20. 100 base pair ladder (product 451 bases); Wells 2-7 stone marten(S1-S6) DNA extracts; Wells 8-14 pine marten DNA extracts(P1-P6); Well 15-empty;Wells 16 and 17 negative controls; Wells 16 and 17 negative controls; Wells 18 and 19 positive controls.

Sequencing was performed in one direction using the DL575R primer. 380 bases of sequence were obtained from marten DNA extracts. Sequences were analysed using Sequence Scanner Software v2 (Life Technologies, 2012). Ambiguous base calls or low peaks at the beginning of each sequence were removed and a sequence of 350 bases remained. All the pine marten mtDNA d-loop sequences obtained from DNA extracts from Nietoperek were identical. Across the regions where the sequences in this study overlap sequences (300 bases) from studies on pine marten phylogeography it was found that the pine marten in Nietoperek belong to the haplogroup 1a (Davison, *et al*. 2001) and the sequence is identical over this region to haplotype " Hap b" from the same study (Figure 3.25).

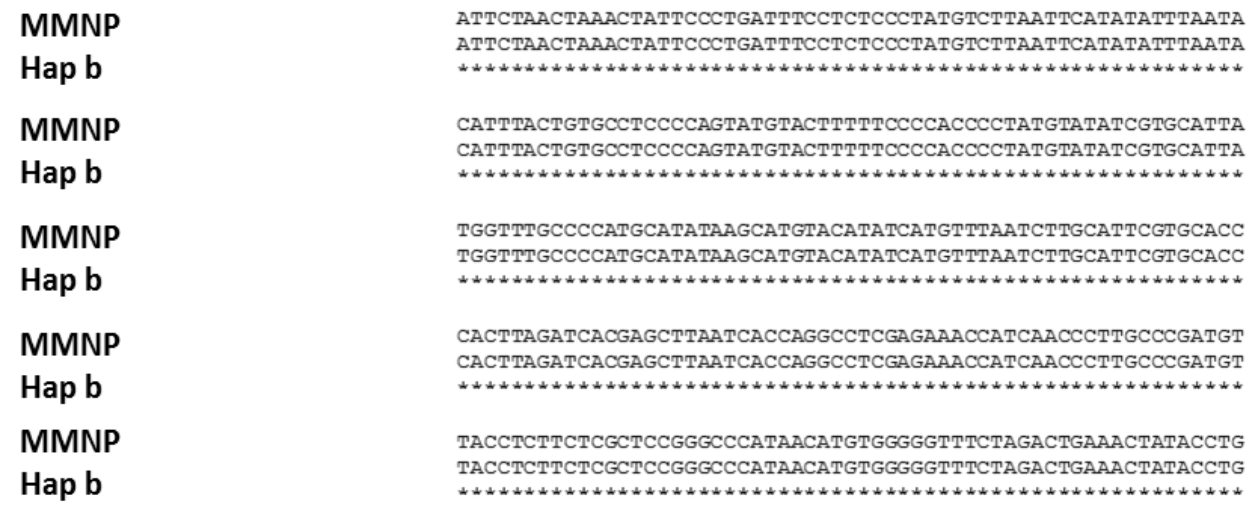


Figure 3.25 Mitochondrial d-loop pairwise alignment in Clustal W2 of pine marten sequence from Nietoperek (MMNP) and haplotype "Hap b" sequence from Davison *et al*.,(2001).

All the stone marten mtDNA d-loop sequences obtained from DNA extracts from Nietoperek were also identical. However the sequence was not identical to any available mitochondrial d-loop stone marten sequences. The overlapping region was 218 bases. When sequences were aligned it was found that there was a single mismatch between Nietoperek, Poland, stone marten sequences and sequences from stone marten found in both Greece and Bulgaria. This sequence therefore represents a new haplotype different to all available sequences as shown in Figure 3.26.

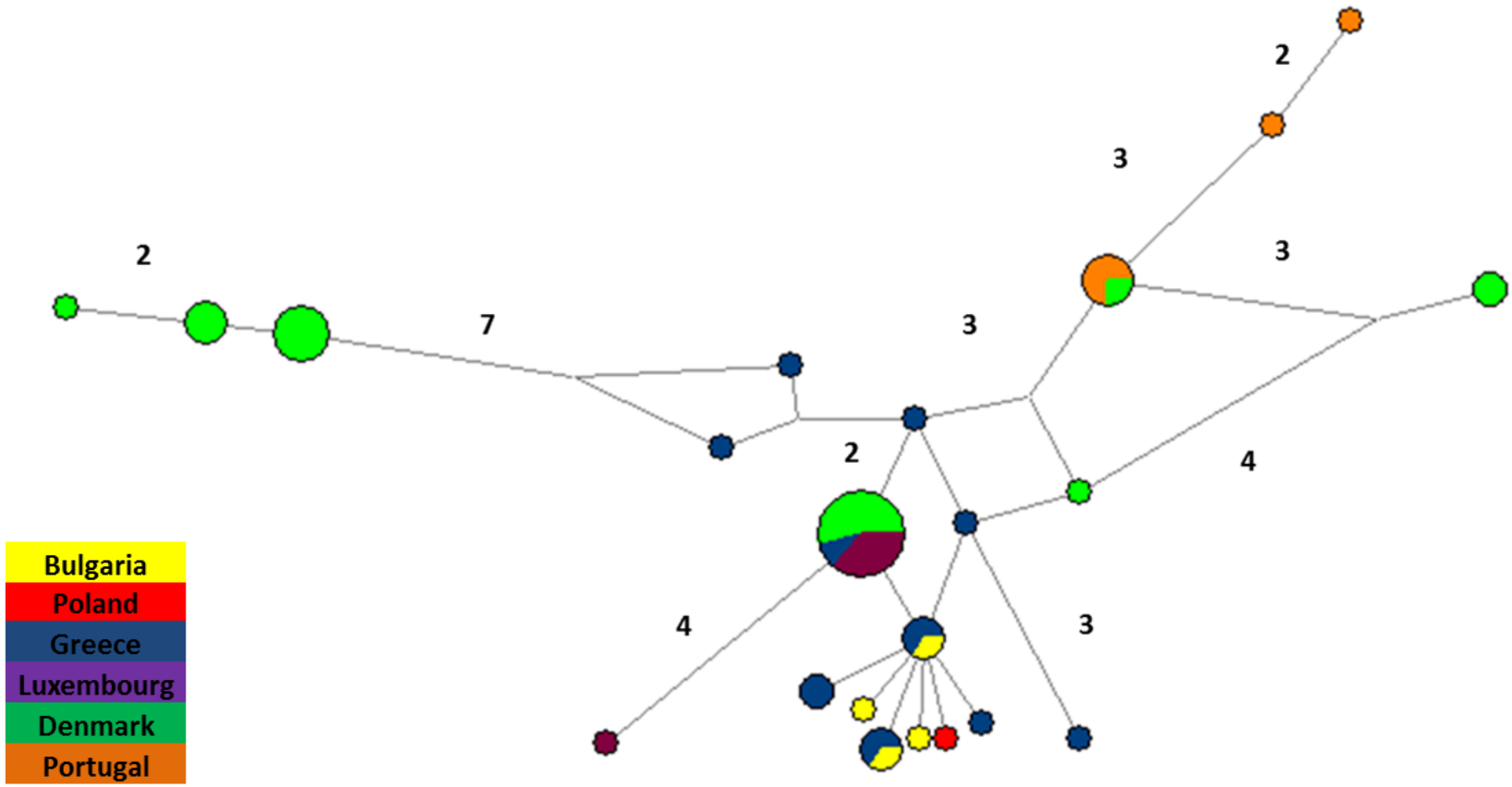


Figure 3.26 Median Joining Network constructed from stone marten d-loop sequences (218 bases) in Network 4.6.

Haplotypes differ by a single nucleotide except where shown. Individual sequences have been colour coded by country of origin.

3.4 Discussion

3.4.1 Scat surveys

Seven scat surveys were carried out during this project which covered the entire survey area both above and below ground. The survey period spanned three years from September 2012 to May 2014. More than 300 scats from each of these calendar years were collected suggesting a sufficient sample size for scat population studies.

Of the 1785 scats collected 659 (36.9 %) were collected above ground while 1126 (63.1%) were collected in the tunnels. 30 samples were collected in the tunnels which on closer examination were not scats. The above ground portion of the survey area was sampled along forest tracks and roads. However, the area between these tracks was not surveyed and marten scats deposited between tracks will go undetected. In the tunnels which are linear, there is no alternative area in which a marten may be depositing scats and so it is possible that a higher proportion of the total scat population of the tunnels was collected. It is also possible that tourist pressure may lead marten to avoid busy pathways above ground when depositing scats.

3.4.2 Molecular analysis of scat DNA extracts collected in Nietoperek

3.4.2.1 Identification of pine marten and stone marten in scat DNA extracts

Of the 1785 scats assayed using the TaqMan® MGB probe based qPCR assay to detect pine marten and stone marten 1216 scat DNA extracts were positive; 414 pine marten scats (34%) and 802 stone marten scats (66%) (Table 3.19). The average C_T value obtained for both species was similar for pine marten and stone marten DNA extracts at 27.4 +/- 2.03 and 26.89 +/- 2.44 respectively. It was originally assumed that scats collected in the tunnels may contain DNA that was more degraded than those found on the surface. There was a difference in the quality found above and below ground but it was not as pronounced as expected with an average C_T values above and below ground 27.06 (+/- 1.63) and 28.89 (+/- 1.44) respectively. The difference in DNA quality is more pronounced in pine marten scats collected in the tunnels when analysed alone with only 54 of the 223 pine marten scats collected in the tunnel having a C_T value <=30.0 and an average C_T value of 30.05 (+/- 2.07).

Table 3.19 Summary of results from the detection of pine marten and stone marten scat DNA using TaqMan® MGB probe based qPCR.

Species	Pine marten	Stone marten	Negative
Total n=1785	414	802	569
Above ground	191	229	267
Tunnels	223	573	332

3.4.2.2 Sex determination of marten DNA extracts

Of the 501 scat DNA extracts with a C_T value less than 30.0 in the species assay 490 extracts were successfully sex typed. The sex determination assay worked equally well with pine marten and stone marten DNA extracts and also with DNA extracts from scats collected both above ground and in the tunnels with the success rate ranging from 94.4% for pine marten scats found in the tunnels to 98.8% for pine marten scats collected above ground. However the proportion of scats selected for sex typing, both from each species and from above and below ground, varied considerably from 48.4 % of stone marten scats found above ground to only 22.9% of pine marten scats collected in the tunnels (Table 3.11). It may be that at least some of the scats identified as being from pine marten had remained in the tunnels for a long period of time before collection and this may have contributed to a lower DNA quality.

3.4.2.3 Microsatellite analysis of pine marten and stone marten scat DNA extracts

With non-invasively collected samples there is inevitably a degree of degradation of the DNA. Scat samples in particular may be degraded but they also may contain PCR inhibitors in the form of plant polysaccharides, for example, depending on the diet of the animal. Every effort must therefore be made to optimise methodologies when dealing with scat DNA extracts. Four different enzymes were trialled in the amplification of the microsatellite panels. Hot Start PCR-to-Gel Taq PCR Master Mix 2X (Amresco) was selected on the basis of having the least background noise, showing consistent amplification, peak height, and clarity of peaks and being the most cost effective of the 4 enzymes trialled.

Through the screening of MMX C_T values 199 scat DNA extracts were selected for microsatellite analysis. Of these 199 scat DNA extracts 95 were successfully amplified across

all loci giving a success rate of 47% of all samples selected for genotyping. The results identified 41 individual animals; 20 pine marten and 21 stone marten (Table 3.15 and Table 3.16). The 47% success rate with genotyping scat DNA from Nietoperek is considerably lower than the success rate obtained when genotyping DNA extracted from hair samples from the control study in Corbally Wood where 92% of the samples selected through the same screening process were successfully amplified at all loci (section 2.4.2.5.2).

The 20 pine marten individuals are represented by 45 scat DNA extracts, while the 21 stone marten individuals are represented by 50 stone marten scat DNA extracts. While these figures are similar it is worth noting that the 50 stone marten scat DNA extracts represent 6.2 % of the total number of stone marten scats collected while 45 pine marten scats represent 10.8% of the total number of pine marten scats collected. During the species qPCR assay pine marten scat DNA extracts had a poorer average C_T value only when found in the tunnels and the average C_T value for both species was similar. The higher proportion of pine marten scat DNA extracts yielding full genotypes may be due to differences in efficiency in the loci used in the two different panels of markers. Any future work may consider a comparison of the efficiency of both panels of microsatellite markers.

3.4.3 Scat densities throughout the survey site

3.4.3.1 Above ground surveys

The number of scats collected above ground varied considerably between surveys (Table 3.8). With above ground scat surveys there are variables that must be taken into consideration. Volunteer numbers and degree of expertise varied slightly between surveys. Initial surveys also did not cover the entire above ground survey site so there is some variation in sampling effort. Overhead conditions during scat collection may also have been a factor with heavy rain on some days of some surveys. Conditions underfoot also changed during the surveys. For example the understory was much more developed in spring than in winter and during the autumn surveys fallen leaves on the forest tracks may have covered scats and made it more difficult for volunteers to locate them (Davison *et al*. 2002). The pattern of scat deposition may also change with habitat characteristics (Sadlier *et al.* 2004). In other species it has also been found that scat deposition rates are influenced, not only by season, but also by diet (Andelt, 1984) with scat deposition rates increasing when an animals diet contains a lot of fruit.

Scat density also varies from season to season and Velander (1986) found there could be a 100 fold higher scat density on forest trails in July than in January. This variation in scat denity appears to be confirmed in this study with scat density above ground of 2.48 / km in May 2014 compared with only 0.48 / km collected in January 2013. The difference in scat densities is not as pronounced in this study but this survey site is relatively small in comparison to Velander's who incorporated multiple studies.

The above ground pine marten scat density varied considerably with survey and section of survey area. Most pine marten scats were found in the Southern Forest throughout surveys with approximately 150 pine marten scats collected in the 3.55 km^2 Southern Forest section (Figure 3.13 A). Few pine marten scats were collected in the Middle Section as would be expected due to the habitat and relatively few scats collected in the North Forest. This may have been due to open understory but also may have been a product of survey effort as the Southern Forest was surveyed more often than the Northern Forest as most of the Northern Forest is not above the tunnel system. Survey success varied with 28 pine marten scats collected in September 2012, 36 in May 2013 and 33 collected in September 2013. However the number of scats collected above ground in January 2013 that could be identified as pine marten was only 6 scats. This may be due to a decrease in above ground scat density during winter (Birks *et al.* 2004) but during this survey heavy snow cover severely limited the above ground survey effort.

The above ground stone marten scat density also varied considerably with survey period. 233 stone marten scats were collected above ground (Figure 3.13 B). The above ground survey area consisted of approximaltley 20 km^2 which gives an average above ground stone marten scat density of 11.5 scats / km^2. However, scat distribution was patchy and most stone marten scats were either collected in the Southern Forest or the Middle Section. While pine marten scats were mostly collected within the forested areas the stone marten scats were largely found along the edges of these forested areas. Stone marten scats were found throughout the Middle Section along tracks and at bunkers which contrasts with the pine marten scats distribution in this section which was mainly limited to the small copses in, and around bunker locations possibly due to the paucity of tree cover in much of this section. Survey success also varied for stone marten scats. 15 stone marten scats were collected above ground in September 2012 while 52 were collected the following September. However, the September 2012 survey only covered the forested areas and did not cover the Middle Section so this may account for the difference in numbers of scats collected. Again in January 2013

only 8 scats were found above ground that could be identified as being from a stone marten and the reasons for this may be as discussed for the pine marten scats.

3.4.3.2 Below ground surveys

From January 2013 onwards any survey underground covered the entire system. Some of the variables present in the above ground surveys do not have an effect on the below ground surveys. For example, overhead conditions were not a problem and underfoot conditions did not vary from season to season except in a small number of sub-sections which were prone to flooding. While scats that have been in the tunnels for some time may vary morphologically from above ground scats the linear nature of this portion of the survey site makes it easier for volunteers to collected most, if not all, of the deposited scats. Table 3.20 shows the scat densities found in each section in total and by species. The average scat density for each survey is included. Only surveys from January 2013 onwards are included to avoid potential bias with the Southern Forest being covered more frequently in previous surveys.

Table 3.20 Scat densities in the tunnels by total, species, section and survey; sm= stone marten; pm=pine marten; Sys Avg= Average scat density throughout the tunnel system. Density figures shown are the number of scats per km of tunnel.

Survey	section	sm	pm	Total	Survey	section	sm	pm	Total
Jan-13	1	0.00	5.97	5.97	May-13	1	0.00	2.39	2.39
	2	0.00	0.00	0.00		2	0.00	1.01	1.01
	3	0.00	11.84	11.84		3	1.75	0.44	2.19
	4	0.00	0.61	0.61		4	0.00	0.00	0.00
	5	5.92	0.62	6.54		5	0.93	0.93	1.87
	6	0.00	0.39	0.39		6	5.48	0.00	5.48
	7	0.94	1.62	2.56		7	4.71	0.13	4.85
	8	2.94	1.47	4.41		8	9.06	2.20	11.27
	9	1.20	4.50	5.71		9	2.40	0.00	2.40
Sys Avg	4.22				Sys Avg	4.19			
Survey	section	sm	pm	Total	Survey	section	sm	pm	Total
Sep-13	1	0.00	1.49	1.49	Jan-14	1	0.00	1.49	1.49
	2	0.00	0.00	0.00		2	0.00	0.00	0.00
	3	0.00	9.21	9.21		3	1.32	0.88	2.19
	4	0.61	0.00	0.61		4	0.00	0.00	0.00
	5	3.74	1.87	5.61		5	3.43	0.31	3.74
	6	0.00	0.00	0.00		6	0.00	0.00	0.00
	7	6.19	0.67	6.87		7	2.29	0.27	2.56
	8	8.57	1.47	10.04		8	2.45	0.49	2.94
	9	3.60	0.90	4.50		9	9.31	0.60	9.91
Sys Avg	5.09				Sys Avg	2.88			

Survey	section	sm	pm	Total
May-14	1	0.00	0.00	0.00
	2	0.00	0.00	0.00
	3	0.00	0.00	0.00
	4	0.00	0.00	0.00
	5	3.74	0.00	3.74
	6	0.00	0.00	0.00
	7	7.68	0.13	7.81
	8	2.94	0.24	3.18
	9	5.11	0.60	5.71
Sys Avg	3.42			

The highest total marten scat density was observed during the September 2013 survey where on average 5.09 scats were collected per km of tunnel. The lowest total scat density was recorded in January 14 with an average of only 2.88 scats / km of tunnel. The high average density of scats found in the tunnels in September 2013 is heavily reliant on high scat densities in section 8 (stone marten) section 7 (stone marten) and section 3 (pine marten). In January 2014 the collected scat density in these 3 particular sections is approximately 4 times lower which may be as a result of the marten depositing scats less in winter as previously suggested for above ground surveys. The scats collected in January 2014 did not reach the laboratory until May2014 and this may have had a negative effect on the integrity of the scat DNA. The scats densities in January 2013 are lower in section 7 and 8 but relatively high in section 3 but it must be noted that as this was the first survey covering this section that these scats may have accumulated over time.

The scat density in section 3 during the January 2013 survey was the highest overall scat density for any one section in a survey at 11.84 scats collected / km of tunnel. While, as mentioned, these scats may be an accumulation, it is interesting perhaps that they were all from pine marten. Forested area above section 3 consists of only 15% of the total area so it could be expected that at least some of the scats found would be from stone marten.

While there is considerable variation in scat densities from section to section as well as from survey to survey the overall average scat density appears relatively constant at an average 3.97 scats / km of tunnel (S.D +/- 0.75).

3.4.4 Distribution of scats positive for marten DNA in Nietoperek

3.4.4.1 Above ground scat distribution

Only pine marten scats were found in the Northern Forest (Figure 3.27 A). In the Middle Section most of the scats collected were stone marten scats but pine marten scats were also collected. In the Southern Forest again there was a mixture of pine marten and stone marten scats found. Overall the majority of pine marten scats were found in forested areas while most of the stone marten scats were collected in the open areas (Figure 3.27 B).

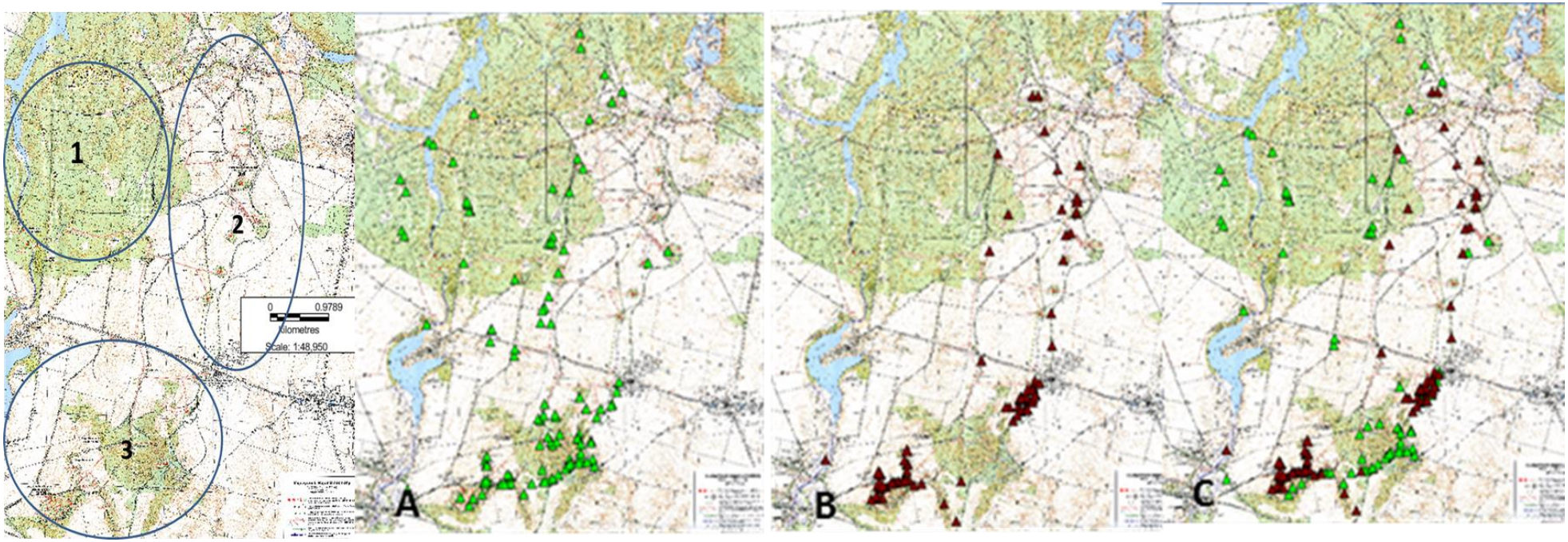


Figure 3.27 (A) combination of Figure 3.3 showing the breakdown of the above ground survey site and Figure 3.13 (A-C) showing the location of pine marten and stone marten scats above ground. 1= Northern Forest, 2= Middle Section, 3= Southern Forest (A) pine marten (B) stone marten (C) both species, pine marten ▲ stone marten ▲ .

The results shown in Figure 3.27 are in agreement with what is known about marten habitats from previous studies and while pine marten and stone marten are sympatric across most of continental Europe, only one, or the other of the species was found at a local scale by Rosellini, *et al*., (2008). Their study area covered 67 km2 in a region of Spain where pine marten and stone marten distribution ranges are known to overlap but they only found pine marten in their study area. However, it must be noted that while the flora is diverse in their study area, it consisted almost entirely of forest and fragmented habitat, like that found in Nietoperek, may result in a different species distribution pattern. Nietoperek is situated in a highly fragmented landscape. In the ecotones, where different habitats intersect, scats from both species were collected. A mixture of scats from both species in the ecotones of a highly fragmented landscape was also found in a study of pine marten and stone marten near Rogow in central Poland (Pilot *et al*. 2007). From these two studies it is evident that while pine marten, stone marten and also fox, which belongs to the same guild of meso-carnivores as the marten, are sympatric throughout much of continental Europe where their ranges overlap (I.U.C.N, 2013 ; Lanski & Heltai, 2011; Wikenros *et al.* 2014), the degree of sympatry between these species is variable. This variability may be related to the degree of habitat fragmentation in an area. The following section examines the above ground distribution of scats identified as being from pine marten, stone marten and fox throughout the survey period with consideration to the level of sympatry or segregation observed.

Figure 3.28 shows the location of all pine marten, stone marten and fox scats collected throughout the entire survey. As the numbers of scats identified as being from badger or polecat was so low they were omitted from any pattern investigations. Four sections of the above ground survey area have been highlighted and are labelled A-D. Section A of Figure 3.28 shows scats from all three species present in the ecotones where the northern forest meets the middle section of the survey area. Section B shows a portion of the middle section with forest islands covering bunkers directly above section 5 of the tunnel system. Both pine marten and stone marten scats were collected in these forest islands. Sections C and D both show areas in the southern portion of the survey area and in these forest fragments scats from all three species were found. To investigate this apparent habitat sharing further single surveyor scat surveys were repeated on 5 consecutive days around PzW 766 during the May 2014 survey and after molecular analysis it was confirmed that scats from all three species had been deposited in this forest fragment over a two day period. This data corresponds

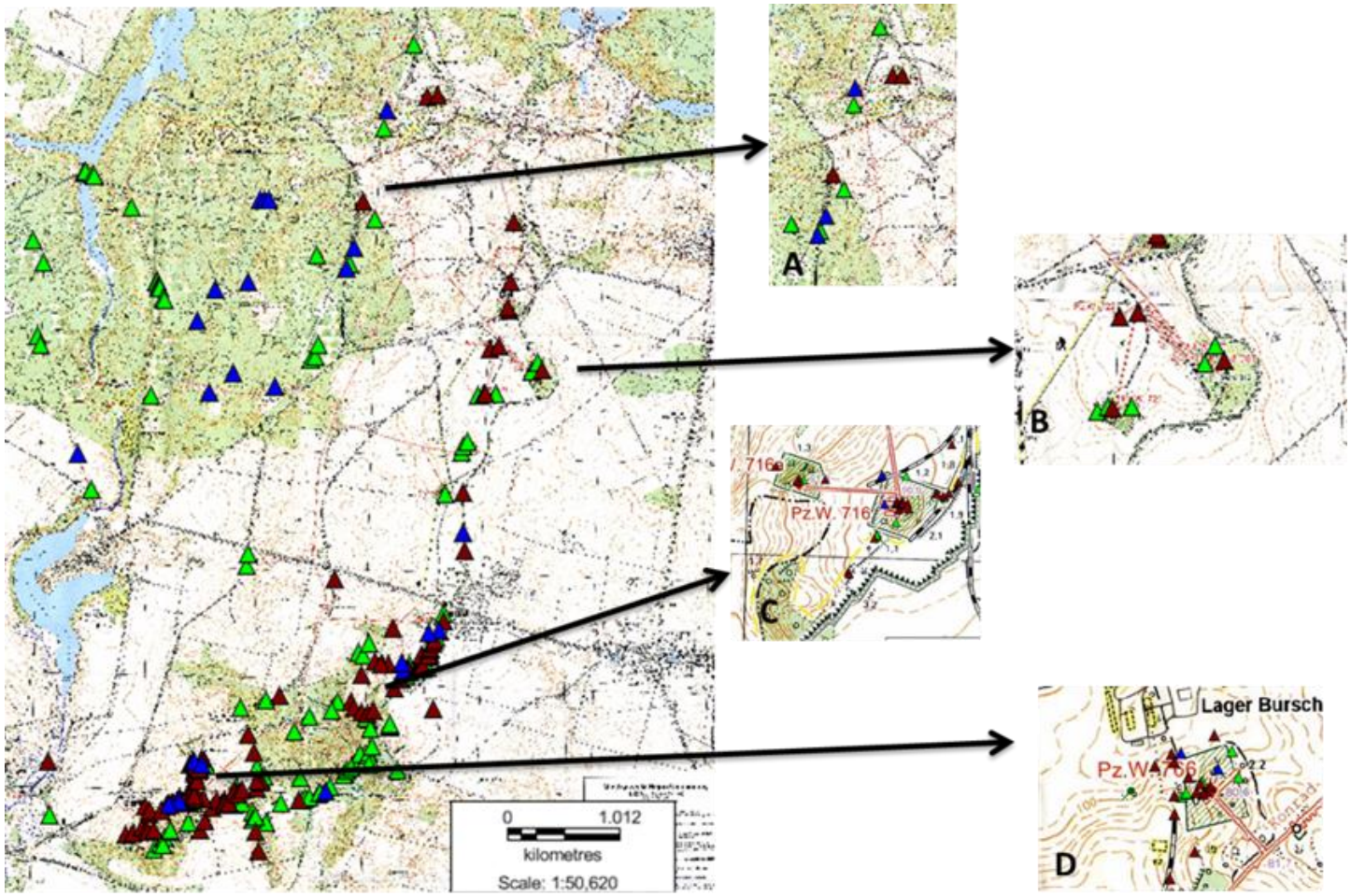


Figure 3.28 Location of all pine marten, ▲ stone marten, ▲ and fox ▲ scats collected above ground; A= area 1 and 2 intersect, B=forest islands above section 5, C= forest islands containing PzW 716 and 716a, D=forested area containing PzW 766.

largely to what was found by Pilot *et al*., (2007) with small fragments of forest, or areas where the habitats meet being used by all three species.

Figure 3.29 shows the Northern Forest and the Southern Forest portions of the survey site highlighting areas where scats from only one species of animal were ever identified. The Southern Forest is smaller and has been magnified for clarity. It can be seen that there is a relatively large area of the Northern Forest (4.5 km2) and a smaller section of the Southern Forest (0.9 km2) where only pine marten scats were found. Similarly there is an area in the centre of the Northern Forest (3.2 km2) where only fox scats were collected. Pine marten scats were found either side of this area but not within.

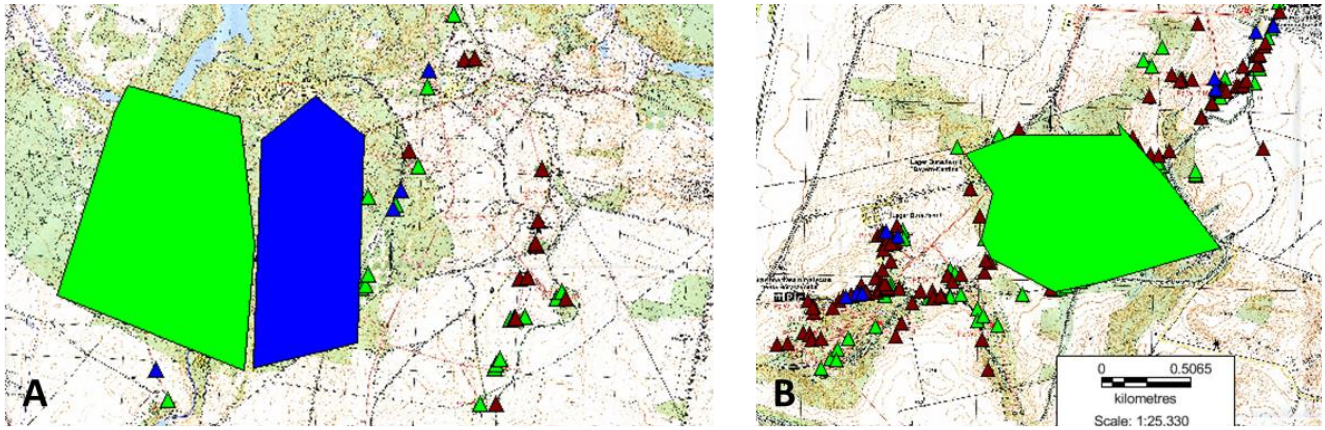


Figure 3.29 Polygons surrounding areas where scats from a single species only were collected; A= Northern forest B= Southern forest; pine marten fox .

This data corresponds to the findings of Rosellini, *et al.,* (2008) who also found that even though pine marten and stone marten were sympatric over large parts of their ranges that on a local scale, habitat segregation was evident. To the right of the Northern Forest however, near where the terrain opens up into arable land, scats from all three species were again collected suggesting that in fragmented habitat or zones between the two habitats there is a tolerance exhibited by each species for the other two that is not present in the more uniform forest habitat. This may be an example of temporal sharing of a habitat. It has been shown that the invasive american mink in the UK, which is predominately nocturnal in the absence of predators, will adapt its behaviour, adopting an avoidance mechanism where they will become diurnal in areas where otter and polecat populations are on the increase (Harrington *et al.* 2009). However it has also been suggested that small mammal abundance is higher in habitat edges (Salek *et al*. 2010) and ground nesting birds may be targeted by predators more

frequenlty in the ecotones where the nest may be visible from a further distance than if deep in a forest (Anglestam, 1986). This increase in prey availability in these areas may result in an increased tolerance of these three competing predators. This suggestion must come with the caveat however that it has been found that for marten and fox in an agricultural landscape in Europe, there is virtually and unlimited source of primary and secondary prey availability so any resource partitioning or sharing would be almost impossible to prove and interspecific differences in feeding habits may be a result of individual patterns rather than niche segregation (Lanski & Heltai, 2011). However, the results obtained here may warrant further investigation to determine if there is a habitat fragment-size threshold above which these meso-predators cease to share a habitat and once again become segregated.

3.4.4.2 Below ground marten scat distribution

3.4.4.3 Overall distribution of pine marten and stone marten scats below ground

Figure 3.30 shows the distribution of scats below ground. Scats from both species were collected throughout the entire tunnel system with only pine marten scats collected in the far north (Figure 3.30 A). This may be due to the north of the system being covered with woodland above ground. In the central part of the system where the above ground area is more open, most of the scats collected turned out to be from stone marten (Figure 3.30 B). In the southern section there was a mix of both pine marten and stone marten scats that largely corresponded to above ground distribution. No fox DNA was detected in any scat collected in the tunnels.

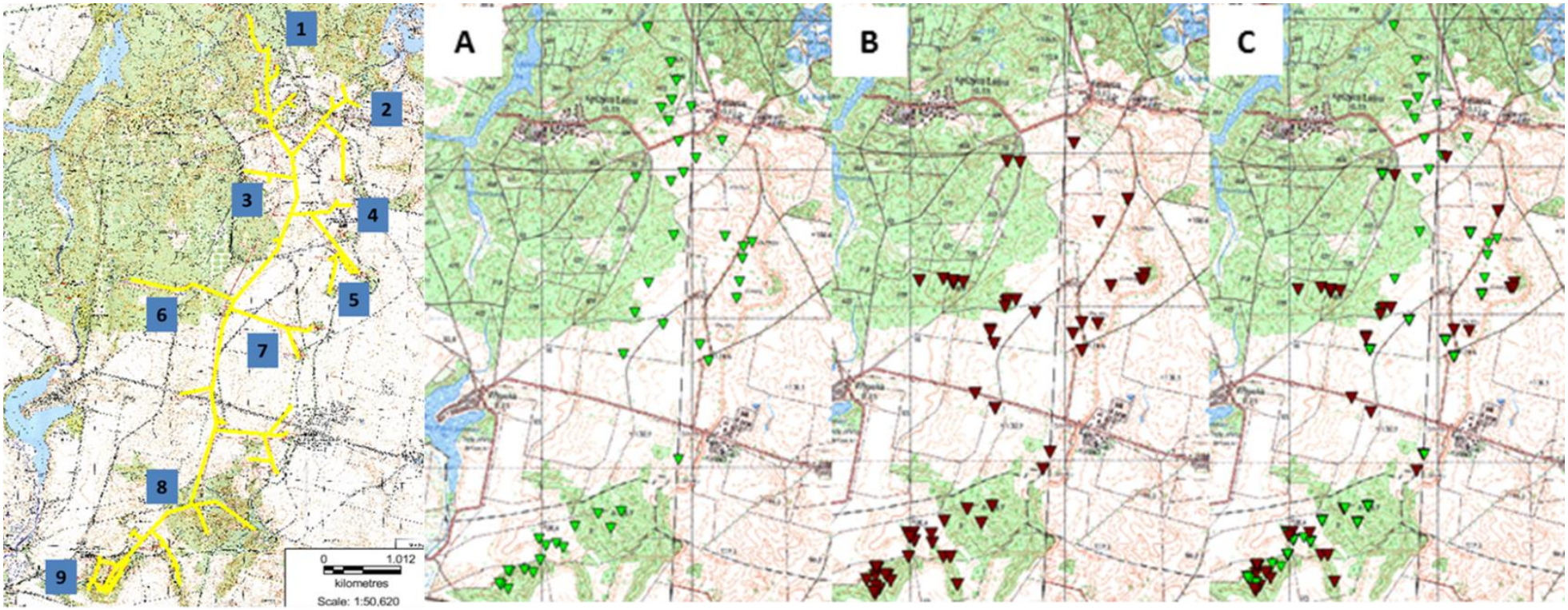


Figure 3.30 Combination of Figure 3.1 showing the breakdown of the tunnels into sections and Figure 3.14 (A-C) showing the position of scats collected from pine marten, ▼ stone marten, ▼ and from both species.

3.4.4.4 Comparison of marten scat distribution above and below ground

In section 1 of the system all scats identified were from pine marten (Figure 3.14 A). The area directly above section 1 is covered in forest and also contained only pine marten scats (Figure 3.13 A).

Section 2 of the system contained no scats from either species. The area above section 2 consists of small fragments of woodland and farmland in which scats from both species were collected, although the number of scats collected above section 2 was low (n=7).

Section 3 was found to contain scats from both marten species. Pine marten and stone marten scats were also collected in the fragmented landscape above section 3. Section 4 also contained no marten scats. The area above section 4 also produced very few scats. This area was mostly open farmland with very few wildlife corridors in evidence to provide cover for marten. While stone marten prefer open areas they still require some cover in the form of low brushwood or hedges, or high grassland and webs of vegetation to facilitate normal movement and ranging activity (Cervinka *et al.* 2013). It may be that stone marten were not present in this area as a result of this lack of cover. There are only a few tracks present above section 4 so it may also be that no scats were found due to the relatively small area covered above ground in this portion of the survey site.

Section 5 contains scats from both pine marten and stone marten (Figure 3.14), as does the corresponding area above ground (Figure 3.13). The area above section 5 is mostly open farmland but also contains 3 bunkers which are surrounded by woodland. The dragons teeth which follow the line of the system in this area are covered in dense vegetation along this portion of the survey site potentially providing cover for both marten species.

Section 6.4 only contained stone marten scats during one survey period (n=14) (3.13 B) and no pine marten scats were found in this section of tunnels. The entrance to section 6 is at surface level is prone to flooding (Figure 3.31). This tunnel slowly drops from ground level to 50 m below the surface and the absence of scats, with one exception, may be due to scats being washed away by floodwaters. 50 - 75 mm of floodwater were reported lying in large portions of section 6 during two separate surveys. The area above section 6 is divided between forested area in the west and open area in the east. Only a small number of scats from both species were found in the above ground area over section 6. In the open area, again there were few tracks to survey, the buildings that still exist around the forest entrance mean

that this portion of the woodland is not harvested. The trees in this area are well established and the understory is sparse under a dense canopy leaving little cover for pine marten.

Figure 3.31 Evidence of flooding at the entrance to section 6 during the September 2013 survey.

Section 7 contained scats from both species with the majority of scats being from stone marten. This may be as expected as most of the corresponding area above section 7 consists of open farmland with small areas of woodland in evidence around some of section 7's bunkers and so scats from both species were also found above ground, again with the majority of scats being from stone marten.

Section 8 also contained both pine marten and stone marten scats with the majority of scats collected being from stone marten. Above section 8 is a mixture of open area, fragmented forest and one larger forested area. Mostly stone marten scats were found in the open farmland and only pine marten scats were found in the forested area with, once again, a mixture of scats from both species found in the ecotones in the fragmented habitat. The open area over section 8 contains a segment of the dragons teeth. This segment of the dragons teeth has a footpath alongside it and tourists regularly use this area. However scats were still found at the base of, and on top of these constructs during all surveys except the May 2014. It may be noteworthy that this survey was the first where extensive strimming of the grass and weeds around the dragon's teeth was observed. Figure 3.32 (B) shows the dragons teeth around section 8 in the summer of 2014 while Figure 3.32 (A) shows how much more overgrown this area was the previous summer. The removal of this growth which could be

used as cover by marten, may be the reason no scats were found here during the May 2014 survey.

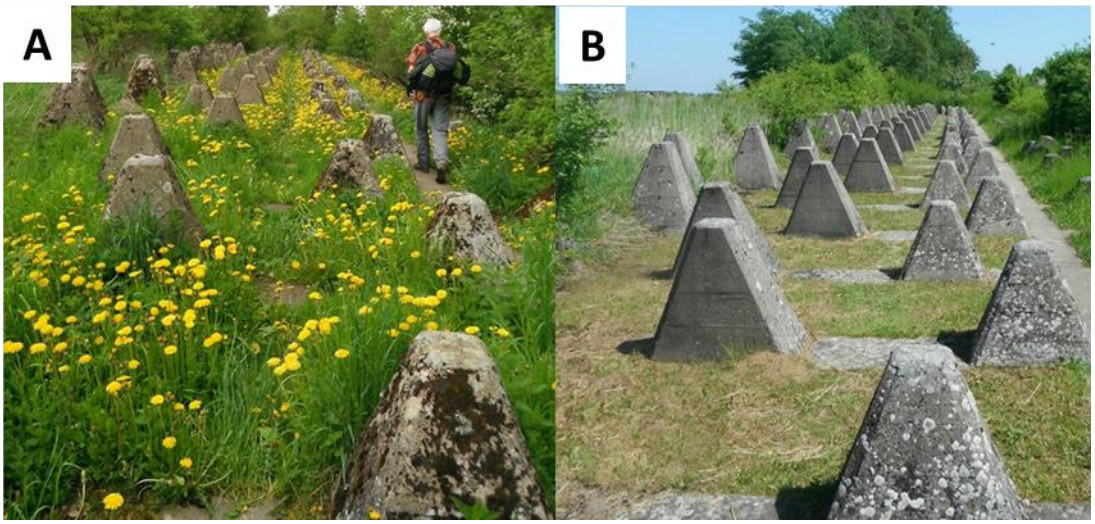


Figure 3.32 Segment of Dragon's teeth above section 8; A= May 2013, B= May2014.

3.4.4.5 Distribution of male and female marten scats throughout Nietoperek

Table 3.21 summarises the scats identified as being from male and female pine marten and stone marten collected both above and below ground throughout all surveys. Scats from both male and female pine marten and stone marten were collected above ground and in the tunnels. The majority of male pine marten scats were collected above ground while the same number of female pine marten scats was collected above and below ground. The ratio of above ground to below ground male scats is different for stone marten with the majority of stone marten scats collected in the tunnels. Likewise, for female stone marten with approximately twice as many female scats collected in the tunnels than on the surface. More female scats from both species were collected in the tunnels than scats from males. This may be due to more females from both species entering the tunnels or from females entering the tunnels with a higher frequency than males or from variation in scatting behaviour from marten of different sexes (Barja *et al*. 2011).

Table 3.21 Summary of all scats sex typed from all surveys in Nietoperek.

Sex	Pine marten male	Pine marten female	Stone marten male	Stone marten female
Total	67	72	122	229
Above ground	52	36	38	73
Tunnels	15	36	84	156

3.4.5 Scat densities throughout the tunnel system

3.4.5.1 Relating pine marten and stone marten scat densities to bat densities in the tunnels

Once it had been established that scats from both pine marten and stone marten were present throughout the system the next step was to see if the density of scats in any section was related in any way to the abundance of bats located in that particular section each year. The average bat densities and scat densities for both marten species was calculated and plotted with higher densities represented by larger symbols (Figure 3.33). Bat densities were sourced from the annual January surveys 2012-2015 (R.E.P.D., 2015). The average bat densities shown include all bat species found in the tunnels but this plot was repeated for each of the 3 most common species individually (Appendix III).

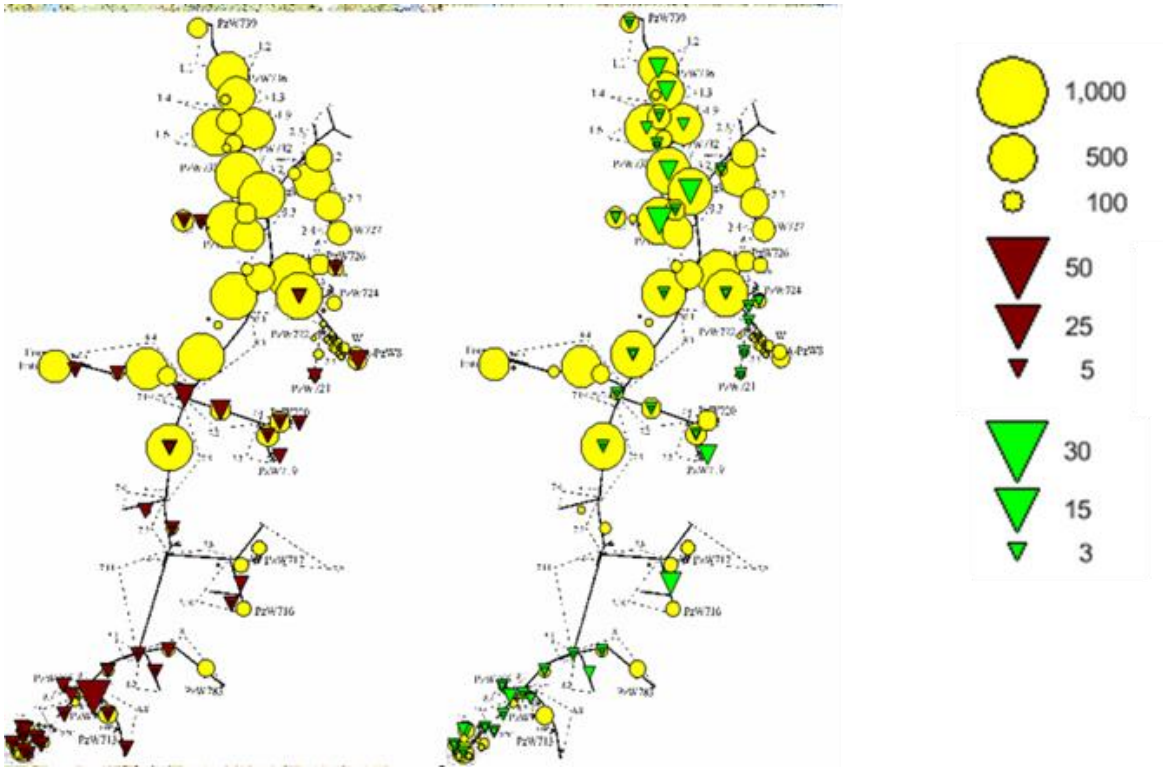


Figure 3.33 Average bat densities, ● stone marten scat density, ▼ pine marten scat density ▼ throughout the tunnel system.

On initial examination, there appears to be a relationship between bat density and scat density in different areas for both marten species. These were sections 1, 3 and 7. However, there were also areas of high scat density in sections of the system where there were low bat densities. These were sections 5, 8 and 9 for both species. It was noted by visual examination of the data that many of the scats appeared to be deposited near access points in section 8 so this potential pattern was investigated throughout the system.

3.4.5.2 Proximity of scat locations to human access points

Sections 2, 4 and 6 were omitted from this analysis due to a paucity of scats while section 9 was excluded as this section is small and circular and the position of a scat is never more than approximately 50 m from a man-made access point. In the remaining sections however it can be seen there is a clear relationship between the number of scats collected in an area and its proximity to an access point (Figure 3.34).

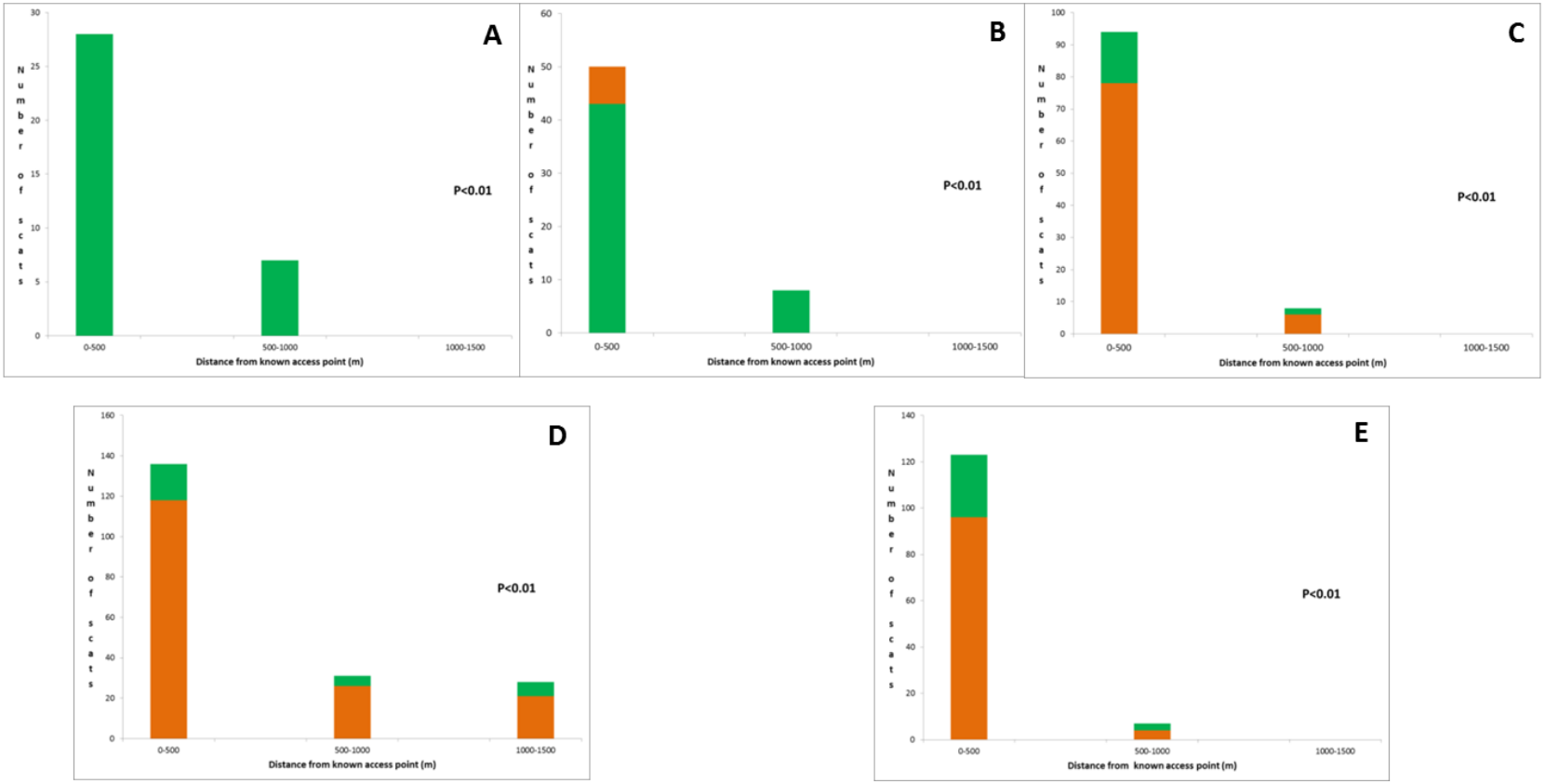


Figure 3.34 Proximity of marten scats to known human access points throughout the tunnel system; A= section 1, B= section 3, C= section 5, D= section 7, E= section 8, pine marten stone marten .

The fact that marten are depositing scat close to access points may be an indication that they are not venturing very far into the tunnels. It may also give some insight into how the marten are navigating these tunnels which at 50 m below the surface are in total darkness. Marten may be depositing these scats close to the access points as a form of chemical auto-communication as has been observed in other work with marten (de Monte & Roeder, 1990). Navigation in total darkness would also be aided by keeping close to the walls of the tunnels and this may be why most scats collected are in close proximity to the tunnel walls.

3.4.5.3 Species variation across surveys in different sections of the tunnels

Some scats were collected before the surveys of the entire system began and as part of smaller surveys. These scats have been omitted from the variation analysis. Section 2 and section 4 were again omitted from analysis due to a paucity of scats. In the remaining sections of the tunnels there was a difference in both the number of scats collected during each survey and in some cases there was also a difference in the species responsible for these scats. A summary of this variation may be seen in Figure 3.35.

Section 1 (Figure 3.35 A) only contained pine marten scats but there was variation to the scat abundance found across survey periods with 20 pine marten scats found during the initial survey of this section with the remaining surveys being relatively consistent, finding between 5 and 8 scats. It may have been as this was the first survey of section 1 that these scats have accumulated over time.

Section 3 (Figure 3.35 B) contained scats from both pine marten and stone marten with the majority of scats being from pine marten. 28 pine marten scats were found in section 3 in January 2013 with only 1 pine marten scat found the following May. Again the high number in January 2013 may have been due to an accumulation of scats over time but in September 2013 21 pine marten scats were again found in this section suggesting an increase in pine marten activity in this section during this period. During the period between May and September there are no bats hibernating in the system suggesting pine marten are visiting the tunnels for reasons other than predation of hibernating bats. The number of scats from pine marten found in section 3 in subsequent surveys was low but there were scats found during each survey. This suggests a relatively constant occupation of section 3 by the pine marten in the area. Stone marten scats were only found in small numbers and only during January.

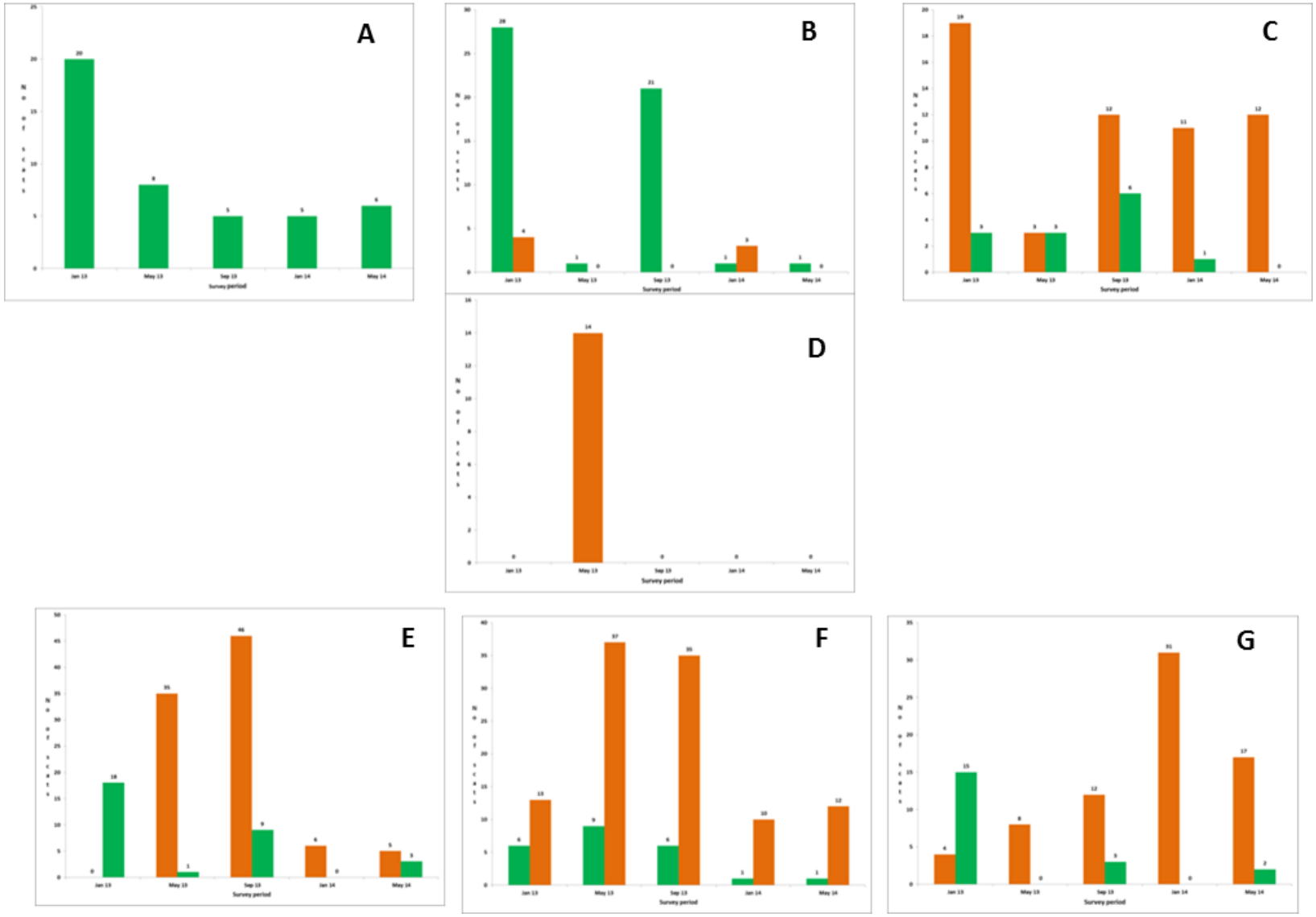


Figure 3.35 Variation in scat abundance by section for all surveys covering the entire tunnel system; A= section 1, B= section 3, C= section 5, D= section 6, E= section 7, F= section 8, G= section 9 pine marten, stone marten .

Section 5 (Figure 3.35 C) contained scats from both pine marten and stone marten. The majority of scats were from stone marten but scats from both species were found during 4 of the 5 surveys of the entire system. In January 2014, 19 (86%) of the scats found in section 5 were from stone marten compared to 3 (14%) from pine marten. This ratio changed during the September 2013 survey when the pine marten scat abundance in section 5 increased to 33%. However, the presence of pine marten scats decreased thereafter with no pine marten scats being found in section 5 during the May 2014 survey. Both species have been found in the area directly above section 5 and this may reflect a shifting in species' above ground ranges over time.

Section 6.4 (Figure 3.35 D) only ever contained stone marten scats and these scats were only found during the May 2013 survey. It is possible that stone marten had never entered this section before the May 2013 survey detected them there but it is more probable that, as this section is prone to flooding, that any scats, from either marten species, that had previously been deposited had been washed away.

There is considerable variation in scat abundance as well as marten species presence evident in section 7 (Figure 3.35 E). It must be noted that section 7 was one of the sections that had been previously surveyed by volunteers conducting bat surveys and so no accumulation of scats over time has taken place (sections 7, 8 and 9). The January 2013 survey found 18 pine marten scats and no stone marten scats. However, during the May 2013 survey it was mostly stone marten scats that were found (n=35) with only a single pine marten scat found. This again may be a product of changes in territories above ground or of species ranges expanding and contracting. In May 2014 only a small number of scats from either species was found in this section but the ratio of 5 stone marten scats and 3 pine marten scats may suggest that there may be resource partitioning taking place and section 7 may be a shared habitat.

Section 8 (Figure 3.35 F) contained mostly stone marten scats which were collected during each of the surveys. However a small number of pine marten scats were detected in section 8 during each survey again suggesting the presence of both species in this section all year long.

Evidence of stone marten could also be found in section 9 (Figure 3.35 G) during each of the 5 surveys with the number of stone marten scats collected gradually increasing before falling off slightly in May 2014. Pine marten scats were only found in section 9 during 3 of the 5 surveys. The January 2013 survey found 15 pine marten scats and this number decreased throughout the project period to only 2 pine marten scats collected in section 9 during the

May 2014 survey. Again, there may be a shift in above ground territories as this has phenomenon has been seen in a number of sections.

The constant presence of marten in some sections contrasted with sporadic finding of scats from either species in a particular section may be a result of territories changing, or species ranges shifting; it may be that both marten are sharing the tunnels or visiting them at different times; it may be that with only the aid of torches and a limited time allowed into the tunnels that not all scats have been collected, although every effort has been made to do this. It may also be that pine marten and stone marten have different motives for entering different sections of the tunnels at different times. This may explain why in some sections there appears to be a constant presence from one species; with only occasional occupancy of the tunnels from the other as seen in section 3 and section 9. What is clear from this data is that marten are present in the tunnels all year long in varying abundance and are not just entering the tunnels during the winter months to predate on hibernating bats.

3.4.6 Distribution of individual marten throughout the Nietoperek survey site April 2011- May 2014

This section will discuss the distribution of scats from some of the individual animals in space and time throughout the survey area and duration of the sample gathering portion of this project. Any individual that was only detected from a single scat was omitted from this analysis as there is limited inference to be made from these single points in space and time other than these scats may be from transient animals or were simply not detected in other surveys. Throughout the entire survey period a total of 41 individual animals were identified comprising of 20 pine marten; 8 males and 12 females, and 21 stone marten; 10 females and 11 males. Of the 95 scat DNA extracts yielding genotypes, 54 (57%) were collected in the tunnels. These 54 scat DNA extracts found in the tunnels were from 10 stone marten and 9 pine marten. The highest minimum number alive values for pine marten were found during the May 2013. Seven individual pine marten were detected during this period. The highest minimum number alive values for stone marten were found equally during the January 2013, May 2013 and May 2014 survey periods. Six individual stone marten were detected during each of these surveys. Therefore although 41 individual marten were detected in the survey area over the entire survey period only 13 marten at most were ever detected during any single survey. Changes in the composition of the marten populations in and around Nietoperek over time may be due to kits being born and not dispersing very far,

compensating for mortalities in the resident populations. Alternatively, the changes in marten individual composition in the Nietoperek population may reflect the fact that the study site is only a very small part of a large mosaic of fragmented forest and farmland, resident to much larger pine marten and stone marten populations.

17 of the 21 stone marten were detected at least once in the system comprising 7 (41%) females and 10 (59%) males. Stone marten individuals were detected in the system a total of 32 times. Although more males were detected, the difference in sex with respect to scat frequency is less obvious. Of the 32 detections of individual stone marten in the tunnels 15, or 46 %, were female. Of the 20 pine marten identified 14 were detected at least once in the system 8 (57%) were female and 6 (43 %) were male. Of the 24 detections of individual pine martens in tunnels 15 (63%) were female and 9 (37%) were male. While the number of individuals' from each sex entering the tunnels were similar, female individuals are detected more often suggesting more frequent visits to the system. As female pine marten and stone marten have been shown to target smaller, easier accessible prey than their male counterparts (Loy *et al*. 2004) the higher than expected frequency of female individual detection in the system may be due to females predating on low hanging bats in hibernation or daily torpor, or to scavenge on dead bats as the escape effort in each case would be zero. This hypothesis is reinforced by the majority of scats yielding sex typing data from the tunnels were female (Table 3.21). It is however important to note the small sample size and the detection of P6, a female pine marten a total of 5 times may lead to a bias towards female frequency. The next two sections discuss the distribution of scats from individual marten and there interactions, if any, with the tunnel system. Some animals were detected in the survey area throughout the project. Some were only ever detected above ground; some were only detected in scats from the tunnels and others were detected above and below ground. Interactions with the below ground portion of the survey area varied between individual animals. Details of each individual animals interactions with the tunnel system and the the above ground territories can be seen in Appendix IV of this document.

3.4.6.1 Individual marten territories as defined by scat locations

In the absence of positive hair sample data, scat DNA was used for genotyping. This not only resulted in a reduced quality of DNA sample but also removed a degree of resolution of the data that would be obtained from getting genotyping data from hair samples from a fixed location during a specific timeframe. However, as individuals from both species were identified with multiple recaptures across surveys above and below ground the following section will discuss the data obtained with respect to the animal's minimum territory. Quadrants encompassing all scats identified from a single individual are used.

Figure 3.36 shows the positions of all scats from each individual stone marten detected more than once, joined together by a quadrant to denote a possible territory. It may be seen that no overlapping of male territories occurs with stone marten which is in agreement with previous studies on stone marten (Herr *et al*. 2010). Territorial loyalty is also shown with individual stone martens detected within an oval (Figure 3.36) not detected in any other part of the survey site.

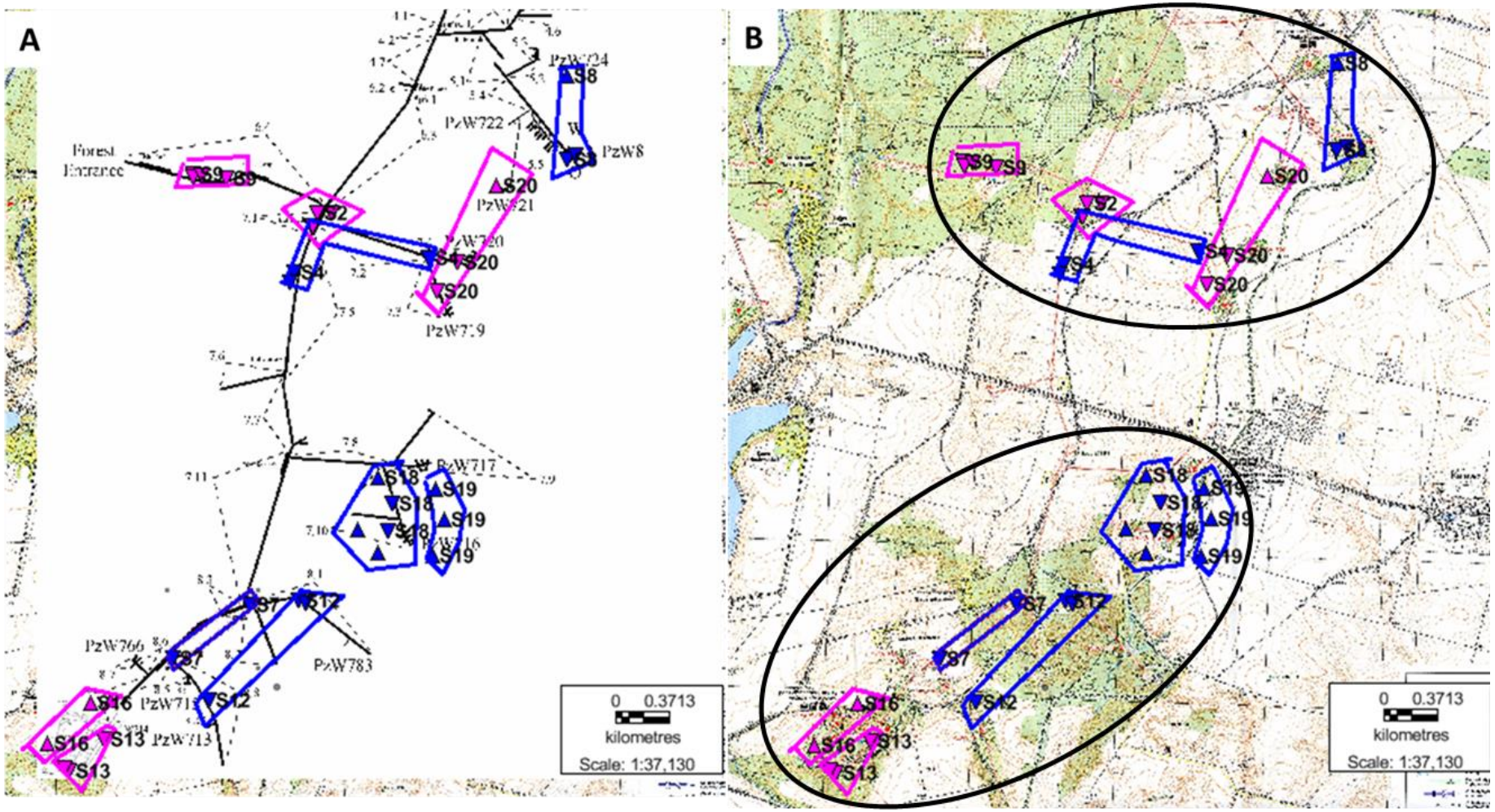


Figure 3.36 Location of minimum individual stone marten territories; male ▢ female ▢ , territorial loyalty is highlighted by oval ◯.

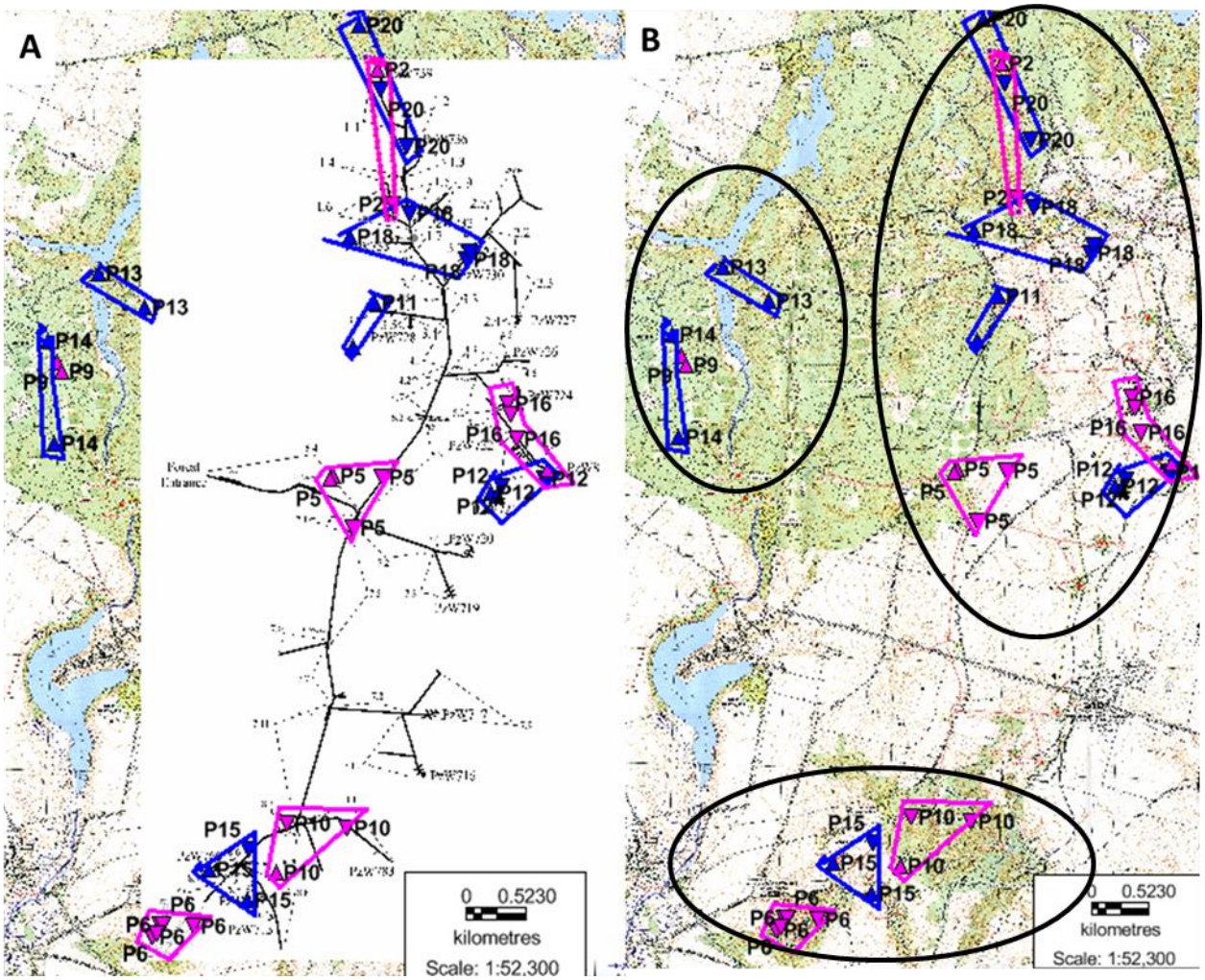


Figure 3.37 Location of minimum individual pine marten territories; male ▢ female ▢ territorial loyalty is highlighted by oval ○.

Figure 3.37 shows the positions of all scats from each individual pine marten joined together by a quadrant to denote a possible territory. Once again, while the territories of some of the females overlap with those of the males there is no overlapping of territory among male pine marten. Territorial loyalty is again in evidence with animals tending to remain in the same general area. For example, P13, P14 and P9 were only ever found in the Northern Forest. Likewise animals found in the Middle Section of the survey site above and below ground were only ever found in this area and finally the animals found above and below ground in the Southern Forest were only ever found in the Southern Forest.

3.4.7 Haplotype analysis

Pine marten mtDNA d-loop sequences from previous studies on pine marten phylogeography were compared with the sequences obtained in this study across 300 bases and assigned the haplogroup 1a (Davison *et al.* 2001). The sequence obtained from Nietoperek pine marten DNA extracts was identical across this region with the haplotype "Hap b" (Davison, *et al.,* 2001). Given the location of the survey site in Nietoperek an allocation of a "Hap b" is consistent with expectations. Figure 3.38 shows the mtDNA lineages of pine marten in Europe from Davison, *et al.,* (2001). While data from Poland is absent from this study it may be seen that the haplogroup 1a is found in countries bordering Poland on either side. Moreover samples to which Davison, *et al.,* (2001) attributed the "Hap b" haplotype were found throughout Europe including in Germany and the Czech Republic which border Poland to the east and south respectively.

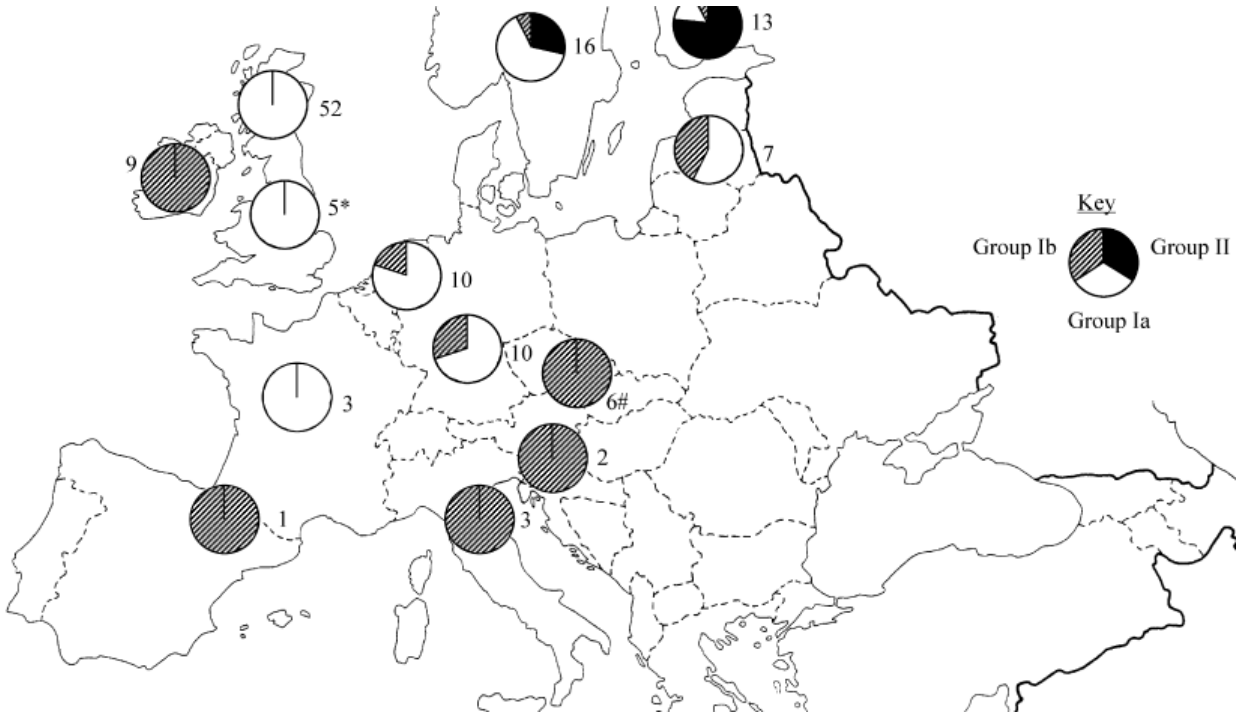


Figure 3.38 Distribution of pine marten mitochondrial lineages in Europe (Davison *et al.* 2001).

The sequences obtained from the stone marten individual scat DNA extracts did not show 100% identity to any available mtDNA d-loop sequence. After alignment and trimming of the non-overlapping ends a median joining network was constructed from the 218 bp regions (Figure 3.26). It can be seen that there is a single base mismatch between the Nietoperek

stone marten sequences and those from haplotypes found in Greece and Bulgaria. These countries are relatively distant geographically from Poland. Stone marten range expansion in Europe occurred much later than that of the pine marten with only one late glacial sub-fossil of stone marten on record (Sommer & Benecke, 2004). This may account for the apparent lack of phylogenetic structure in stone marten mtDNA but at present there is a paucity of phylogeographic studies on stone marten available for comparison (Vergara *et al.* 2015). The new haplotype has been lodged in GenBank under Accession number KT726937.

4 DIETARY ANALYSIS OF SCAT SAMPLES COLLECTED IN AND AROUND THE NIETOPEREK BAT RESERVE, POLAND

4.1 Introduction

Bat species have only occasionally been found in the diet of both pine marten and stone marten (Tryjanowsky, 1997; Tothe-Apathy, 1998). However, the non-invasive approaches and molecular and morphological techniques used to detect other prey items in marten scats, as well as to detect other prey in the faeces of other animals, will be similar. Therefore, the beginning of this section will discuss some examples of these techniques when applied various animals.

Purcell *et al.*(2004) utilised 3 separate regions of the mitochondrial genome when developing a molecular method for the genetic identification of salmonid prey from Pacific harbour seal scats. The 3 regions used were the d-loop, a section of the 16 S ribosomal gene and a section that spanned three genes; the cytochrome oxidase III, t-RNA glycine and ND 3 genes. Within this region, there was very little intraspecific variation while there was significant interspecific variation among different salmonid species making it an ideal tool or target for use in differentiating salmonid species using DNA extracted from bones isolated from faecal samples. For each of the 7 species of salmonid one individual was sequenced for the 16 S rRNA and COIII / ND 3 regions. Manual sequencing was performed and cut-sites for restriction enzymes identified. RFLP was performed and the products visualised on a gel. Morphological examination identified bones from all gathered scats that belonged to the genus *Oncorhynchus* and these were then selected for DNA analysis to determine which species they were. The results showed that there was sufficient variation in both the d-loop and COIII / ND 3 regions to distinguish between all the salmonid species in the study and 6 restriction enzymes that were selected successfully distinguished all species by their point-of-cut. Once the methodology had been verified it was applied to 23 bone samples and 14 of these samples were additionally confirmed by sequencing. Of the total 39 scats examined DNA was successfully amplified from 35 and the study highlighted the effectiveness of sequencing and RFLP analysis of the COIII / ND 3 region as a method of identifying the 7 salmonid species involved in the study. It also highlighted the effectiveness of using a 16 S rRNA primer pair for bones that are not easily identified using morphological methods.

Deagle *et al*.(2005) used molecular scatology for prey analysis using stellar sea lions (*Eumetopias jubatus*) as the predator. This was a captive study and the sea lions were fed known diet. In this study the 3' end of the mitochondrial 16 S ribosomal RNA gene was chosen as a PCR target and in a previous study they had already designed conserved primers that amplified DNA fragments from all of the prey species used in the study (Deagle *et al.*, 2005(b)). A Denaturing Gradient Gel Electrophoresis (DGGE) based method was used to identify species using this region. An attempt at a quantitative estimate of prey consumption was also made using a clone library from fish PCR products to try and quantify the proportion of fish DNA present in 8 samples. PCR products were cloned into a plasmid, transformed, and plated. The technique incorporated a blue / white colour differential. The results showed positively for some species but poorly for others with one species completely absent from results. It was concluded however that this technique may be useful to give a rough estimation of proportion of certain species in the sea lions diet.

The overall conclusion was that while the clone library technique was straightforward it only allowed end-product analysis and recommends the utilisation of qPCR as a possible alternative highlighting its benefits which include rapid simultaneous quantification of DNA from multiple prey species, qualitative comparisons and in this study would have allowed for a fish – squid ratio comparison which is not possible with the clone library.

Bohmann *et al*. (2011) utilised the Roche FLX sequencing platform to deep sequence uniquely 5 ' tagged insect generic barcode cytochrome oxidase I (COI) fragments that were obtained from faecal pellets of two species of African bat; *Chaerophon pumulis* and *Mops condylurus*. As the GenBank and BOLD databases contained a limited quantity of insect COI sequences they used a bio-informatics method known as Molecular Operational Taxonomic Unit (MOTU) analysis to aid in identifying the species of insect that each bat was preying on. The Roche FLX sequencer that was used employs a technique known as next generation sequencing and as such is termed a "high throughput sequencer". It is capable of sequencing 500 Mb of sequence in one run from as many as 1 million sequences generated in parallel. Uniquely tagged primers are used to amplify each specific DNA template source facilitating parallel sequencing of amplicons from all samples while still keeping track of the origin of prey sequence. It was found that there was a scarcity of South African insect information in the BOLD database so bio-informatics was used to assign species to some of the prey detected. So species level analysis was carried out using MOTU as a means of estimating the prey species consumed using the program jMOTU which groups the sequences according to

similarity. The parameters for the degree of similarity are set by the user. The program jMOTU then identifies molecular operational taxonomic units within groups of sequences which can be used as an aid in taxonomic identification. Frequency of detection of each MOTU was calculated using assigned MOTU for each haplotype as a template for species identification. Mean MOTU consumed by each bat was also calculated to see if the two bat species had similar number of MOTU consumed. MOTU accumulation curves were also constructed to estimate dietary richness. After statistical adjustments and analysis it was evident that in over half the samples there was between species consumption of MOTU indicating there is no evidence of resource partitioning between the two bat species. This may be due however to the sheer abundance and diversity of prey available in the area being examined.

Approximately 38 000 bats occupy the hibernation site in Nietoperek each winter and the population may still be growing. After initial occupation it takes several decades of growth until the population meets a maximum level, the numerical value of which is different for various species (Lutsar *et al*. 2000).

Myotis myotis constitute the largest portion of the entire bat population (75.4% in 2015). This contrasts with the findings of (Fuszara *et al*. 1996), who surveyed hibernating bats in underground shelters in central and northeastern Poland. *Myotis myotis* were found in caves, forts and cellars but of the 141 bunkers surveyed they found no *Myotis myotis*. The number of species they did find at each site however was positively correlated with corridor length. There are many long corridors in the Nietoperek bat reserve and these may be responsible for the diversity of bats found down there.

The large number of individual bats as well as the wide diversity of bat species present may make the Nietoperek bat hibernation site an attractive foraging area for marten. In this study, novel qPCR bat species specific primers were designed to detect the presence of bat DNA in pine marten and stone marten scats. This chapter discusses the design of qPCR species specific primers and their use in detecting bat DNA in marten scat DNA extracts. The distribution of marten scats positive for bat DNA both above and below ground is also discussed.

4.2 Method

The locations of positive and negative data are plotted on a map of the area in and around Nietoperek. Acronyms are used instead of the full name of each bat species (Table 4.1) in some cases to maximise the clarity of data displayed on these maps.

Table 4.1 Acronyms of scientific bat names used throughout this document.

Species	Acronym
Myotis myotis	Mymy
Myotis daubentonii	Mdb
Myotis nattereri	Mnat
Myotis dasycneme	Mydsc
Myotis brandtii	Mybr
Myotis bechsteini	Mybch
Myotis mystacinus	Mymyst
Plecotus auritus	Paur
Barbastelle barbastellus	Bb
Eptesicus serotinus	Epser
Nyctalus leisleri	N.leis
Nyctalus noctula	N.noc
Pipistrellus nathusii	P.nat
Pipistrellus pipistrellus	P.pip
Pipistrellus pygmaeus	P.pyg
Plecotus austriacus	P.aus

4.2.1 Design of qPCR primers for detection of bat species DNA in marten scat DNA extracts.

MtDNA sequences from all relevant bat species were accessed in GenBank (NCBI). Accession numbers are shown in Appendix V. The mitochondrial NADH dehydrogenase 1 subunit (ND1), Cytochrome b gene (Cytb) and the Cytochrome Oxidase 1 gene (COI) were all screened for potentially suitable targets for amplification. Sequences for all three regions

were aligned firstly by species using BioEdit (Hall, 1999) to highlight intra-species conserved regions. Once conserved regions had been selected the sequences were aligned with sequences from the remaining bat species found in the area and screened for conserved areas within species that had inter-species variability. Finally, any intra-species conserved regions were aligned with same-region sequences from both pine marten and stone marten and again screened to ensure inter-species variability.

Once possible target regions had been selected, qPCR primers were designed with the aid of Primer Express® 2.0 software (Applied Biosystems). When designing primer pairs for use with qPCR, the amplicon size should be between 50 and 150 bases long. Other parameters considered included primer length, melting temperature, annealing temperature and GC content. Once potential primer pairs had been selected the sequences were entered into Oligo Calc (Kibbe, 2007) to check for the presence of self-dimerization or hairpin structures. Target areas were selected on the basis of conserved sequence area within each species with inter species variation within these areas. The Basic Local Alignment Search Tool® (BLAST) (NCBI) was used as a desktop check for potentially successful amplification of targets as well as checking for non-specific amplification of other bat species and to ensure no amplification of the predator species', or other non-target DNA, likely to be present in a marten scat.

Of the 10 bat species found in the tunnels in Nietoperek, 7 are *Myotis* species comprising the majority of individuals found here each year. Therefore *Myotis* specific primers were designed. Scat DNA extracts were assayed using all non-*Myotis* species primers and the *Myotis* genus specific primers. Only samples that were positive using the *Myotis* genus specific primers were subsequently assayed using the *Myotis* species specific primer pairs (Figure 4.1). This was performed to save on both time and consumables. Controls used to test primers were sourced from known DNA extracts in the Molecular Ecology Research Laboratory in W.I.T with the exception of the *Myotis myotis* control. Control DNA for *Myotis myotis* was obtained on-site from a carcass positively identified by a bat expert during the January 2013 bat census. The skeletal remains was added to S.T.A.R® and treated as if it was a scat sample. Bones from the carcass were crushed in a mortar and pestle and DNA extracted following the protocol used in section 2.3.2. SYBR® Green Dye qPCR assays to test the novel primer pairs on DNA extracts of known origin were performed as described in section 3.2.7 using the appropriate primer pairs.

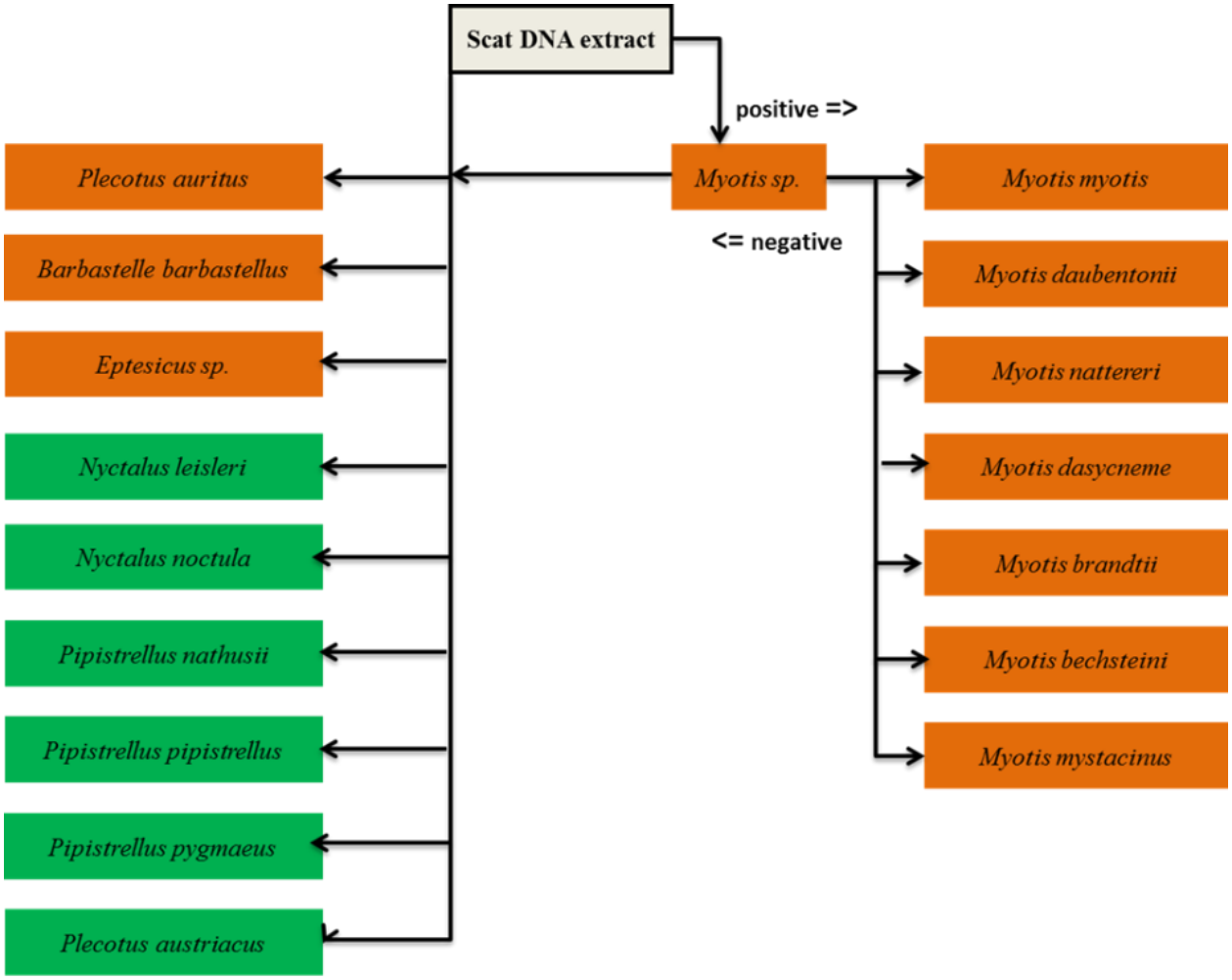


Figure 4.1 Flow diagram of qPCR dietary analysis methodology. Only scat DNA extracts positive using the *Myotis* genus specific primers were assayed for individual *Myotis* species DNA. - bats found in the tunnels -bats only found above ground.

4.2.2 Detection of bat DNA in pine marten and stone marten scat DNA extracts using the SYBR® Green Dye qPCR assay

Only DNA extracts with C_T < 22 in the qPCR TaqMan® MGB probe based marten species assays were initially tested for bat DNA (n=146). Lower C_T values in qPCR indicate higher quantity of starting DNA template and, as both the marten probe based qPCR assay and the bat species qPCR SYBR® Green Dye assay target mitochondrial DNA then the quantity of

predator DNA should be a good indicator of prey DNA quality. Predator DNA will be found in scats in higher quantities than prey DNA (Deagle *et al*. 2005) so qPCR C_T values for bat DNA detection would be expected to be higher. Initially samples with higher C_T values in TaqMan® MGB probe based marten species assays were included for analysis but due to limited time and resources focus was aimed at the best quality DNA extracts. However, the number of samples selected for dietary analysis was later extended by 14 samples to include samples with a higher C_T value in the marten Taqman® MGB probe based qPCR assay which yielded genotypes. This gives a total sample size of 160 marten scats. 25 fox scats and 2 European polecat scats were also included for dietary analysis.

Assays were performed using either existing qPCR primer sets (Table 4.2) which amplify DNA from some of the bat species found in and around Nietoperek or using the primers designed in the course of this project. Assays included bat species not found in the tunnels but present above ground in Nietoperek.

Table 4.2 Existing qPCR primer pairs used to detect bat DNA in marten scat DNA extracts.

Target species	Primer	Sequence	Region	Product	Reference
Barbastella barbastellus	BbarcytbF	CACCTCCTATTCCTACACGAAACA	Cytb	79bp	A. Harrington (unpublished)
	BbarcytbR	GGGTGGAATGGGATTATATCTACG			
Myotis mystacinus	MmystcytbF	TTCCTAGCTATACACTATACGTCAGATACT	Cytb	92bp	A. Harrington (unpublished)
	MmystcytbR	TTACGGCTGAGTCCTACGC			
Myotis brandtii	MbracytbF	CAATTCCGTACATTGGAACAGACCTT	Cytb	75bp	A. Harrington (unpublished)
	MbracytbR	TGTTGACAAAGCTACTTTGACCCG			
Nyctalus leisleri	NleicytbF	TTGGAACAGATCTTGTTGAATGAATC	Cytb	77bp	A. Harrington (unpublished)
	NleicytbR	GAAAGGCGAAAAATCGAGTTAGAGTA			
Nyctalus noctula	NnoccytbF	GCCGACCTTGTTGAGTGAATTTGA	Cytb	75bp	A. Harrington (unpublished)
	NnoccytbF	AAGTGAAAGGCGAAAAATCGAGTTAGG			
Pipistrellus nathusii	PnatcytbF	GGTGGCTTTATCTACAGAAAAACCA	Cytb	81bp	A. Harrington (unpublished)
	PnatcytbF	CAATTTACTCTCCGCAATCCCA			
Pipistrellus pipistrellus	PpipcytbF	AACCGCCTTCAGCTCCGTTACT	Cytb	70bp	A. Harrington (unpublished)
	PpipcytbR	CGTGTAGGTATCGTAGAACTCATCCG			
Pipistrellus pygmaeus	PpygcytbF	GGATCCCTATTAGGCATCTGTCTAGGGCTG	Cytb	92bp	A. Harrington (unpublished)
	PpygcytbR	CTGAAGGCTGTTGCTGTATCTGACGTGTAGTGTATA			
Plecotus austriacus	PauscytbF	CGTATATTGGAACAACTCTAGTAGAATGA	Cytb	83bp	A. Harrington (unpublished)
	PauscytbR	GGAATGCGAAGAATCGAGTC			

The same qPCR protocol was used for all dietary analyses. Each reaction contained; 5 µl of FastStart Universal SYBR Green Master , 0.5 µl of primer mix (forward and reverse) at 0.25 µM final concentration, 2 µl of molecular grade water and 3 µl of template DNA. DNA of known origin was used as a positive control and PCR blanks containing all reagents without template DNA were used to monitor for contamination. qPCR amplifications were performed on the ABI 7300 Real-time PCR system using the standard protocol as described in section 3.5.7.5.2.

4.3 Results

4.3.1 Design of qPCR primers for detection of bat species found in the tunnels in Nietoperek

The ND1 region was targeted with primers pairs to amplify DNA from; *Myotis myotis* (91 bases), *Myotis daubentonii* (55 bases), *Myotis dasycneme* (53 bases), *Plecotus auritus* (51 bases) and *Eptesicus* species (60 bases). It was not possible to design primers that target mtDNA which will successfully discriminate between *Eptesicus serotinus* and *Eptesicus nilssonii* as in this part of Europe these two species share a large portion of mtDNA as a result of a past hybridisation event (Artyushin *et al.* 2009). Therefore the primer pair designed amplifies DNA from both species and any positive samples would be selected for sequencing. An 83 base pair Cyt b region was selected as target for amplification of *Myotis bechsteinii* DNA as it contained the most conserved region within this species and the most variabilty in the target area between other species. Primers that amplify a 94 base region of COI in *Myotis nattereri* were selected as this portion of the COI was highly conserved within species with intraspecies variability. Figure 4.2 shows the alignement of mitochondrial ND1 sequences over the 91 base region which the *Myotis myotis* specific primers will amplify, highlighting the variability of sequences in this region between the different *Myotis* species found in the tunnels. Table 4.3 contains a list of the novel primer pairs designed during this study with the primer sequences, mtDNA target region, and product size included.

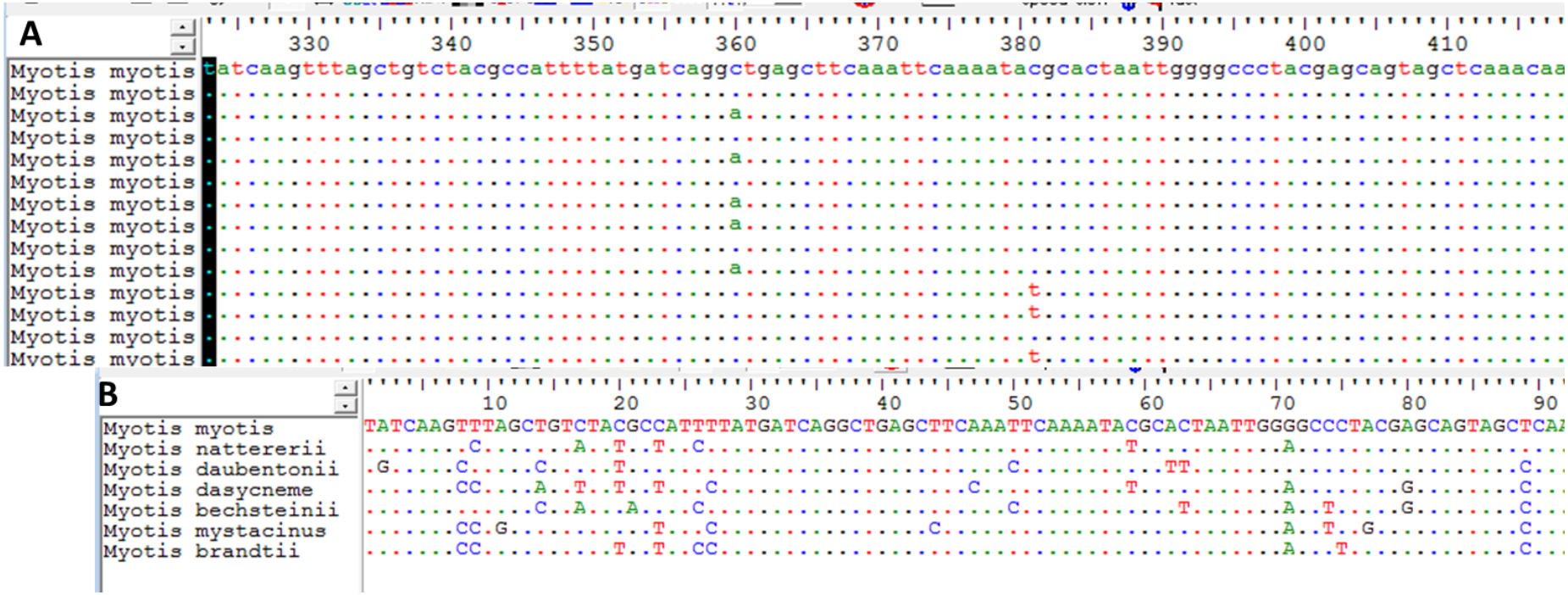


Figure 4.2 Sample alignment in BioEdit of ND 1 sequences from *Myotis* DNA sequences over the 91 base target region (starting base 323 ending base 414) by the primer pair MymyF and MymyR in *Myotis myotis* DNA. A= *Myotis myotis* sequences including the conserved primer regions on both flanks. B= Sequences from all *Myotis* species found in the tunnels including the inter species variability in the primer regions on both flanks. Dots signify identical sequences. Mismatches are noted by the symbol of the differing base.

Table 4.3 List of novel primer pairs designed to detect bat DNA in marten scat DNA extracts using qPCR.

Target species	Primer	Sequence	Region	Product
Myotis sp	MYF1	GATCAGGCTGAGCTTCAAATTCAAAAT	**ND1**	67bp
	MYR1	ATTGTTTGGGCTACTGCTCG		
Myotis myotis	MymyF	TATCAAGTTTAGCTGTCTACGCCATTTT	**ND1**	91bp
	MymyR	TTGAGCTACTGCTCGTAGGG		
Myotis daubentonii	MydbF	AGCCCAAACAATTTCCTACGAAGT	**ND1**	55bp
	MydbR	GTAGGATGGATAGAATAATAATGGCAAGAG		
Myotis bechsteini	MybchF	GGAATCTGCCTAACACTACAAATCACA	**Cytb**	83bp
	MybchR	CATGGAGGTAGCGCAGG		
Myotis nattereri	MynatF	CCCGCACTTTCTCAATATCAAACACC	**COI**	94bp
	MynatR	CGGCAGCTAGAACTGGAAGAGAAA		
Myotis dasycneme	MydscF	CCTACTCTAGCTTTAACCCTAGCTTT	**ND1**	53bp
	MydscR	GGATATGGTATGGGTAGTGGAATTCAT		
Plecotus auritus	BLEF	TCTAGGAGCATACAAGACCCCAA	**ND1**	51bp
	BLER	AGGCTGATGGTGAATATTTCTGGG		
Eptesicus sp.	EptF	CATTATTGCACCCACTTTAGCCC	**ND1**	60bp
	EptR1	GTGGCATGGGTAGTGGAATTCA		

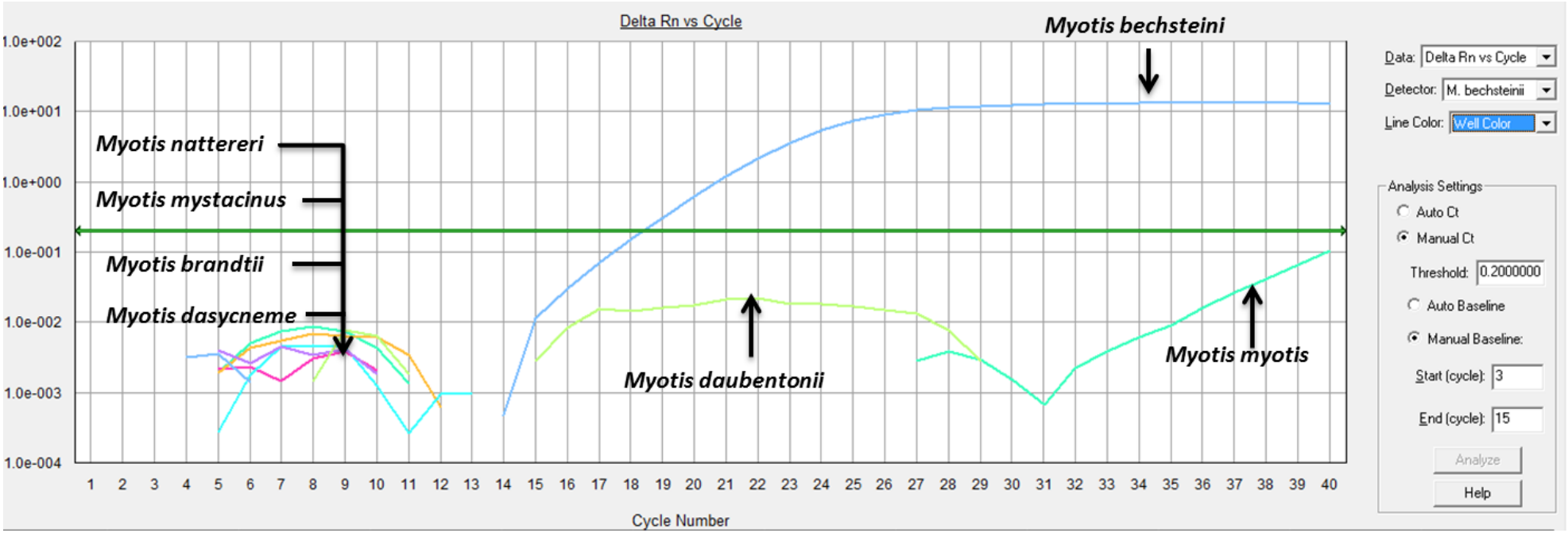


Figure 4.3 Sample plot from qPCR primer trial using MybchF and MybchR to amplify *Myotis bechsteinii* DNA and monitor for cross species amplification. DNA extracts of known origin were used in primer trials.

Figure 4.3 shows a sample qPCR amplification plot from trials of the primer pair MybchF and MybchR designed to target DNA from *Myotis bechsteinii*. The primers successfully amplified DNA from *Myotis bechsteinii* with no cross-amplification of DNA from the other *Myotis* species found in the tunnels.

4.3.2 Detection of bat DNA in pine marten and stone marten scat DNA extracts using the SYBR® Green Dye qPCR assay

Positive samples were defined as DNA extracts in which bat DNA was detected with a C_T value <= 35.0 and with a T_M the same as the positive control (+/- 0.5 °C) using the SYBR® Green Dye qPCR assay. Through screening of the qPCR TaqMan® MGB probe based assays to detect marten DNA, 160 marten samples were selected for dietary analysis.

From screening with the primer pair MYF1 and MYF2 which amplify *Myotis* genus DNA using a SYBR® Green Dye qPCR assay 64 of the 160 marten scat DNA extracts were positive for *Myotis* genus DNA. All 64 of these DNA extracts were then attributed to *Myotis* species in SYBR® Green Dye qPCR assays using *Myotis* species specific primer pairs (Figure 4.4).

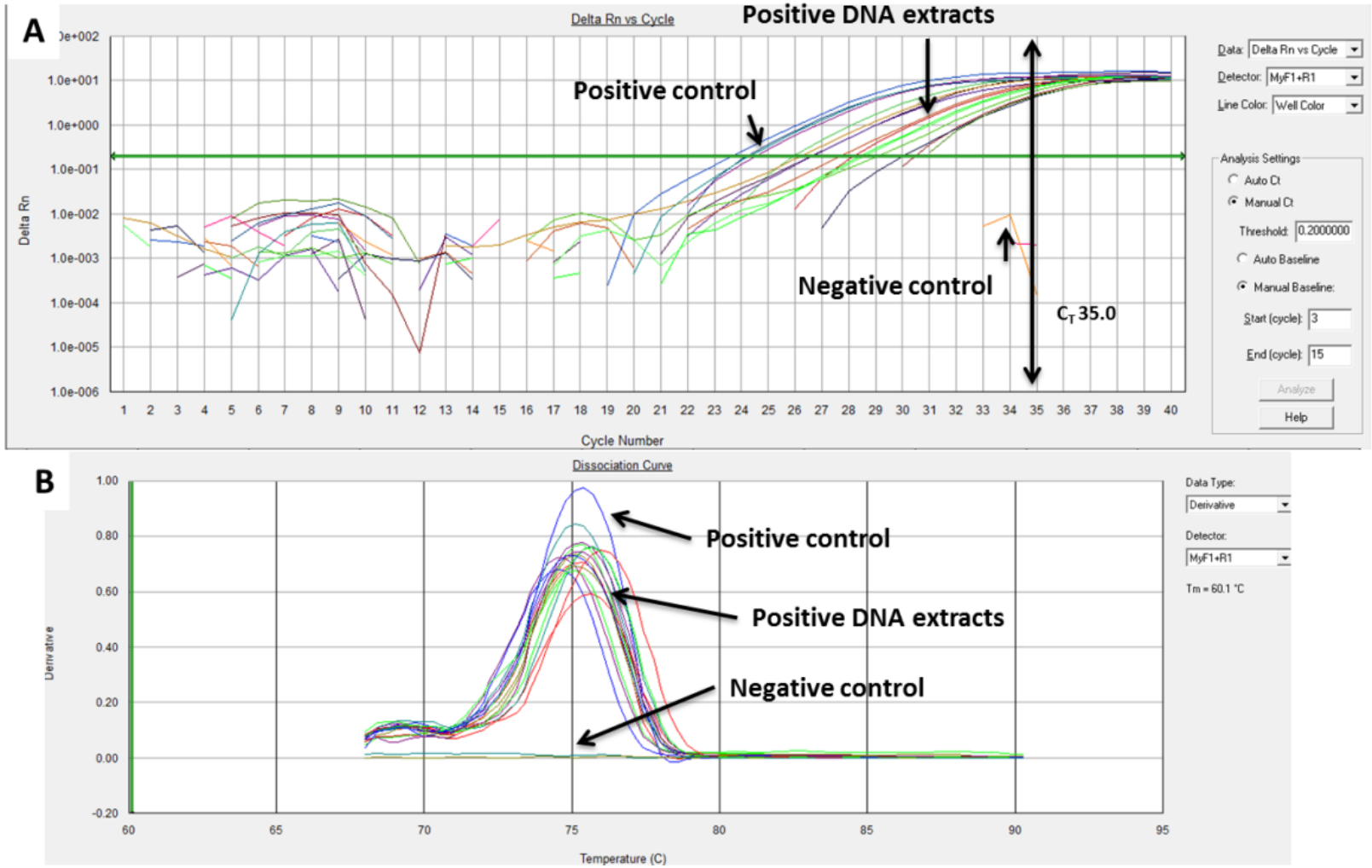


Figure 4.4 (A) Sample plot from SYBR® Green Dye qPCR assay to detect *Myotis* DNA in marten DNA extracts using the primers MYF1 and MYR1. Arrow indicates cut-off for positive DNA extracts (C_T 35.0). (B) Melting temperature analysis of SYBR® Green Dye qPCR assay showing the T_M of positive DNA extracts in relation to the positive control. T_M range 74.8 – 75.8 °C.

Of the 160 marten DNA extracts selected for dietary analysis, 74 were from stone marten while 86 were from pine marten. 37 (50%) of stone marten scat DNA extracts were positive for bat DNA. 32 (37%) pine marten scat DNA extracts were also positive. For marten scat DNA extracts that tested positive for bat DNA, the majority had been collected below ground, with 86% of positive stone marten and 69% of positive pine marten scats collected in the tunnels. 62% of stone marten scats and 78 % of pine marten scats that contained no target DNA during dietary analysis were collected above ground (Table 4.4). No DNA extracts from fox of from European polecat tested positive for any bat species' DNA.

Table 4.4 Summary of pine marten and stone marten scat DNA extracts tested for bat DNA; SM=stone marten, PM=pine marten, above=above ground, below=in the tunnels, Bat+=scat DNA extracts positive for bat DNA, Bat- = scat DNA extracts negative for bat DNA.

	Scats	Bat+ total	Bat+ below	Bat+ above	Bat- total	Bat- below	Bat - above
SM	74	37	32	5	37	14	23
PM	86	32	22	10	54	19	35

Table 4.5 shows a breakdown of the marten scats which were positive during the SYBR® Green Dye qPCR assays for the various bat species found in and around Nietoperek. Only bat species found in the tunnels were detected in pine marten or stone marten scats.

Table 4.5 Marten scats positive for bat DNA by species; SM=stone marten, PM=pine marten, A=above ground, B=tunnels, bat species found in the tunnels. * *Eptesicus sp* refers to both *Eptesicus serotinus* and *Eptesicus nilssonii.*

Bat species	Total	PM	SM	F	EP
Myotis daubentonii	27	12	15	0	0
Myotis myotis	18	7	11	0	0
Myotis nattereri	14	9	5	0	0
Myotis bechsteini	2	0	2	0	0
Myotis mystacinus	3	3	0	0	0
Myotis dasycneme	0	0	0	0	0
Myotis brandtii	0	0	0	0	0
Barbastella barbastellus	3	0	3	0	0
Plecotus auritus	2	1	1	0	0
****Eptesicus sp***	0	0	0	0	0
Nyctalus leisleri	0	0	0	0	
Nyctalus noctula	0	0	0	0	
Pipistrellus nathusii	0	0	0	0	
Pipistrellus pipistrellus	0	0	0	0	
Pipistrellus pygmaeus	0	0	0	0	
Plecotus austriacus	0	0	0	0	

The 160 marten scat DNA extracts which passed screening comprised scats from various survey periods in different numbers (Figure 4.5). This variation ranged from 5 scats in the April 2011 survey to 38 scats collected during the May 2013 survey. 58% of scats selected from this survey period tested positive during dietary analysis. Only 6 marten scats from January 2014 were selected for dietary analysis through the screening process and none of these scat DNA extracts tested positive for any bat species' DNA.

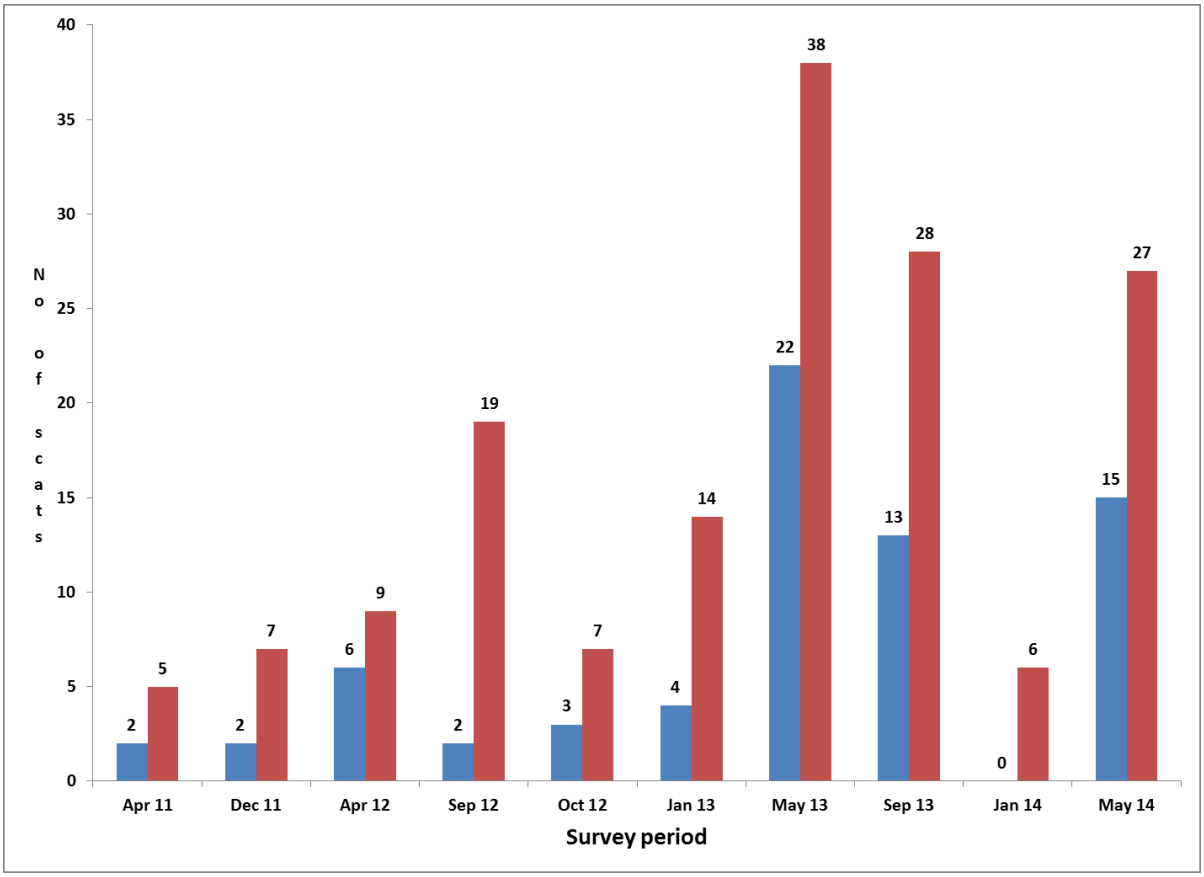


Figure 4.5 Survey breakdown of marten scats tested using SYBR® Green Dye qPCR for bat species' DNA. Total number of scat DNA extracts tested from each survey. Number of scat DNA extracts positive for bat species' DNA.

4.4 Discussion

4.4.1 Design of qPCR primers for detection of bat species DNA in marten scat DNA extracts.

Novel qPCR primers were designed to detect bat DNA in scat DNA extracts. *Myotis* genus specific primers were designed to amplify target DNA from all *Myotis* species found in the tunnels with no cross amplification of non-*Myotis* species DNA. Seven novel species specific primer pairs were successfully designed to target bat species present in the tunnels (Table 4.3) and used with existing primer pairs (Table 4.2) to test marten DNA extracts for the presence of DNA from all bat species found in and around Nietoperek.

4.4.2 Detection of bat DNA in scat DNA extracts

4.4.2.1 Bat DNA present in scat DNA extracts from pine marten and stone marten

A total of 69 of the 160 scat DNA extracts selected for dietary analysis tested positive for bat DNA. The bat species detected in marten DNA extracts can be seen in Table 4.5. The most abundant bat species detected in marten scat DNA extracts was *Myotis daubentonii* at 39% (n=27) while 26% (n=18), and 20% (n=14) were from *Myotis myotis* and *Myotis nattereri* respectively. This is to be expected given these three species constitute most of the entire hibernation population of Nietoperek and are also found hibernating throughout the system during winter censuses (REPD, 2015). These 3 species were found most frequently in scats from both pine marten and stone marten (Table 4.5). Of the other bat species hibernating in the system 5 species were present in scats in small numbers. No scat DNA extract was positive for DNA from *Myotis dasycneme*, *Myotis brandtii* or *Eptesicus serotinus*. The number of individuals of these three species found in the tunnels each year is very low however with average counts of 29, 27 and 10 (2011-2015) individuals respectively from the approximatley 37 000 bats found there annually. No scat DNA extract was positive for bat DNA from any species of bat not found in the tunnels.

Predator DNA will be found in scats in higher quantities than prey DNA (Deagle *et al*. 2005) so qPCR C_T values for bat DNA detection would be expected to be higher. In controlled dietary studies where the amount of a prey item consumed as well as the time at which it has been eaten and the time between and consumption and scat deposition may be recorded, the relationship between quantities of predator DNA and quantities of prey species DNA should remain relatively constant. During this study however, there was no way of knowing when a marten had consumed a bat, or how much of the bat the marten had eaten so considerable variation in the quantities of prey DNA present in marten scat DNA extracts was expected. Figure 4.6 shows a sample set of C_T values from marten species TaqMan® MGB probe based qPCR with their corresponding C_T values when tested for *Myotis* genus DNA using the SYBR® Green Dye qPCR assay.

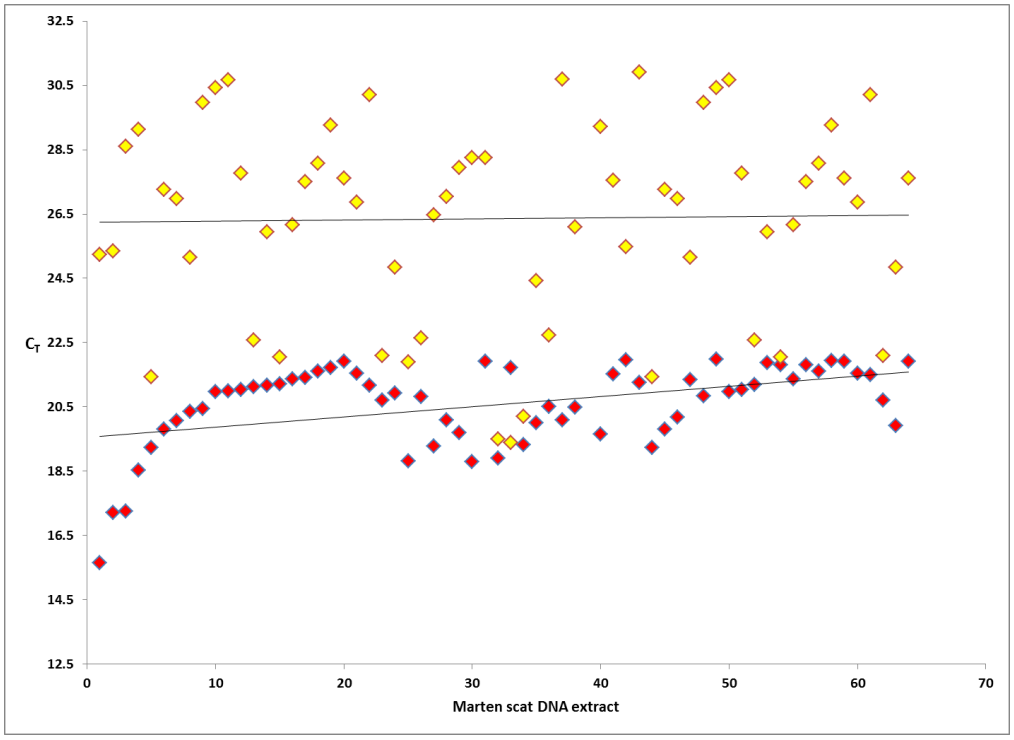


Figure 4.6 Comparison of predator DNA C_T values and prey DNA C_T values. Average ΔC_T = 5.9. ◆ C_T value of DNA extract when assayed for marten DNA using probe based qPCR assay. ◆ C_T value of DNA extract when assayed for *Myotis* genus DNA.

The average ΔC_T for predator and prey DNA was found to be 5.9 cycles. However, as expected, the difference between predator and prey DNA quantity ranged from -2.3 to 11.3 indicating a high degree of variability in the quantity of prey DNA detected in marten DNA extracts. It is worth noting that this was not a controlled dietary study. Therefore it is not possible to tell how much of a particular bat a marten consumed. It is also not possible to tell the length of time between this bat consumption and the marten depositing the scat. In each case; a small amount of bat consumed, or a long period of time between consumption of a bat and the deposition of a collected scat, the bat DNA would be present in smaller quantity.

Of the 27 non-marten scats selected for dietary analysis, 2 were from European polecat and collected in the tunnels. Both scats were negative for DNA from all bat species but this is a very small sample size and no inferences are made from this result. The 25 fox scat DNA extracts were also negative for DNA from all bat species. Both European polecat and fox have been found to predate bats (Malecha, & Antczac, 2013; Dreissens & Siemers, 2010). All scats attributed to foxes were collected above ground (section 3.3.7). The fact that no fox scat contained bat DNA strengthens the suggestion that bat consumption is occurring down in the tunnels.

4.4.2.2 Bat DNA in marten scats identified as being from individual animals

All 95 scat DNA extracts that yielded genotypes in section 3.3.9 were selected for dietary analysis. A total of 49 of the scats identified as being from individual animals tested positive for bat DNA. These 49 scat DNA extracts represented 25 individuals (Figure 4.7). Scats from 10 pine marten individuals out of a total of 20 individuals identified contained bat DNA. Scats from 15 stone marten out of a total of 21 individuals identified throughout the project contained bat DNA.

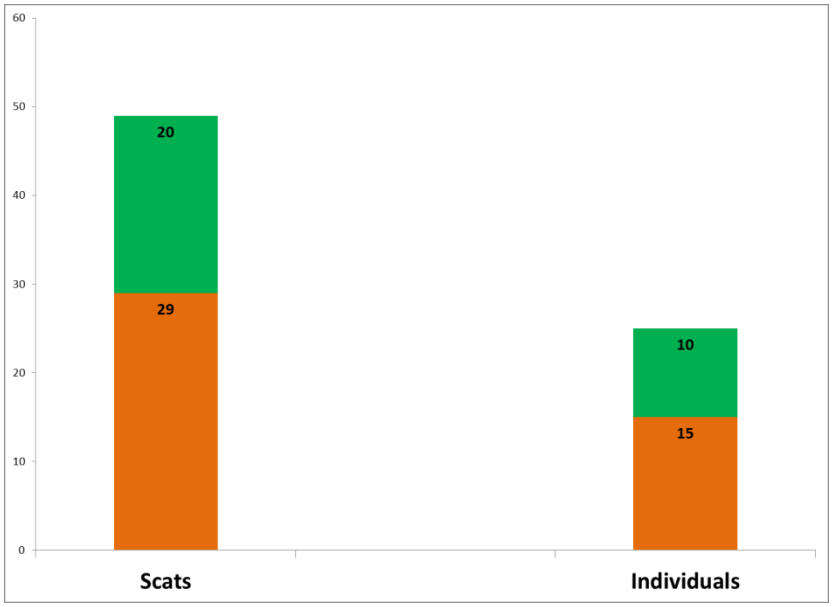


Figure 4.7 Scats from individual marten which tested positive for bat DNA during qPCR pine marten stone marten. Wilson prevalence estimate = 0.592 for scats and 0.6 for individuals C.I = 0.95.

Figure 4.8 shows the sex ratio of marten scat DNA extracts positive for bat DNA. Of the 49 scat DNA extracts identified as being from individual marten 30 (61%) were from female animals. The proportion of stone marten scats from males and females which tested positive for bat DNA was similar with slightly more male scats 55% containing bat DNA. Of the 20 pine marten scat DNA extracts attributable to an individual animal, 17 (85%) were from females (Figure 4.8 A). When compared with the sex ratio of individual animals responsible for each scat DNA extract containing bat DNA, again slightly more female individuals' scat DNA extracts contain bat DNA. With stone marten individuals, it appears more males are responsible for bat consumption than females with 9 male stone marten found to have consumed bats and only 6 female stone marten. The sex bias evident in the total pine marten

genotyped scats containing bat DNA is also present in the number of individuals responsible for these scats with the 3 male pine marten scats (Figure 4.8 A) representing only 2 male pine marten (Figure 4.8 B) and the 17 female pine marten scats representing 8 female pine marten individuals. It is not clear why there is a sex bias in one species and not in the other but perhaps this sex bias reflects differing factors leading to each species' presence in the tunnels.

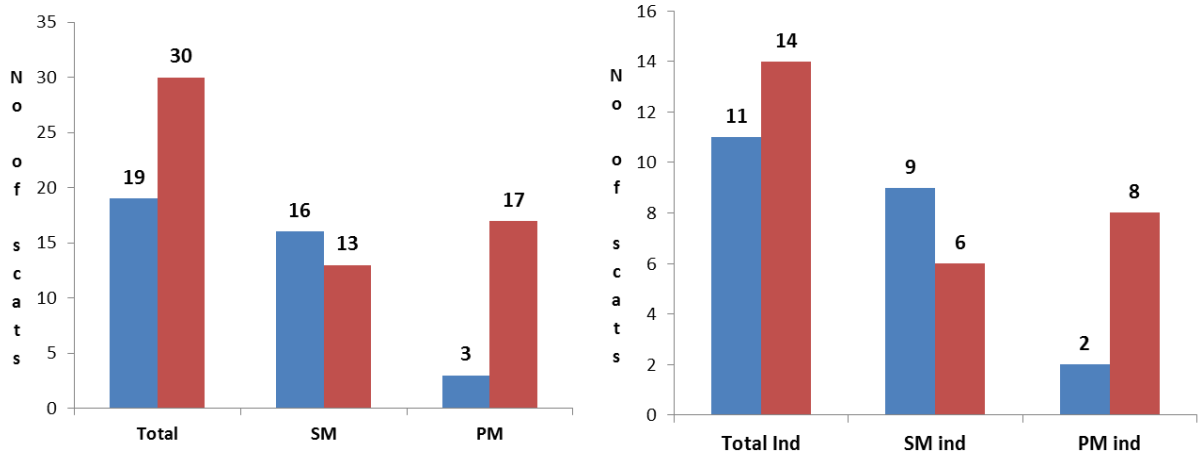


Figure 4.8 (A) Sex ratio of scat DNA extracts from individual marten positive for bat DNA, Total= The total number of scats identified as being from an individual animal which contain bat DNA,SM= number of stone marten scats, PM=number of pine marten scats (B) Sex ratio of marten individuals responsible for depositing scats containing bat DNA, Total Ind = The total number of individual marten in whose scats bat DNA was detected, SM Ind= number of stone marten individuals, PM Ind= number of pine marten individuals; male, female.

The number of individual animals with scat DNA extracts containing bat DNA (Figure 4.9) mirrors the variation in the total positive DNA population by survey (Figure 4.5). In the earlier surveys only stone marten individuals were identified whose scats were found to contain bat DNA. This may however be a product of the areas these surveys covered which had more stone marten scats present. From January 2013 onwards when the entire system was surveyed, individuals from both pine marten and stone marten were identified as having consumed bats with similar frequency throughout the survey period.

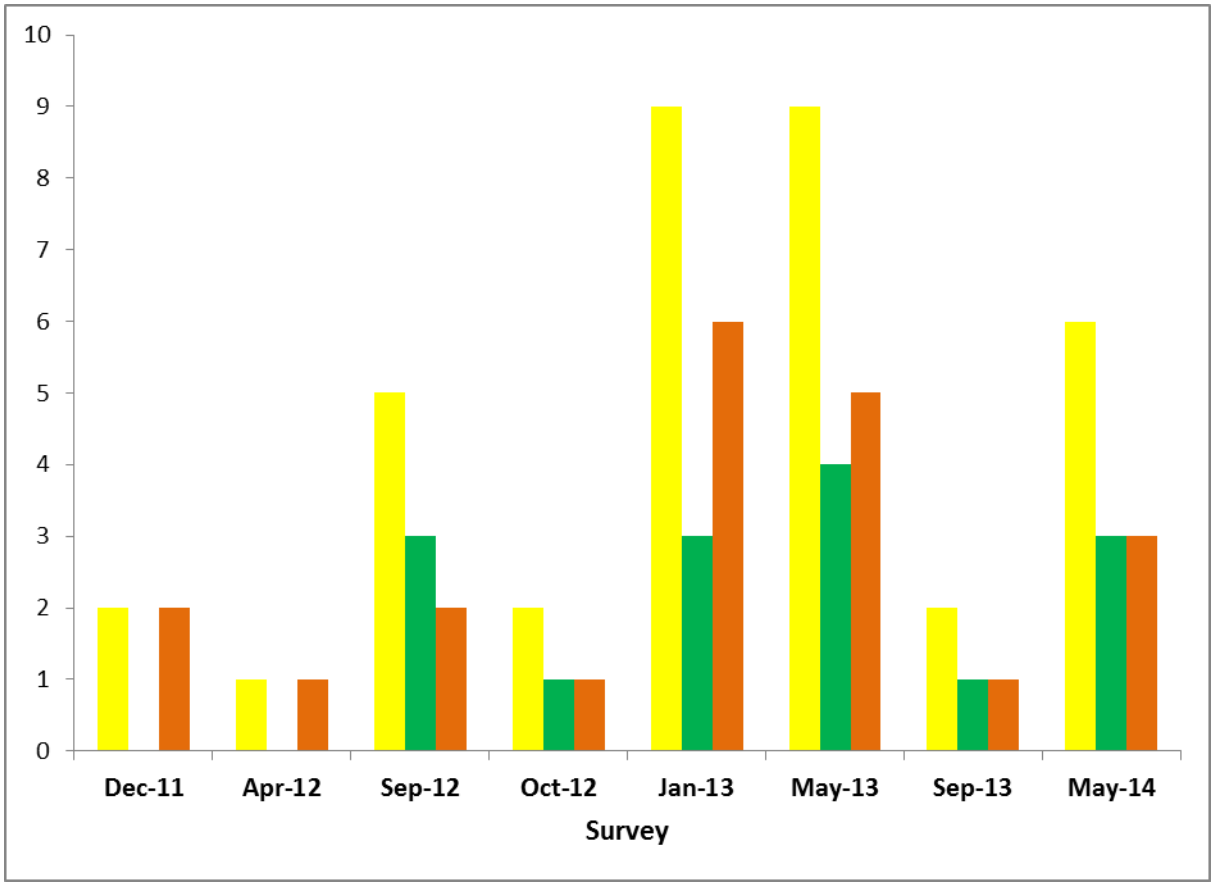


Figure 4.9 Individual marten identified as consuming bats during each survey Total animals pine marten stone marten, January 2014 results show no marten scat containing bat DNA.

From the distribution of scats identified as being from individual animals in section 3.3.10 not all identified individuals were detected during each survey. Some individuals were only detected during a single survey and others were detected sporadically throughout the project timeframe. Scats from both pine marten and stone individuals that were found to contain bat DNA mirror this pattern (Table 4.6). For example scats from S13 only contained bat DNA during a single survey even though the animal was detected in more than one survey. There were scats from S16 positive for bat DNA in December 2011 and again in January 2013 suggesting marten consumption, by predation or scavenging, is an on-going occurrence. P1 was identified as consuming bats during the September 2012 survey. This animal was only detected during this survey and only in one scat DNA extract. If this animal was a transient pine marten then this result suggests entrance into the tunnels to consume bats may be common to all marten in the survey area. P6 was detected in the tunnels 5 times across three survey periods but scats from P6 contained bat DNA in only two of these survey periods. The fact that scats from P6 have been found in the tunnels and were negative for bat DNA may not be used as a suggestion that this marten was not in the tunnels to consume bats however

as it is possible that any marten scat negative for bat DNA and found in the tunnels was deposited prior to the consumption of a bat. From Table 4.6 it may however be concluded that the individual marten responsible for the consumption of bats varied in both species throughout the survey period.

Table 4.6 Summary of bat DNA positive scat DNA extracts from individual marten by survey, stone marten pine marten.

Survey	Individual stone marten						Individual pine marten			
Dec-11	S16	S13								
Apr-12	S10									
Sep-12	S18	S20					P1	P4	P6	
Oct-12	S11						P5			
Jan-13	S20	S8	S16	S11	S1	S6	P5	P10	P20	
May-13	S2	S3	S18	S9	S15		P6	P12	P2	P3
Sep-13	S21						P16			
Jan-14										
May-14	S5	S20	S18				P16	P18	P20	

4.4.3 Distribution of marten scats assayed for bat DNA

Figure 4.10 shows the distribution of marten scats that contained no bat DNA from any species. Scats that were negative for bat DNA were collected both above ground and in the tunnels. The scats collected in the tunnels which were negative for bat DNA were from both pine marten and stone marten. All scats collected above ground in the Northern Forest (Figure 4.10 A) which is not directly above the tunnel system were negative for bat DNA ($P < 0.001$). This result may serve to re-inforce the initial hypothesis that bat consumption is occurring in the tunnel system and not in the surrounding area above ground.

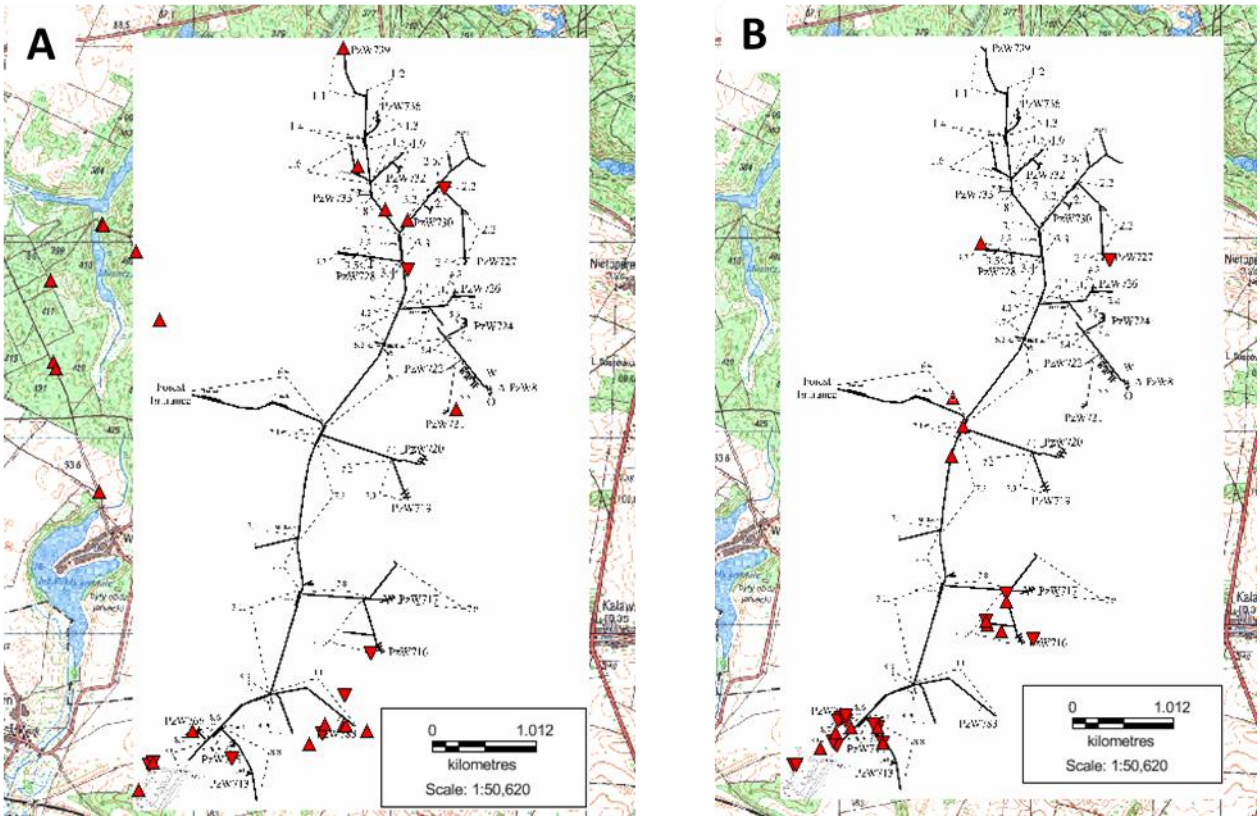


Figure 4.10 Location of scats negative for bat DNA using SYBR® Green Dye qPCR. A= pine marten scats B= stone marten scats ▲ above ground, ▼ tunnels.

The distribution of pine marten and stone marten scats positive for bat DNA can be seen in Figure 4.11 A and B respectively. Scats from both marten species tested positive for bat DNA throughout the tunnel system. Scats from both marten species also tested positive for bat DNA were also collected in areas above ground which are directly above the tunnels. The distribution of scats positive for bat DNA from both marten species largely reflects the marten species distribution discussed in Chapter 2 with positive pine marten scats found in the north and south of the system (Figure 4.11 A) and positive stone marten scats found mostly in the centre and south of the system (Figure 4.11 B). Positive pine marten scats were detected either in or above sections 1,3,5,8 and 9. These are sections that showed high pine marten scat density and indicate pine marten consumption of bats is not limited to any particular section but is prevalent throughout the system.

Stone marten scats from both above and below ground tested positive for bat DNA with the majority of scats being collected in the tunnels. One positive stone marten scat was collected above section 3 but most of the scats were either collected in or around sections 5,6,7,8 or 9. These sections contain most of the total stone marten scats and again indicate that stone marten consumption of bats is prevalent throughout the tunnel system.

The presence of marten scats positive for bat DNA above ground does not preclude the marten having consumed the bat in the tunnels. The fact that most of the positive samples were found in the tunnels however may give some indication to the frequency or longevity of visits to the system as scats containing bat DNA are being deposited while the offending marten is either still in the system or had returned in the period of time it has taken for the bat DNA to pass through its intestinal tract.

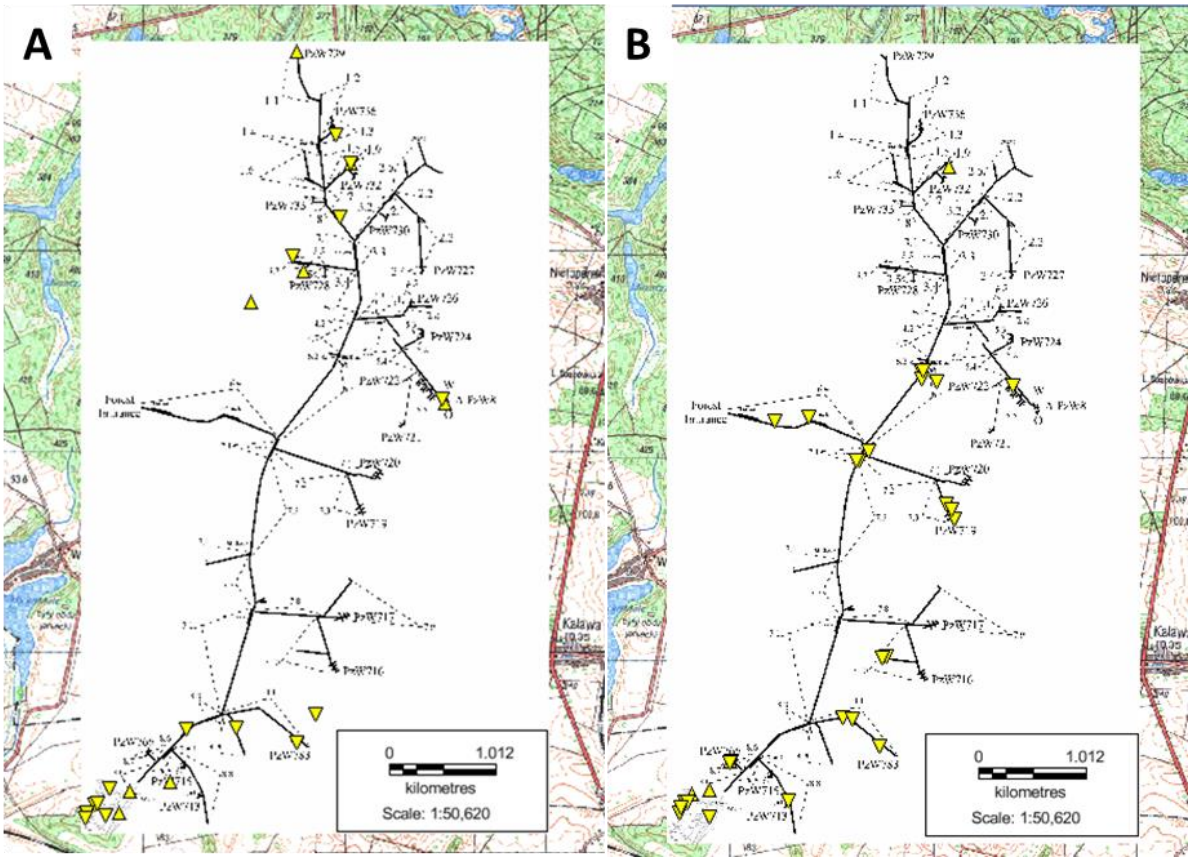


Figure 4.11 Location of marten scats positive for bat DNA, A= pine marten scats, B= stone marten scats ▲ above ground, ▼ tunnels.

4.4.4 Distribution of marten scats positive for bat DNA by bat species

Figure 4.12 shows the location of all marten scats positive for bat DNA by bat species. There is variation between which species are detected in each section.

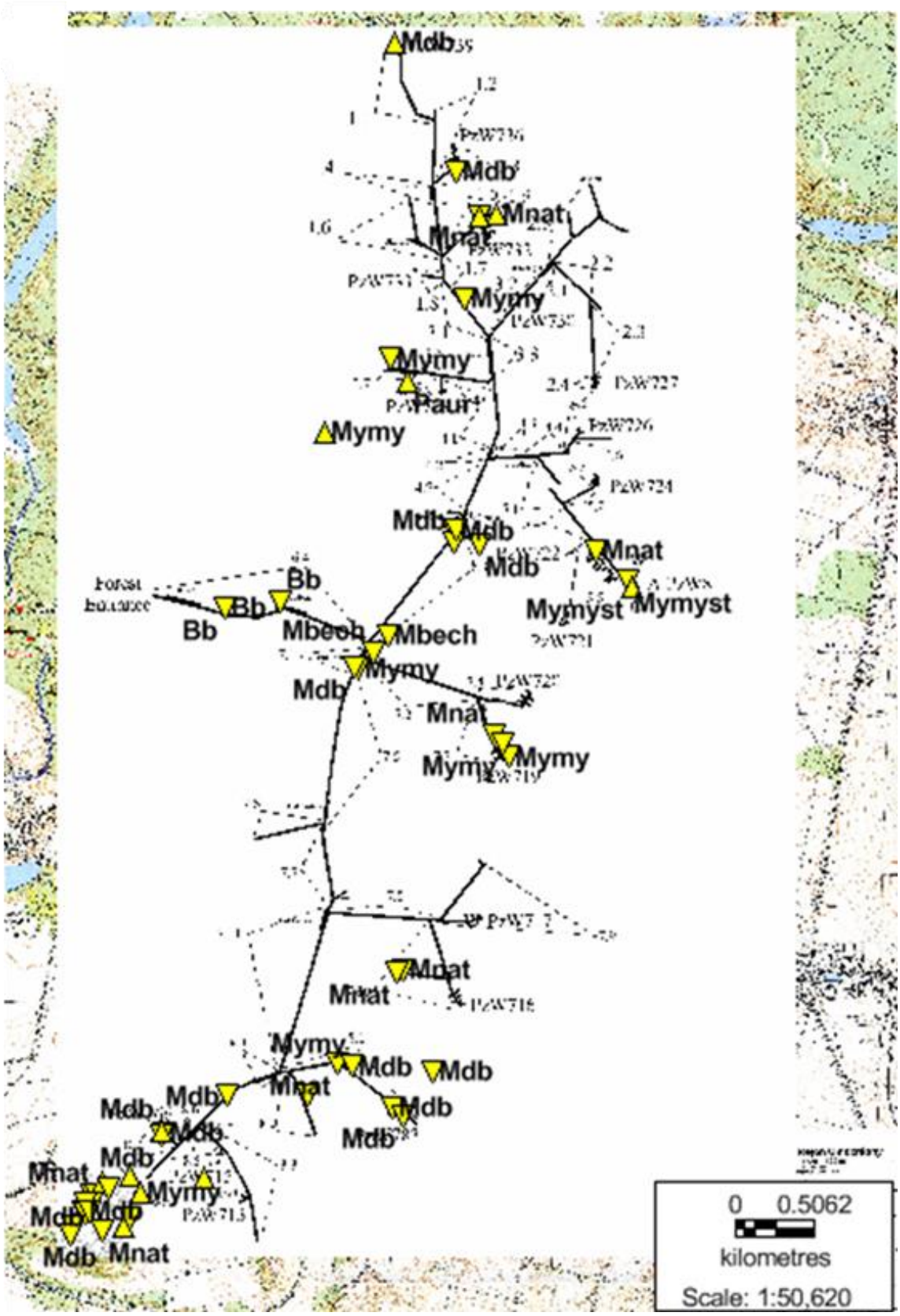


Figure 4.12 Location of scats from both marten species positive for bat DNA by bat species, species acronyms are as in Table 4.1 ▲ above ground, ▼ tunnels.

Myotis daubentonii DNA for example, was detected in scats from the north of the survey area above section 1. It was also detected in scat DNA extracts collected in the centre of the system and again in the far south of the system. *Myotis daubentonii* is found in abundance throughout the tunnel system during annual bat censuses. This is also true for *Myotis nattereri* and *Myotis myotis* and scats containing DNA from both these species were also found throughout the system. On the other hand some species were only ever detected in marten scat DNA extracts from a single section. In section 6.4 three scats were found to contain *Barbastella barbastellus* DNA. Approximatley half the bats from this species that hibernate in the tunnels each year are found in this section (R.F.P.D, 2015). These results suggest that marten consumption of a particular bat species is related to the distribution of this species.

4.4.4.1 Survey and species variation in marten scats positive for bat DNA

Every effort was made to collect all scats in a section of tunnels during each survey so it is reasonable to assume that any scat collected in any particular survey was deposited in the time since the previous survey with the exception of the first survey of any one section.

Survey variation in scat densities in the tunnels as discussed in Chapter 3 is reflected in the number of scats from each survey period which passed the screening process for dietary analysis (Figure 4.5). Only a small number of scats were positive for bat DNA from earlier surveys but it must be noted that the first complete survey of the tunnels was not until January 2013 and previous scat surveys only covered a small area of the system. During these surveys the proportion of scats collected that contain bat DNA is variable. 66% of scats tested from the April 2011 survey contained bat DNA. A high proportion of positive scats was expected for this survey as scats collected during this survey could have been deposited any time from the previous December during a time when bats are hibernating in the tunnels. The proportion of scat DNA extracts positive for bat DNA was found to be much lower for the September 2012 survey where only 10.5% of samples tested contained bat DNA. This result is in partial agreement with the initial hypothesis that marten are entering the tunnels to prey on hibernating bats as the proportion is much lower. However 2 of the 19 scats tested did contain bat DNA so some consumption of bats has occurred at a time when bats are not hibernating. As expected, the proportion of scats positive for bat DNA increased during surveys in which the collected scats may have been deposited during bat hibernation with 58% of scats from the May 2013 survey testing positive for bat DNA. Any scat collected

during this survey would have been deposited at any time from the previous January with bat hibernation continuing until spring. These higher proportions over the winter months were again in agreement with the initial hypothesis on why marten were entering the tunnels.

However, of the scats tested from the September 2013 survey, 13 of the 28 (46%) were found to contain bat DNA. This result is contrary to the initial hypothesis and suggests that marten, whether through predation or scavenging are consuming bats in the system at times of year when the bats are not hibernating. It is worth noting that bats are present in the tunnels all year long. While the majority of the bats hibernating in the system do not stay in Nietoperek all year there are resident bats from various species in the tunnels and there is a *Myotis myotis* colony of approximately 3000 bats that use a portion of the far south of the system as a maternity roost.

No marten scat collected during the January 2014 survey tested positive for bat DNA. As discussed in Chapter 3, the scat density in the tunnels for both marten species was lowest during this survey (Table 3.20). While samples were positive for both pine marten and stone marten DNA, few had marten species qPCR assay C_T values less than 22.0 (n=6).

There was also some variation in the bat species detected within pine marten and stone marten scat DNA extracts. *Myotis daubentonii* occurs with nearly the same frequency in scats from both species; *Myotis myotis* occur slightly more in stone marten scats and *Myotis nattereri* occurs more in pine marten scats. *Barbastella barbastellus* and *Myotis bechsteini* DNA was only found in stone marten scats and *Myotis mystacinus* was only detected in pine marten scats but it is noteworthy that the sample size is small for these species. *Plecotus auritus* DNA was detected in one pine marten scat and one stone marten scat (Table 4.5).

4.4.5 The impact on the bat population of Nietoperek from the presence of marten in the tunnels

The highest number of pine marten and stone marten scats found to contain bat DNA occurred in 2013 with 39 marten scats collected and found to contain DNA from one of the bat species present in the tunnels (Figure 4.5). It is noteworthy that the total number of scat DNA extracts which contained bat DNA as well as the total scat numbers positive for each bat species are very small and therefore only limited inferences can be made. However, this section will discuss the potential impact of marten consumption on bat species hibernating in Nietoperek.

Assuming a single bat per marten scat 39 positive scats would reflect a detected consumption of approximately 0.1% of the total bat population in Nietoperek. The only calendar year covered by this survey was 2013 and during surveys conducted in 2013 a total of 557 scats were collected that were positvely identified as being from pine marten or stone marten (section 3.3.4). 413 of these scats were collectd in the tunnels. Even assuming each one of these scats represented a bat consumption event, the number of bats consumed would still only reflect approximately 1.1% of the annual bat population present in Nietoperek during hibernation. DNA from *Myotis myotis*, *Myotis daubentonii* and *Myotis nattereri* occurred most frequently in marten scat DNA extracts (Table 4.5). While this result suggests that marten are consuming more of these species, the total number of each of these species found in the tunnels is considerably higher than the other bat species present and the percentage found in scats of the total numbers of each species is 0.06, 0.59 and 0.38% respectively (Table 4.7). Although the numbers of bats from these three species found in marten DNA extracts are all low, there is variation in the number of bats consumed by marten compared to the number of bats from each species present in the tunnels. The 18 *Myotis myotis* positive scat DNA extracts compared to the 29111 Myotis myotis present is a ratio of 0.0006 while the ratio for *Myotis daubentonii* and *Myotis nattereri* are higher at 0.006 and 0.004 respectively. While these numbers are all low the results suggest a preference for non- *Myotis myotis* bat species and may be indicative of predation rather than scavenging given the tendancy of these species to roost lower down than *Myotis myotis.*

Myotis daubentonii have been on the decline in Nietoperek since 1991 where there were 17000 individuals recorded, to 5000 individuals in 2005 (Sachanowicz & Ciechanowski, 2005), to around 4000 in 2011 and 4500 recorded in 2015 (R.E.P.D, 2015). Given the

frequency of *Myotis daubentonii* detection in marten scats, the fact that this species hibernates low to the ground (Kokurewicz, 2004) and the sighting of scratch marks in the system below positions known as *Myotis daubentonii* hibernation spots, it may appear that marten consumption of this species of bat is having a negative effect on its presence in Nietoperek. However, the numbers of *Myotis daubentonii* have not been in decline during the timeline of this project with an increase in numbers from 4000 in 2011 to 4500 in 2015. This suggests marten consumption was not responsible for previous decreases in *Myotis daubentonii*. Additionally, *Myotis daubentonii* has been increasing in other areas of Europe and in Poland with the Szachownica Cave showing an upward trend in *Myotis daubentonii* numbers (Lesinski *et al*. 2010) indicating a possible shift in hibernation location rather than a decline. It has been suggested that the apparent decline in *Myotis daubentonii* in some parts of Poland reflect changes in the numbers in local populations only (Fuszara *et al*. 1996). Future work in this area could include a marten abundance survey as well as a dietary study in and around the Szachownica Cave to investigate this further.

The next most common species of bats found in the tunnels are *Barbastella barbastellus* and *Plecotus auritus.* Again the number of marten DNA extracts positive for DNA from these species is low when compared to the total number of each species found in the tunnels. The percentage of the overall population of *Myotis bechsteinii* and *Myotis mystacinus* which occurred in marten DNA extracts is higher at 5.8 and 3.5% respectively but these species are found rarely in the tunnels and represent only a small number of positive marten scat DNA extracts; 2 positives for *Myotis bechsteinii* and 3 positives for *Myotis mystacinus*.

Table 4.7 Average total bat numbers recorded in the tunnels during annual censuses (2011-2015) and the percentage of each species population present in marten scat DNA extracts.

Species	Average no of each bat species found in the tunnels (2011-2015)	%
Myotis myotis	29111	0.06
Myotis daubentonii	4549	0.59
Myotis nattereri	3590	0.38
Plecotus auritus	461	0.4
Barbastella barbastellus	677	0.4
Myotis mystacinus	58	3.5
Myotis bechsteinii	34	5.8
Myotis brandtii	58	0
Myotis dasycneme	29	0
Eptesicus serotinus	9	0

The *Myotis bechsteinii* population in Nietoperek is low and the species is considered endangered and one of Poland's rarest breeding bat species (Sachanowicz *et al*. 2006). However, it is possible that both these scats contain DNA from the same bat. The two marten scat DNA extracts positive for *Myotis bechsteinii* DNA were both from stone marten scats and both scats were collected from a cluster of scats during the May 2013 survey suggesting they may possibly be from the same animal.

The results of these dietary analyses confirm marten are consuming bats but can give no insight into whether this consumption is a result of predation or scavenging. While the proportion of scat DNA extracts positive for bat DNA is relatively high (43%) the highest proportion of the overall bat population of Nietoperek detected in marten scats in any year is comparatively low at 0.1%, suggesting that while marten consumption of bats may be widespread, any impact from possible predation is low. This figure (0.1%) compares favourably with the figures found by Ruczynski *et al*., (2005) when assessing the non-predator non-accident mortality rates of bats inhabiting man-made shelters in Torun, Northern Poland. In this study it was found that the mortality rate from non-predation for *Myotis daubentonii* was very similar at 0.6%. The result for *Myotis nattereri* was again similar at 0.4% and and for *Myotis myotis* it was found to be 0.1% which is higher than the

number of positive marten scat DNA extracts containing DNA from this species. There were no results available from this study for comparison for the remaining species, and while the *Myotis bechsteinii* figure of 6% is high the overall sample size is small (n=34) and any scat containing DNA from this species would have a significant impact (Lower limit 0.8460 Upper limit 0.999 C.I 95%) on the overall percentages. The data from Torun is from non-predator related mortality and so a direct comparison may not be made between these two data sets but a comparison of the figures themselves shows that both in Torun, and Nietoperek the respective results are very low suggesting the impact in both cases is also very low. While a large proportion of the marten scats showed the presence of bat DNA, this figure is low in comparison to the resident bat population numbers. It may be concluded then, that if it is predation, and not simply scavenging on bat carcasses that is occurring, the impact on the bat population appears to be low. With the information available it is not possible to tell if it is predation or scavenging. The use of remote cameras positioned in the tunnels where the scratch marks have been found below hibernating bat positions, and in areas where scats positive for bat DNA have been collected, would potentially determine which of these things is occurring or if marten are both scavenging and predating bats.

As discussed in Chapter 3, marten in Nietoperek are in total darkness when in the tunnels and may be depositing scats near access points as a form of chemical auto-communication to navigate the tunnels without sight. Many species also use semio-chemistry as an olfactory mode of both predator and prey detection (Charpentier *et al.*2008) and it has been found that the presence alone of a predator in a habitat may have a negative effect on a prey population (Sheehy & Lawton, 2014). Therefore the presence of marten in the tunnels may have a negative effect on the bat population even if there is no predation occurring. It has been suggested however that bats have either lost, never possessed, or do not use the ability to recognise predator scents. Dreissens & Siemers, (2010) monitored the reactions of *Myotis myotis* to chemical compounds found in fox and mustelid faeces, and no avoidance of these chemical predator cues was observed. Possible reasons proposed for this phenomenon included; the hibernation sites often being in low risk areas deep in caves or high in trees, the volent nature of the animal, low predation pressure, and the fact that olfactory cues provide less temporal information on predation risk as the scent of a predator may linger in an area long after the predator has left. Therefore, the presence alone of marten in the tunnels should not have a negative effect on the resident bat population.

4.4.6 Possible reasons for marten presence in the tunnels

Reports of bats converging on the tunnels in Nietoperek over winter date back to the early nineteen seventies (Urbanczyk, 1981) and even from this date it had been suggested that marten may be predating these bats during their hibernation (Urbanczyk 1981; Bekker, 1988; Romanowski & Lesinski, 1991; Tryjanowsky, 1997; Tothe-Apathy, 1998). The results of this study confirm the presence of marten in the tunnels as well as the presence of bat species in both martens diet. It is not known however if marten are entering the tunnels specifically to consume bats or if bat consumption occurs while marten are in the tunnels for alternative reasons. This section will discuss other possible reasons for marten venturing into the tunnels. Possible reasons will be discussed separately for ease of description but none of the proposed scenarios are mutually exclusive. It is likely that the reason for marten presence in the tunnels is multi-factorial and it may be that a combination of any, or all, of these factors contributed to the martens initial, and maintained presence in the tunnel system.

While this, and other studies, confirm the presence of bat species found in the tunnels in pine marten and stone marten scats this may not be a product of predation alone and may be the result of marten scavenging on dead bats as both marten species are known to scavenge (Branji, 1995; Selva, *et al.* 2005). However, for reasons discussed earlier it is impossible, at present, to place camera traps in the tunnels which could prove conclusively if the consumption of bats is a product of predation, scavenging, or both. The fact that bat DNA is present in marten scat DNA extracts all year round does not preclude the possibilty of predation as the marten may equally be predating bats in daily torpor in times of the year when the bats are not hibernating.

The temperature in the tunnels is a constant 10 °C all year long and given that the winters in Poland may be severe marten may be entering the tunnels to keep warm in winter. This is particularly relevant to the stone marten whose distribution does not cross into northern European Russia or the Scandinavian Peninsula where the winters are particularly severe (Proulx *et al*. 2004). In summer temperatures can reach above 30° C and equally the marten may be entering the tunnels in summer to keep cool.

While marten have been found not to exhibit increased vigilance when feeding in areas where foxes are abundant (Wikenros, *et al*., 2014) marten are subject to intra-guild predation by foxes (Lindstrom, *et al*., 1995, Storch, *et al.*, 1990). There is only one documented case of

"reversed" intra-guild predation where a marten was found to have killed a young fox (Brzeziński *et al*. 2014). It may be then, that marten presence in the tunnels is a form predator avoidance as no evidence of foxes were found in the tunnels during our study. Predator avoidance may also be expanded to include avoidance of other larger carnivores that may be found in the area including wolves, whose scats were also not present in the tunnels.

The tunnels not only appear to be devoid of other predators but to a large extent are free from human presence most of the time with tours and "Bunker men" in certain areas at times but most of the system is closed off to the the public. The linear tunnels may then be a path of least resistance for the marten through their territory, avoiding not only predators but also tourists, hunters and the need to cross potentially dangerous roads.

It may be that marten will enter tunnels or caves because they occur in their territory. Marten have been found to venture into caves previously (Obuch, 2012). It has also been documented that both pine marten and stone marten are curious in nature, a trait that has lead the latter to cause damage to automobiles in some countries in Europe. It is possible that some individual animals are more curious than others, and it has been suggested that individual animal personality traits like boldness may lead to greater food intake rates (Biro & Stamps, 2008), which may be the case for marten that enter the tunnels as they have access to a food resource that animals which remain on the surface do not. The results indicate that approximatley half the individual marten identified have accessed the tunnels. Of the other half, some may have been transient animals as they were only detected above ground on a single occasion but others that were detected during multiple surveys may lack the boldness of their tunnel visiting counterparts.

Once the marten has found a source of food in a particular part of its territory it may return to this site in the hope of a repeat meal. This has been seen with camera traps showing pine marten returning to hair tubes repeatedly, long after the bait has been taken (*Pers obs*) and with both pine marten and stone marten returning to sites where they have previously killed birds (Goszczynski *et al.* 2007). This apparent memory of potential food sources may lead a marten to return again and again to the tunnels even at times when bats are not hibernating. Given the degree of marten activity in the system there may have been a form of cultural transmission developed similar to that of the blue-tits (*Parus caeruleus*) learning to open milk bottles in the UK in the forties and the great-tits (*Parus major*) that have been shown to actively seek out, kill and eat pipistrelle bats in a cave in Hungary (Estok *et al.* 2010).

5 OVERALL CONCLUSIONS AND FUTURE WORK

The hair tube survey performed in Corbally Wood was successful in yielding samples for molecular analysis. The various parameters including tube positioning, bait used, and the latency of initial detection period trialled, all proved effective. From this survey, DNA extracted from the collected hair samples was used for molecular analysis to successfully identify the species and sex of the animals that left the hair samples using qPCR and to identify individual animals throughout the survey site by amplification of a panel of microsatellite markers. The positioning of the hair tubes gave insight into the distribution of these individuals throughout Corbally Wood (Figure 2.10). The results from this survey confirm the methods employed can be used effectively to study a population of marten using non-invasively collected samples.

The hair tube survey in Nietoperek was not a success however, and failed to yield any hair samples. As seen in Corbally Wood, the method was effective in obtaining hair samples from marten and so can be eliminated as a reason for why this survey failed. Other possible reasons for marten not using the hair tubes in Nietoperek are discussed in section 2.5.1 and consideration of these factors is required before a repeat hair survey is performed. The results from the parallel hair tube surveys currently underway in Ireland and Poland may reveal why the hair tube survey in Nietoperek was unsuccessful. At the time of writing however, these results are not yet available.

In the absence of hair samples, scat surveys were performed to gather samples and molecular techniques performed on scat DNA extracts to study the pine marten and stone marten populations in Nietoperek. Scat DNA extracts proved to contain sufficient analysable DNA for species and sex identification by qPCR and for individual animal identification by microsatellite analysis; although due to the degraded nature of scat DNA the genotyping success rate was low at 47.7% of screened DNA extracts when compared to other studies using scat DNA extracts that obtained a genotyping success rate of 66.67% (Ruiz-Gonzalez *et al*. 2014) after screening. From the molecular analysis of scat DNA extracts it was determined that pine marten and stone marten are found throughout the survey site in Nietoperek, both above and below ground in varying abundance and distribution (Figure 3.27 & Figure 3.30). Patterns of scat distribution in the tunnels suggest no relationship between scat density and bat densities (Figure 3.33). Marten are depositing scats close to access points

(Figure 3.34). This may be as a form of chemical auto-communication or it may suggest the marten are not venturing far into the system.

Scats from both male and female marten were identified above ground and in the tunnels (Figure 3.17 and Figure 3.18). Results also show that marten are present in the tunnels all year long and not only during the winter when the bats are hibernating (Figure 3.35). Individual animals were identified from both species with approximately half the individuals detected in the entire survey area found to enter the tunnels (Figure 3.22 & Figure 3.23). Even with the small number of scat DNA extracts which genotyped successfully it was possible to observe that territoriality was largely conserved above and below ground and that intra-sexual territorial exclusion was also in evidence (Figure 3.52 & Figure 3.53). Forty one individual marten were identified over the survey but the number of individuals identified during any one survey was much smaller suggesting that the individuals in these populations are part of larger, dynamic pine marten and stone marten populations. In areas with uniform habitat, intra-species territoriality was in evidence between pine marten, stone marten and foxes with the more fragmented areas being shared by all three species (Figure 3.28).

The genotyping success rate of the hair DNA extracts, screened using MMX results from the probe based qPCR assay, from Corbally Wood (92%) was considerably higher than the success rate when using similarly screened scat DNA extracts from Nietoperek (47%). A successful hair tube survey in this area therefore may yield improved quality DNA samples giving a higher genotyping success rate which would lead to more detailed information on the genetics of both marten populations in the area. Control over the location and time when a sample is collected using hair tube surveys would also give increased resolution to any future populations studies in the survey site. It is therefore proposed that the design of a successful hair tube survey approach should be a high priority in any future non-invasive studies of marten in Nietoperek.

qPCR was used successfully to detect bat DNA in marten scat DNA extracts using both existing and novel primer pairs designed as part of this project (Table 4.4). Both marten were found to be consuming bats throughout the system and the more common bat species found in the tunnels were found in scat DNA extracts more frequently. The ratio of *Myotis myotis* bat DNA found in marten scat DNA extracts compared to the total numbers present is lower than the ratio of *Myotis daubentonii* and *Myotis nattereri* detected in marten scats and compared with their respective population sizes. This suggests there may be a preference

among marten for non *Myotis myotis* species and may indicate predation of lower roosting species rather than scavenging. Bat consumption by marten occurs throughout the system (Figure 4.12). The fact that only bats found in the tunnels were found in marten scats strongly suggests the marten are eating bats down in the system. Some individual marten were shown to be consuming bats throughout the survey period (Table 4.6). While the consumption of bats by marten appears widespread, the impact on the resident bat population for the duration of this project appears to be low (Table 4.7). These results may be used as a reference for future studies in the area. As the hibernating bat population in Nietoperek is monitored annually, any decreases in the numbers of a bat species or in the entire population may warrant a re-survey to investigate if marten consumption of bats is the cause of this decrease.

Along with marten DNA and, in some cases bat DNA, the DNA of any food item consumed recently by the individual animal may also be contained within the scat DNA extract. A more comprehensive dietary study may therefore be undertaken; initially using the scats collected during this project, and could incorporate scats from future surveys. Detection of prey species, insects, and plant items may again be performed using qPCR techniques to target individual species expected to be found in marten diets. However, dietary composition studies may be difficult in species like the marten that consume a wide variety of prey and separate qPCR primer pairs may have to be designed for each prey item leading to pressures on time and costings. A more effective approach may be to utilise Next Generation Sequencing (NGS) similar to the work of Bohmann, *et al.* (2011) who sequenced fragments of prey DNA from bat pellets and used bio-informatics software to perform species level identification. This approach could be incorporated into any further studies on the marten in Nietoperek, allowing both dietary composition and comparison studies to be undertaken.

There was also 74 fox DNA extracts inadvertently collected during this project (Figure 3.15) and inclusion of these scats into any future dietary studies would allow a comparison of the diets of all three sympatric species in and around Nietoperek. Results contained in this project suggest foxes are not entering the tunnels (Figure 3.15) and are not consuming bat species (Table 4.5). Foxes have been found to predate bats in other studies (Hutson *et al*. 2001). Comparison of complete dietary study results between scat DNA extracts from individual animals confirmed to be consuming bats, with those containing no bat DNA, may be informative; as well as a comparison between all three species with respect to dietary niche, breadth and degree of overlap.

The use of remote cameras in the tunnels was not trialled during this project due to the risk of theft or vandalism reported by previous researchers in the system (H. Schofield & T. Kokurewicz, *Pers. comm.*). However, it may be possible to set up cameras in parts of the system, far removed from the areas accessible to tourists. Regular checking of these cameras would be necessary to avoid losing data due to theft, but if permission was granted to access these areas on a regular basis, even over a short period of time, it may be possible to determine if marten are predating or scavenging bats.

In conclusion, in the absence of hair DNA samples from Nietoperek, scat DNA extracts were successfully used with molecular techniques to study the genetics of the resident pine marten and stone marten populations. Any future studies on the marten populations in and around Nietoperek should aim at incorporating hair tube surveys into the survey methodology. Once the hair tubes are successful above ground, adaptations may be made to allow their use in the tunnels without endangering the resident bat population. The increased resolution obtained from hair DNA samples obtained at a predefined space and time can be supplemented with DNA extracted from scats which, as shown in this project will also yield information about the identification of the offending species, sex and individual animal. Scat DNA extracts should also be used for a comprehensive comparative dietary analysis of both marten species and possibly include scats from foxes.

APPENDIX I- SECTIONS AND SUB-SECTIONS OF THE NIETOPEREK TUNNELS

Length of tunnels and sub-sections. Included are bunkers (PzW) located in each sub-section

Section	1									2				
Sub-section	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.1	2.2	2.3	2.4	2.5
Length (km)	0.679	0.478	0.32	0.13	0.31	0.519	0.191	0.139	0.585	0.086	0.406	0.449	0.08	0.965
Total Length (km)	3.351									1.986				
PzW	739		736					733	732				727	
Section	3							4						
Sub-section	3.1	3.2	3.3	3.4	3.5	3.6	3.7	4.1	4.2	4.3	4.4	4.5	4.6	4.7
Length (km)	0.387	0.622	0.249	0.315	0.273	0.27	0.167	0.435	0.048	0.208	0.264	0.149	0.186	0.351
Total Length (km)	2.283							1.64						
PzW		730			728								726	
Section	5					6								
Sub-section	5.1	5.2	5.3	5.4	5.5	6.1	6.2	6.3	6.4					
Length (km)	0.313	0.189	0.16	0.2698	2.277	0.071	0.0822	0.9858	1.416					
Total Length (km)	3.2088					2.555								
PzW			724		722,721,A8				A64					
Section	7													
Sub-section	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	7.9	7.10	7.11			
Length (km)	0.2182	0.7505	0.4471	0.4128	0.944	0.4442	0.708	0.9333	0.4299	1.0427	1.095			
Total Length (km)	7.426													
PzW			719	720					717	716				
Section	8													
Sub-section	8.1	8.2	8.3	8.4	8.5	8.6	8.7	8.8	8.9	8.10				
Length (km)	1.0518	0.4608	0.1718	0.6444	0.2109	0.1044	0.162	0.8516	0.2807	0.1435				
Total Length (km)	4.0819													
PzW	783						766	A1	715	713				
Section	9													
Sub-section	9.1	9.2.1	9.2.2	9.2.3	9.2.4	9.2.5	9.2.6	9.2.7	9.2.8	9.2.9				
Length (km)	0.401	0.1062	0.3676	0.0997	0.0827	0.1612	0.1509	0.2322	0.2386	0.0828				
Total Length (km)	1.9229													
PzW	714						PzT .1	PzT.2	PzT.3		Nord			
Section	9													
Sub-section	9.2.10	9.2.11	9.2.12	9.2.13	9.2.14	9.2.15	9.2.16							
Length (km)	0.0818	0.0927	0.0736	0.1297	0.0661	0.6365	0.3267							
Total Length (km)	1.4071													
PzW														
Overall length (km)	29.86													

APPENDIX II-INDIVIDUAL SURVEYS OF NIETOPEREK (SEPTEMBER 2012-MAY 2014)

September 2012

Above Ground

The above ground survey route 1 covered from Petla Boryszynska (Boryszyn loop) along the tracks indicated on the map in Figure 1 to the museum in Pniewo. The route consisted mainly of tracks within the forest. A large field was surveyed along the headlands in the direction of the museum. The total distance for route 1 was 4.8 km. This route was covered twice on this trip with three days between surveys.

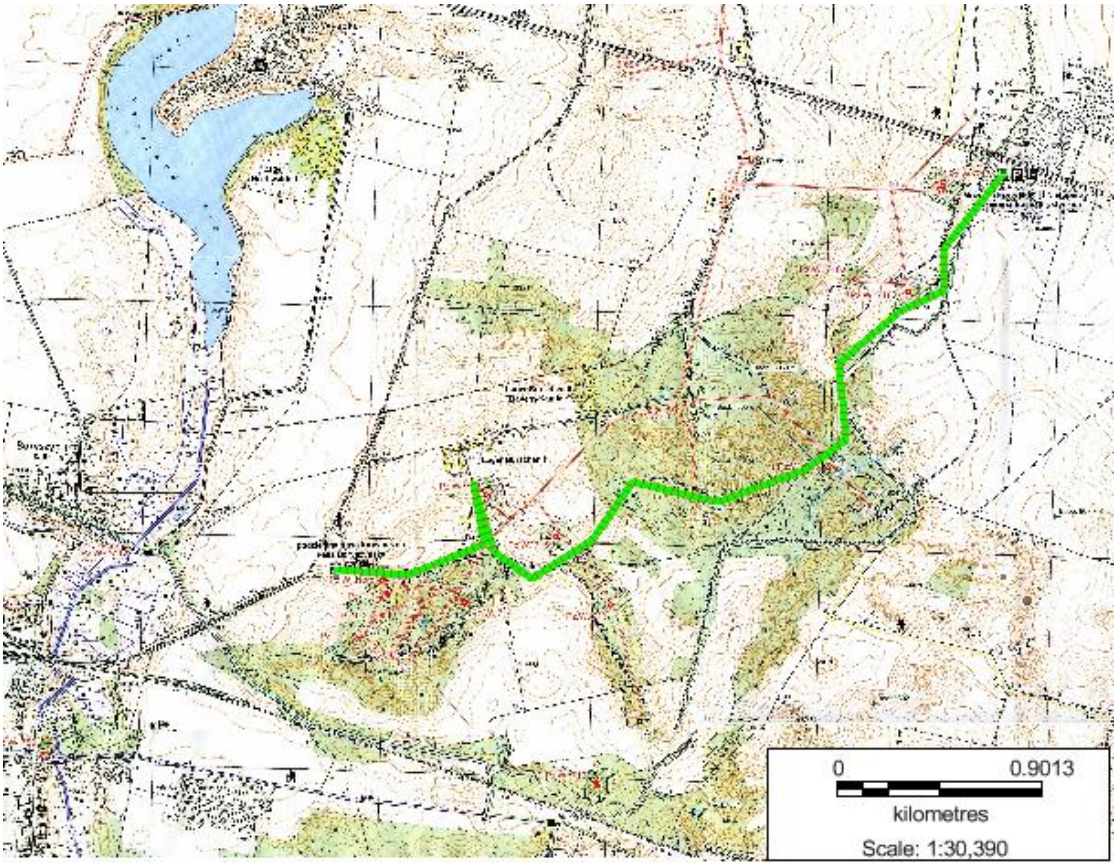


Figure 1. Above ground route 1 taken in September 2012

Survey route 2 as shown in Figure 2, was north of Wysoka towards the Forest Entrance. This route was along tracks in managed forest. The tracks varied in composition from compressed stone to sand tracks. This survey route was also covered twice with 3 days between surveys. Route 2 covered a distance of 9.78 km.

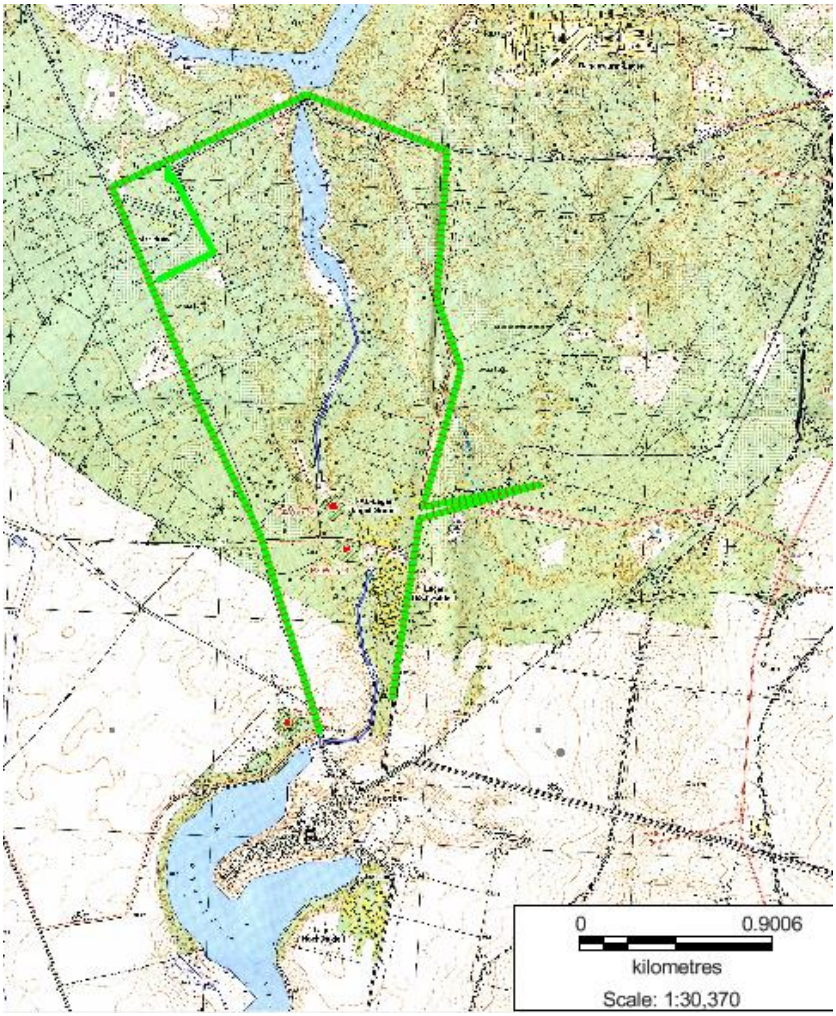


Figure 2. Survey route 2 covered in September 2012

Below Ground

All of section 9 of the tunnels and the southern portion of section 8 were surveyed during September 2012. Figure 3 shows the area covered in underground during this survey

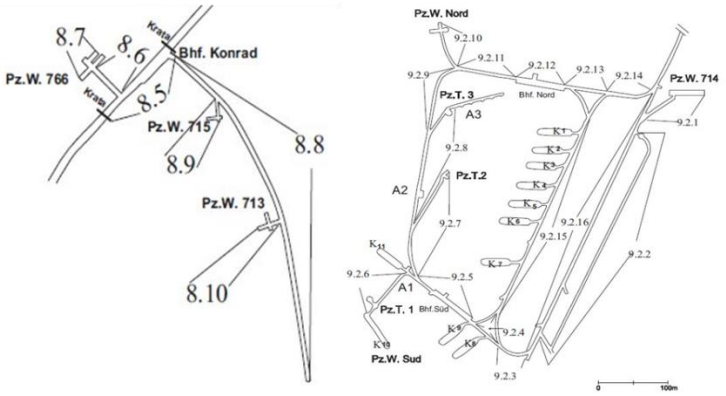


Figure 3. Areas surveyed below ground in September 2012

October 2012

This scat survey was incorporated as part of a trip to set up baited hair tubes. The survey route followed the line of the existing hair tubes as well as the route along which the new hair tubes were erected (Figure 4). The distance covered during this survey was 8.7 km.

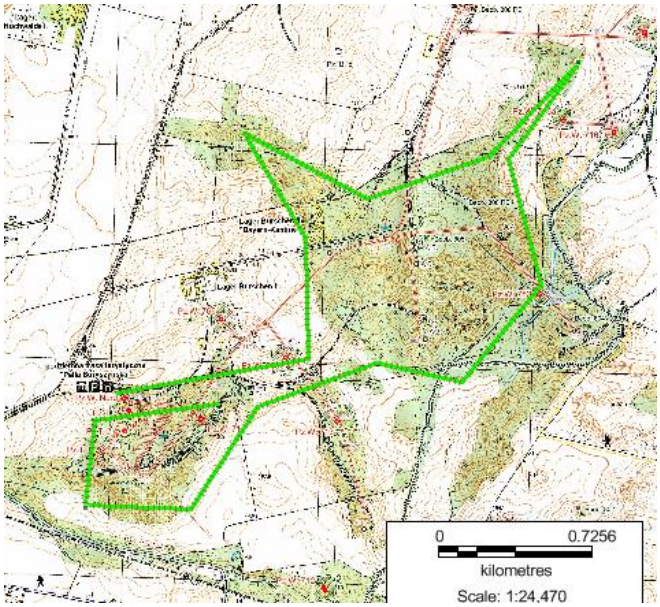


Figure 4. Area covered during the October 2012 survey.

January 2013

Above Ground

Deep snow cover and poor weather conditions prohibited any extensive above ground survey work on this trip. A small survey, from Borszyn entrance to bunker PzW766, was performed where a small number of scats were found.

Below Ground

A portion of section 1 was flooded and surveying was impossible in this area but the rest of entire system was surveyed. The entire survey of the underground tunnels was performed in 1 day in conjunction with the annual bat survey (Figure 5).

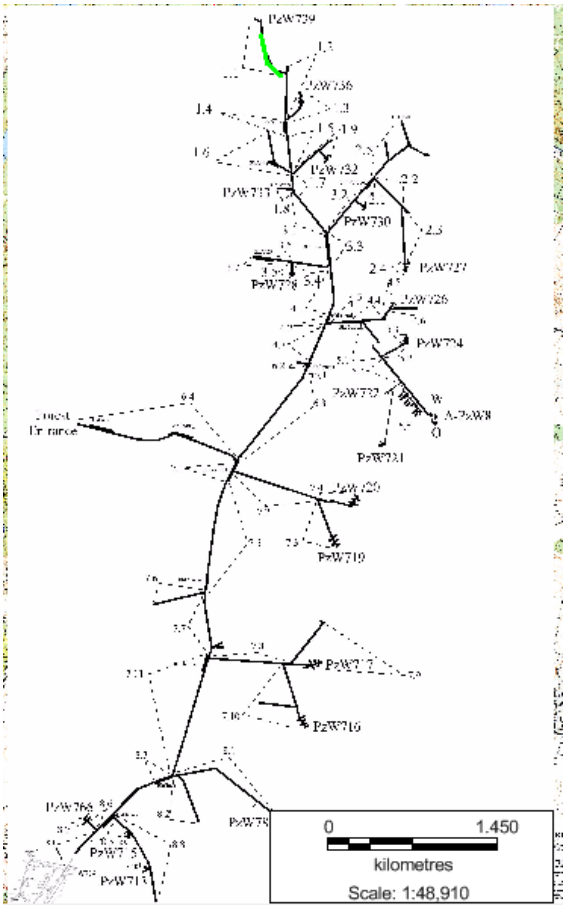


Figure 5. Area covered underground in January 2013 (Area flooded).

May 2013

Above ground

The above ground survey in May 2013 covered the same areas as the survey in September 2012 but also covered a wider area of the forest north of Wysoka and an area of arable land directly above the tunnel system that contains a number of intact bunkers. The track marked on Figure 6 was followed and each bunker along the track was also surveyed for scats. The scat survey in the Northern Forest covered a distance of 10.16 km. The distance covered in the Middle Section was 5.19 km and the survey in the Southern Forest covered a distance of 4.6 km.

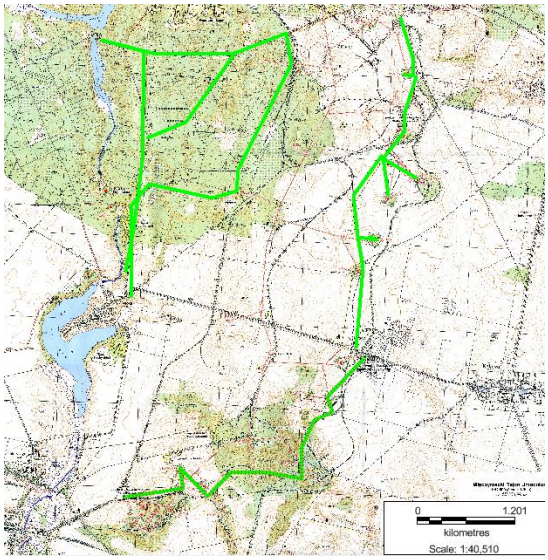


Figure 6. Above ground survey routes for May 2013

Below Ground

A portion of section 1 and a portion of section 3 were flooded and as such were not surveyed. The remainder of the system was surveyed in full (Figure 7). Sections 8 and 9 were surveyed on day 1 of the underground survey. The northern sections were surveyed on day 2.

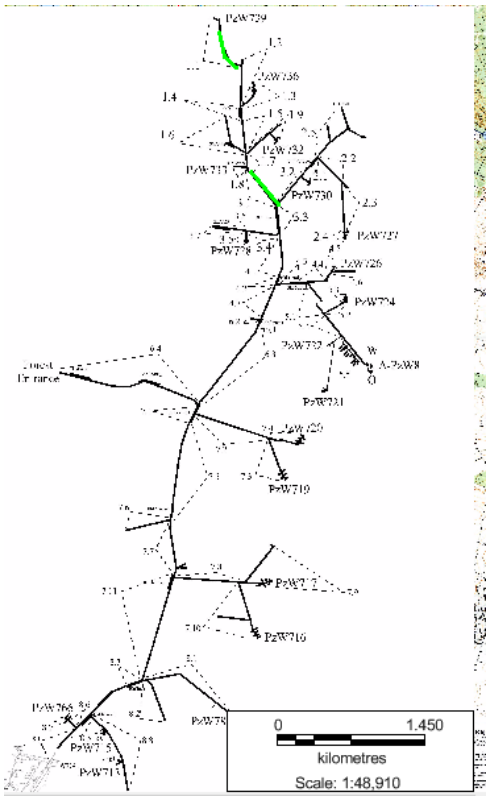


Figure 7. Area surveyed underground May 2013 (Areas flooded).

September 2013

Above ground

An above ground survey was conducted in areas that correspond to the tunnel system below ground. Figure 8 shows the areas covered in the Northern Forest and Middle Section. Two routes were taken in the Northern Forest. The northernmost route covered 5.18 km and the other route covered 8.22 km. The distance covered during the survey of the Middle Section was 8.69 km. Figure 9 shows the areas covered in the Southern Forest. The distance covered by this route was 16.3 km.

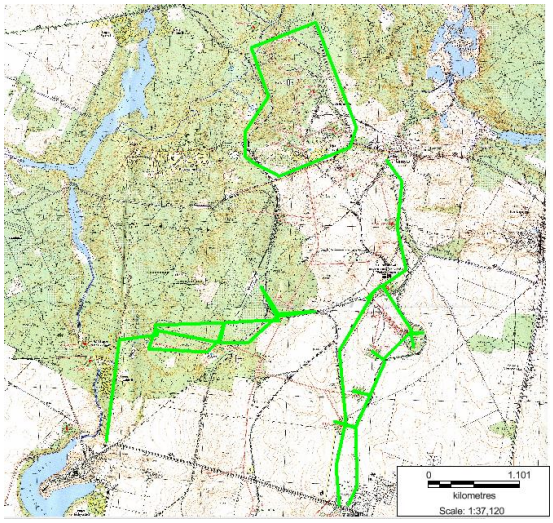


Figure 8. Routes in the Northern Forest survey area covered in September 2013

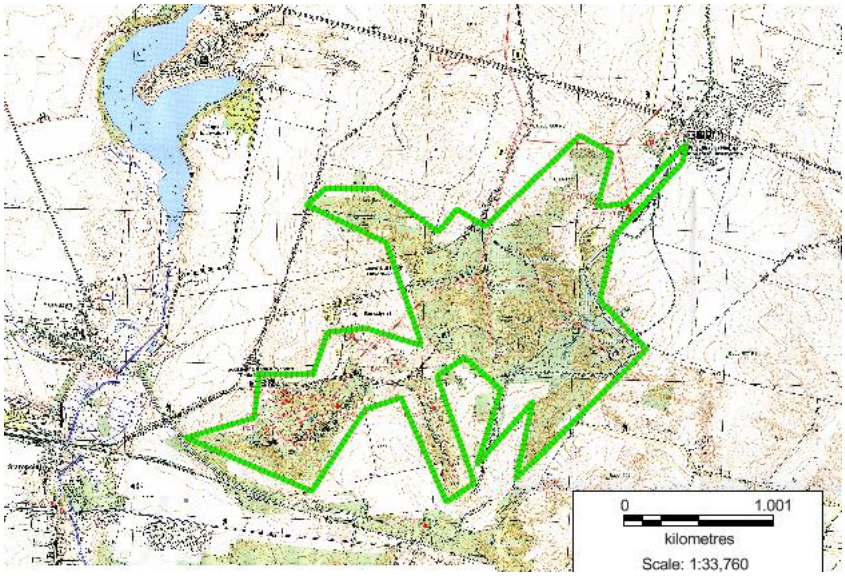


Figure 9. Routes in the Southern Forest survey area covered in September 2013

Below ground

The below ground survey in September 2013 covered the entire tunnel system with no reported areas flooded.

January 2014

Below ground

In January 2014 the system was surveyed with the aid of volunteers from the VWT and students from Wrocław University of Environmental and Life Sciences. Access to the system proved difficult during this survey and the survey was performed over a number of days. However, the entire system was again covered.

May 2014

Above ground

This survey session was divided into five main routes. Route 1 covered the Northern Forest and Middle Section and where possible, followed tracks and roads that crossed over, or followed the line of the system below (Figure 10). This survey covered a distance of 18.5 km.

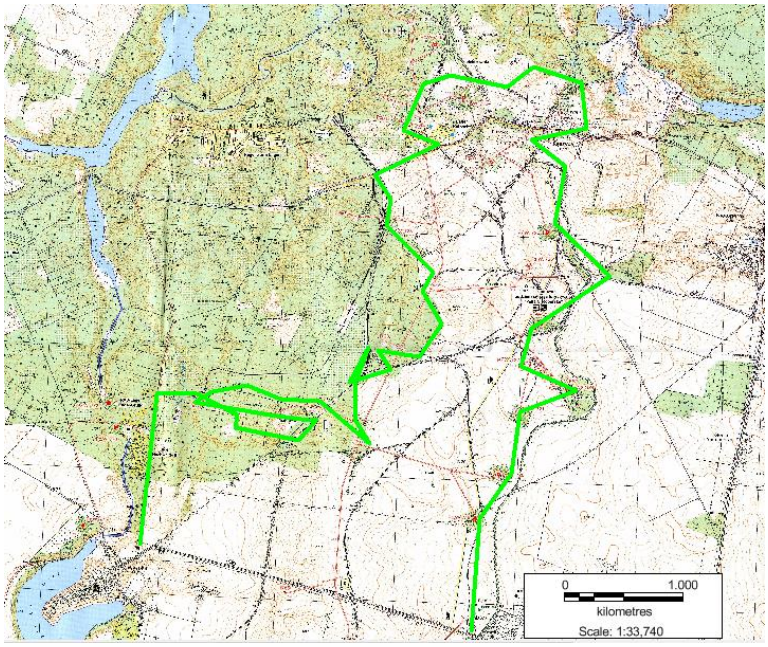


Figure 10. May 2014 Route 1

Route 2 covered an area above section 1 of the system (Figure 11). This survey route covered a distance of 9.84 km.

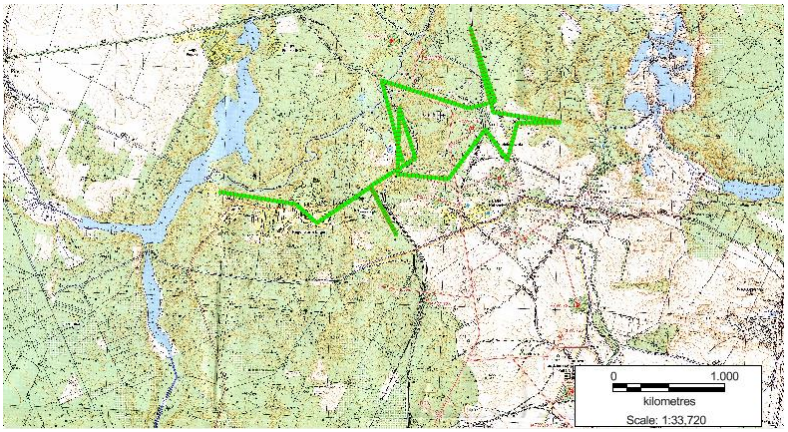


Figure 11. May 2014 Route 2

Route 3 was in the Southern Forest. This survey session incorporated the linear dragon's teeth and followed this feature as far as the museum in Pniewo (Figure 12). The distance covered in this survey was 6.64 km.

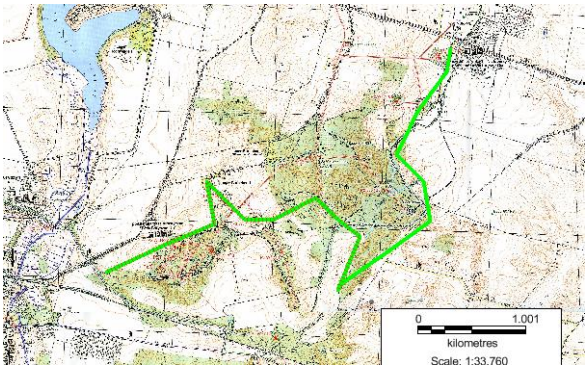


Figure 12. May 2014 Route 3

Route 4 covered the Southern Forest route covered in previous surveys but also visited any forest islands that may contain an access point or bunker (Figure 13). This survey route covered a distance of 8.6 km.

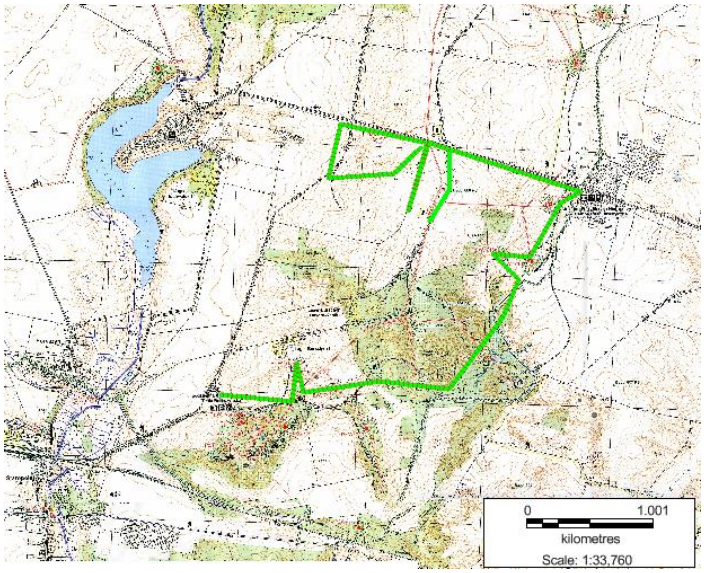


Figure 13. May 2014 Route 4

Route 5 followed the route usually taken in the Southern Forest and covered a distance of 4.9 km. This route was surveyed on 5 consecutive days and any fresh scats collected (Figure 14). Scats from both species had been found in this area and this survey was designed to see if the marten were using this area at approximately the same time.

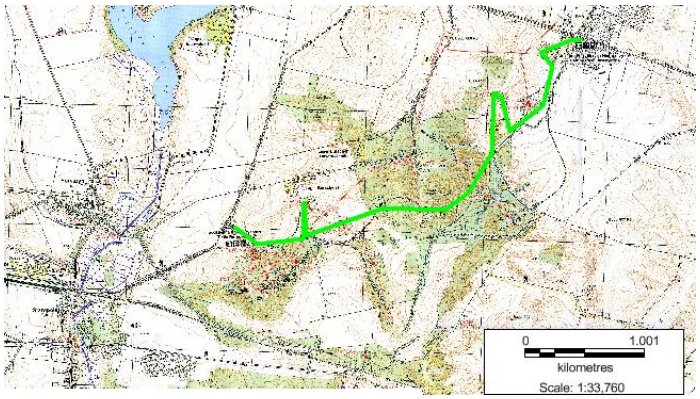
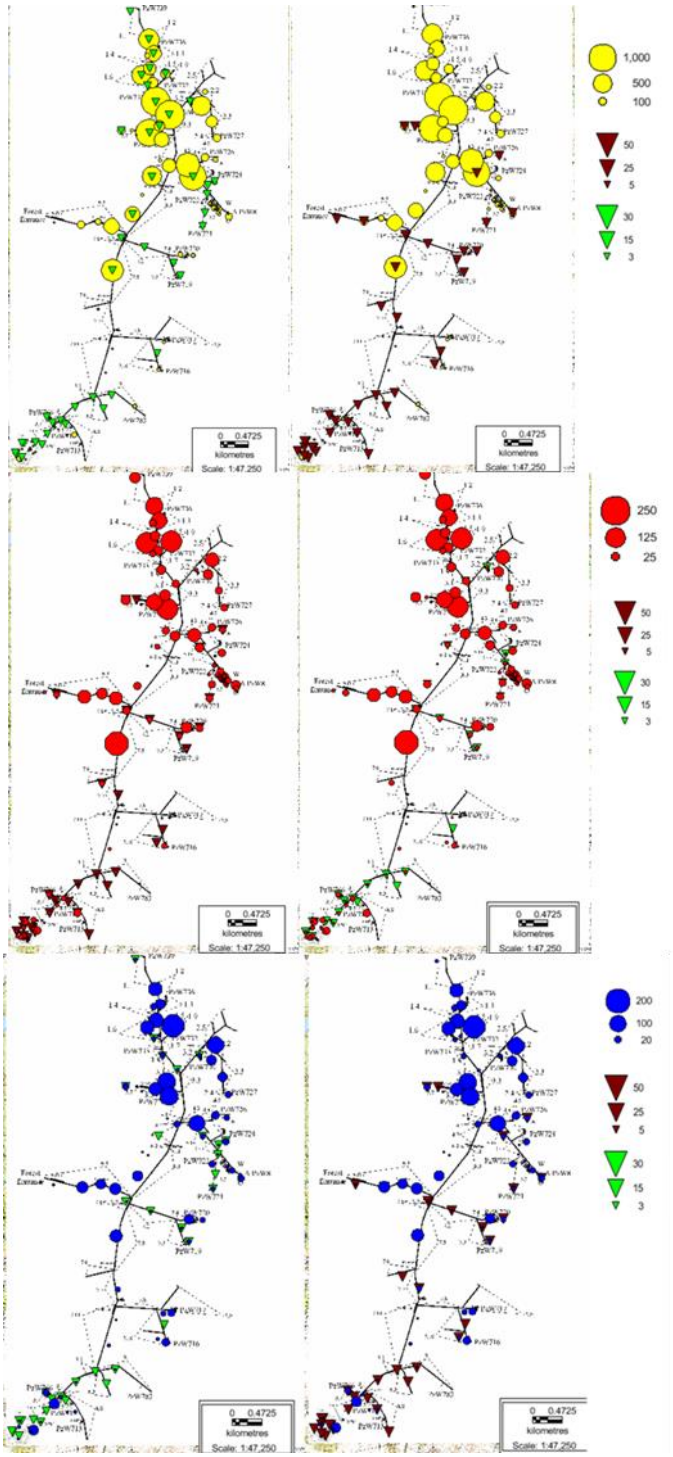


Figure 14. May 2014 Route 5

Below ground

During the May 2014 survey period the entire system was again surveyed with the exception of sub-sections 1.1 and 1.2 which were flooded and deemed unsafe to survey.

APPENDIX III-MARTEN SCAT DENSITIES AND DENSITIES OF THE 3 MOST COMMON BAT SPECIES FOUND IN THE TUNNELS



Bat densities; ***Myotis myotis*** **●** ***Myotis daubentonii*** **●** ***Myotis nattereri*** **●,Scat densities; pine marten ▼ stone marten ▼**

APPENDIX IV SUMMARY OF INDIVIDUAL MARTEN DISTRIBUTION

Summary of individual stone marten scat distribution in Nietoperek

S2 and S3 are both female stone marten (Figure 1).S2 was only ever detected below ground and S3 was only ever detected above ground. Scats identified as being from both these individuals were all collected during the same survey period in May 2013. While initially it appears that two female stone marten are sharing a territory the fact that one animal was only ever detected above ground and one animal was only ever detected in the tunnels indicates there may not be a rigid conservation of territories between the above and below ground habitats.

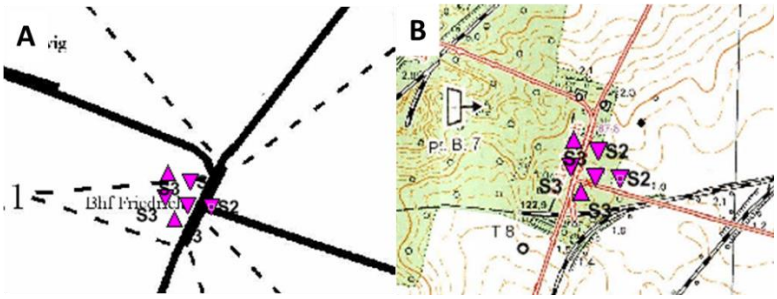


Figure 1. Location of scats from S2 and S3, two female stone marten; tunnels above ground A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Figure 2 shows the locations of scats from S9, a female stone marten. S9 was only detected in May 2013 and only detected below ground and only in section 6.4. This is part of section 6 which is prone to flooding so this may be the reason S9 was not detected in other surveys. Due to flooding, it may be possible for scats to also get washed into the tunnels but the scats which yielded genotypes for S9 were from a cluster of 11 scats which were collected from a raised area at the side of the tunnel making this scenario unlikely. From Figure 3.37 B it can be seen that scats from S9 were detected in the tunnels below an area which is covered in forest. Stone marten scats were not found above ground in this area. However, some of the long arm of section 6.4 is under open farmland, as is some of the rest of section 6 (41%) as can be seen in Table 1.

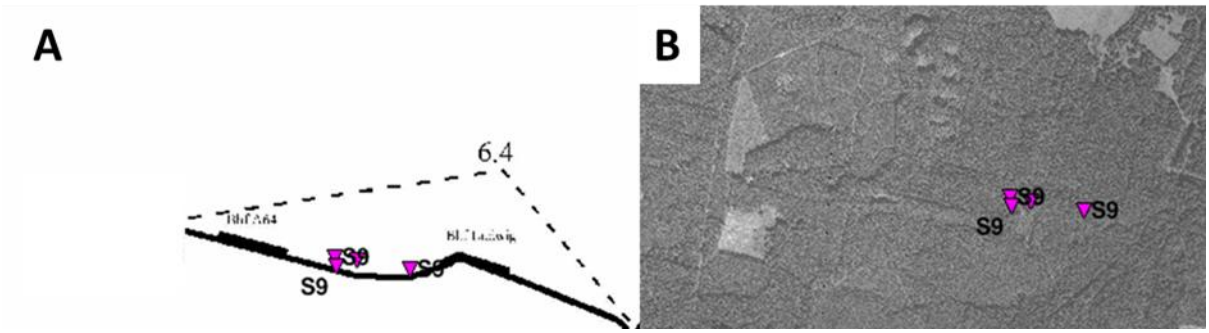


Figure 2. Location of scats from S9, a female stone marten; tunnels ▼; A=the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Table 1. The percentage of the survey area above each tunnel section that is covered in forest. The remainder of the above ground area consists of arable land and meadows.

Section	Forest (%)
1	91
2	14.5
3	21
4	12
5	13.5
6	59
7	28
8	86
9	100

S4 is a male stone marten and the position of scats from this animal can be seen in Figure 3. This male was only detected in the tunnels and only in section 7; twice in May 2013 and once during the September 2013 survey 1.2 km away through the tunnels. Both PzW 19 and PzW 20 are close to where these scats were collected and it is possible one or both of these bunkers are used as access points to the system by this individual. At least one stone marten was present in section 7 of the system during this project.

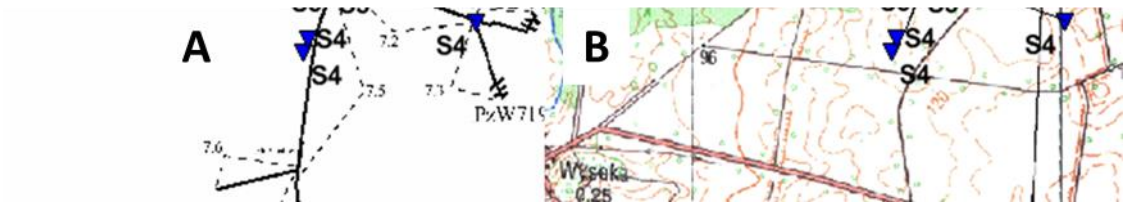


Figure 3. Location of scats from S4, a male stone marten ▼ ; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

S20 is a female stone marten (Figure 4). This individual was detected above PzW 721(Section 5) in May 2014 and also below ground not far from PzW 720 and 719 (in section 7), 16 months previously in January 2013. The distance between PzW719 and 721 is approximately 1 km but to travel between these bunkers underground requires travelling 4.5 km through sections 4 and 6. As no scats were detected in section 4, and all three scats were collected near a bunker, it may be that this individual is not travelling very far into the tunnel

system. Given the scats were collected in both January 2013 and May 2014 this suggests that this was a resident female in this part of the survey area during this timeframe.

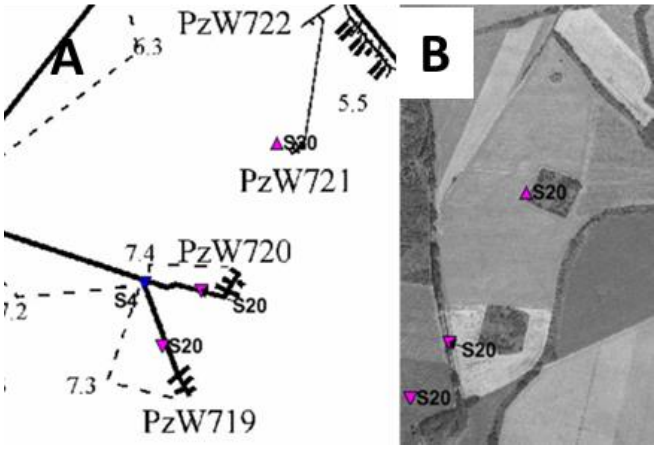


Figure 4. Location of scats from S20, a female stone marten ▼ tunnels, ▲ above ground; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

S8 is a male stone marten (Figure 5). S8 was detected twice in January 2013 below A-PzW8 and twice again in a cluster of scats in September 2013 above ground 0.7 km away above ground close to PzW 724. From Figure 5 (B), it may be seen that the scat found in September 2013 was along a segment of the dragons teeth that has lots of cover and may be used as a wildlife corridor. The short distance between these scats along with the time between detections suggest this was a resident male in the area of the survey site above section 5 from at least January 2013 to September 2013.

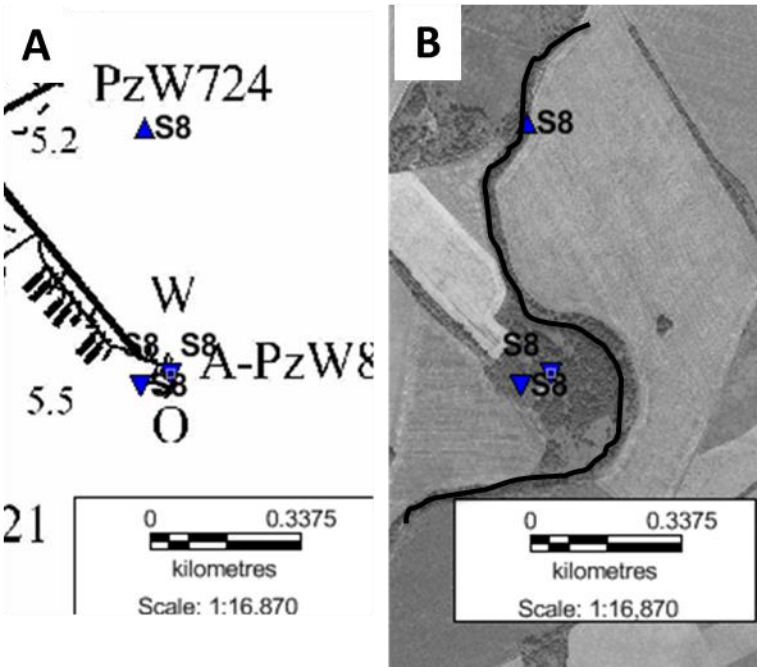


Figure 5. Location of scats from S8, a male stone marten ▼tunnels, ▲above ground; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area, ⁓ line following the path of the dragon's teeth.

From the location data from S8 and S20 it may be concluded that at least 1 male and 1 female stone marten were present in this area during this project.

Figure 6 shows the position of scats from both S18 and S19. The individual S18 is a male stone marten and was detected 6 times, both above (n=3) and below (n=3) ground in 2012, 2013 and 2014 once again suggesting this was a resident male in this part of the survey site during this time. S19 is also a male stone marten and was detected near to S18. S19 was only detected only in May 2014 and was only detected above ground. There are two bunkers; PzW716 and 717 in the area where these marten are detected but as S18 is the only one of the two to be detected below ground, and is detected closer to these bunkers, they may be part of S18's territory and are therefore avoided by S19. From Figure 6 it can be seen that there is no overlap in the position of scats from these two males.

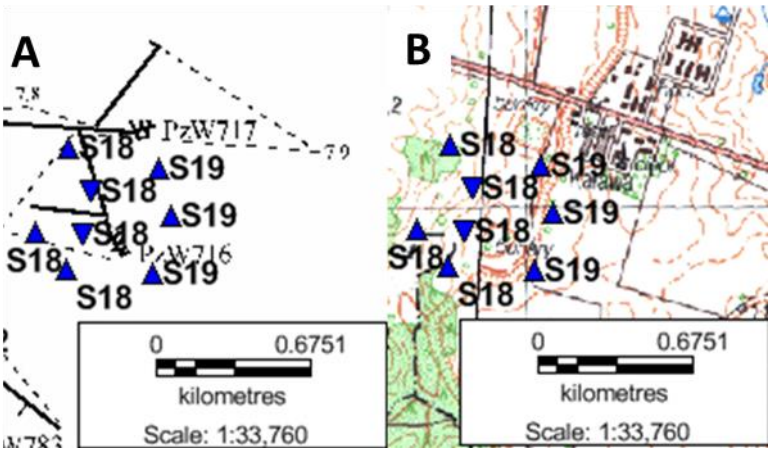


Figure 6. Location of scats from S18 and S19, two male stone marten, tunnels, ▼ above ground ▲ ;A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Both S7 and S12 are male stone marten. While it appears that the underground territories of these two males overlaps (Figure 7) S7 was detected in December 2011 and twice in September 2013 and S12 was detected in September 2013 and again three times in May 2014 so it is possible both territories have evolved over this time and these two individuals never encountered each other. It is worth noting that both S7 and S12 were detected in the tunnels below areas where stone marten are not found and while it was shown earlier that the above and below ground territories for both species are largely conserved that there are exceptions and this is not rigid. It may be concluded from the locations of scats from S7 and S12 that at least two male stone marten were present in section 8 during the entire survey.

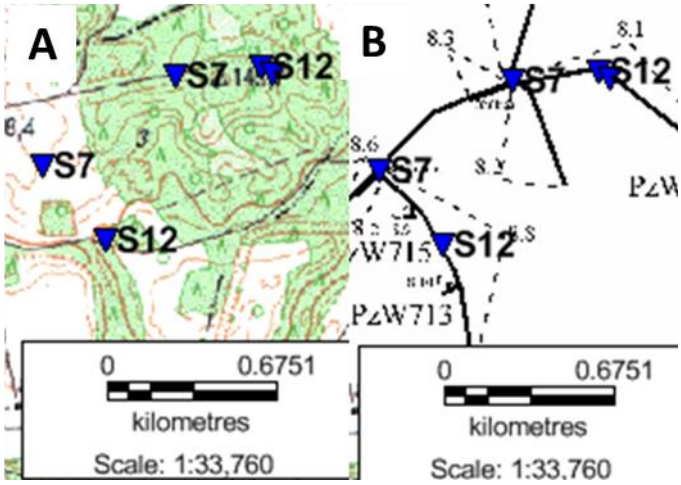


Figure 7. Location of scats from S7 and S12, two male stone marten▼ tunnels▲ above ground; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Figure 8 shows the location of scats collected from S13 and S16. Both these animals are female stone marten. S13 was only ever detected below ground, in December 2011 and September 2012. S16 was only ever detected above ground in December 2011, April 2012 and January 2013. S16 was only ever detected on the outskirts of the area above section 9 and it may be that the access points to section 9 are within the territory of S13. Like S7 and S12 in section 8, S13 was detected in an area below ground that would traditionally be considered pine marten habitat above ground.

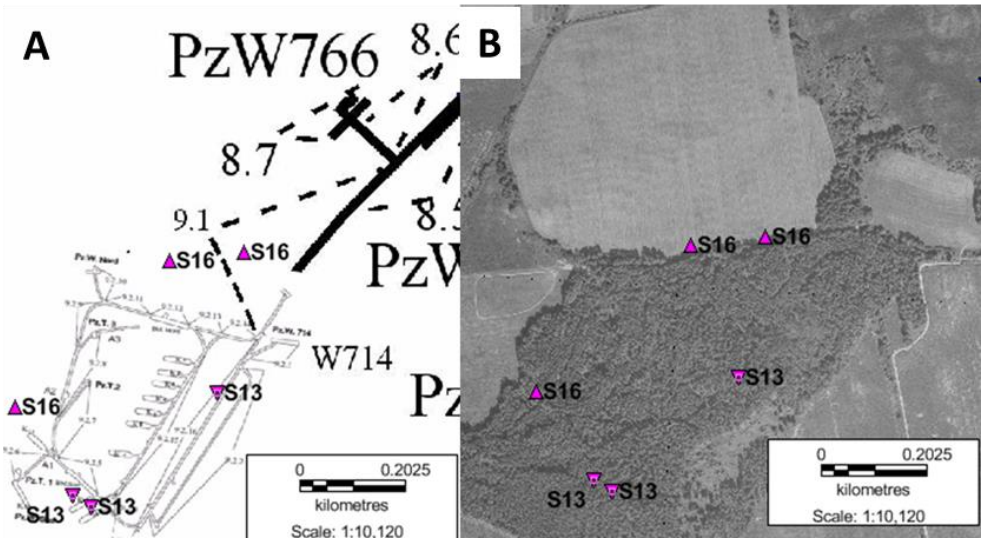


Figure 8. Location of scats from S13 and S16, two female stone marten, tunnels, ▼above ground ▲ ; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Summary of individual pine marten scat distribution in Nietoperek

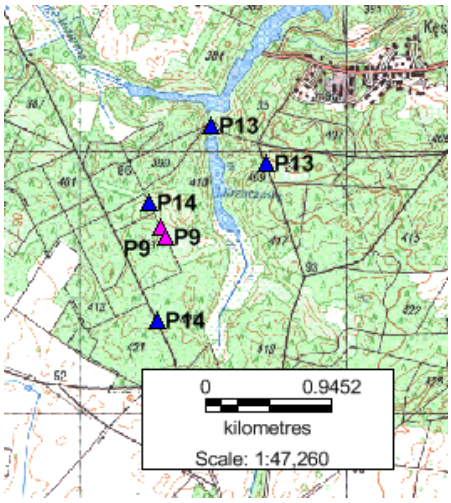


Figure 9. Location of scats from P9, a female pine marten ▲and P13 and P14, two male pine marten ▲

Figure 9 shows the location of scats collected from P9, P13 and P14. P9 is a female pine marten while P13 and P14 are male pine marten. These animals were only ever detected in the western portion of the Northern Forest and were all detected during the September 2012 survey. After September 2012 the effort in above ground sampling focused mainly on the areas directly above the tunnels system, the nearest point of which is over 2 km away from where these scats were collected. There is limited territorial data from these scats but the locations of scats from both males do not suggest any overlapping of territories. It is interesting that in this area the genotyping success rate was much higher than the overall success rate as discussed earlier. A possible explanation for this is during the September 2012 survey, large numbers of dung beetles were witnessed eating scats in the Northern Forest. The soil in this part of the forest is of a sandy nature and 5 beetles were witness breaking up a scat and burying it in approximately 2.5 minutes. Previous studies have shown that invertebrate interference may have an effect on scat survey success rates (Birks *et al*. 2004; Jedrzejewski & Jedrzejewski, 1990). This competition for samples from the dung beetle may result in fewer scats being collected but also may result in fresher scats being collected which possibly yielded better quality DNA.

Figure 10 shows the locations of scats collected from P2 and P20. P2 is a female pine marten and was detected both above and below ground during the May 2013 survey. The above

ground detection was above section 1.1 and the below ground detection was in section 1.6 which is 1.2 km away in a straight line or 2 km through the tunnels.

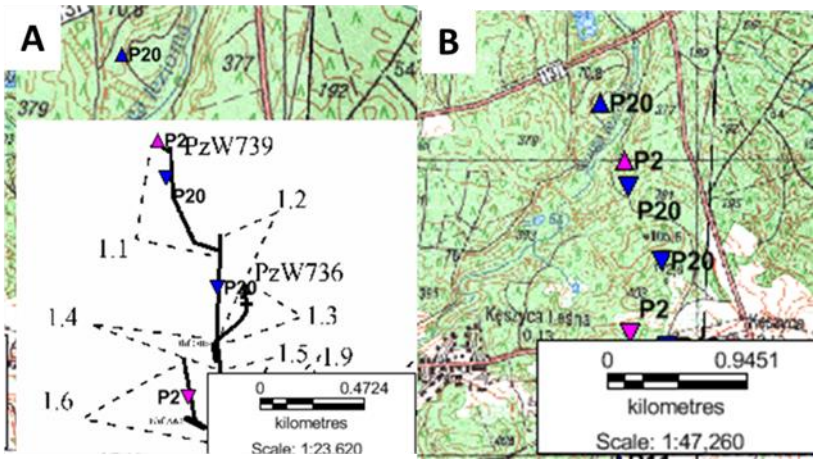


Figure 10. Location of scats from P20, a male pine marten ▲ and P2 a female pine marten, ▲ A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

P2 may have been accessing the tunnels though PzW 739 as the above ground detection was close to this bunker. P20 is a male pine marten and was detected below ground in sections 1.1 and 1.2 during the January 2013 survey. It was detected again above ground north of section 1.1 in May 2014. P20 may have been accessing the system through either, or both, PzW739 and PzW 736 as scats underground were detected close to both. The 15 months between detections of P20 suggest this animal was a resident in this portion of the survey area during this time.

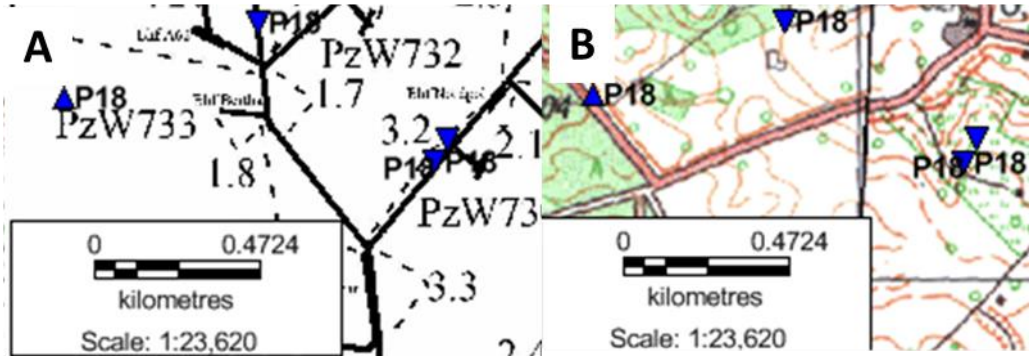


Figure 11. Location of scats from P18, a male pine marten ▲; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

P18 is a male pine marten (Figure 11). P18 was detected a total of 4 times, both above and below ground and during survey periods 8 months apart suggesting this male was also a resident animal in the survey area above section 1 during this time. Below ground detection was in September 2013 and it was also detected in May 2014 approximately 1.15 km away in a straight line or 1.6 km away if travelling along the tunnels and exiting at PzW 733. However two of the scats found in January 2013 were deposited near PzW 730 so this may also be an access point for this individual. These results indicate that at least two male, and one female pine marten were present in section 1 of the system during this project.

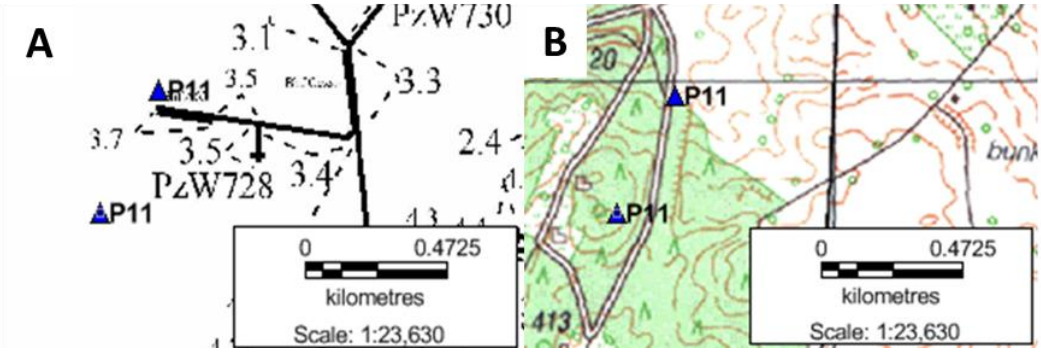


Figure 12. Location of scats from P11, a male pine marten ▲ ; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Figure 12 shows the location of scats collected from P11, a male pine marten. This individual was detected during the April 2011 and May 2013 surveys. Although only detected twice during the project, the timeframe suggests a resident male. P11 was only ever detected above ground despite being directly over the tunnels and close to PzW728.

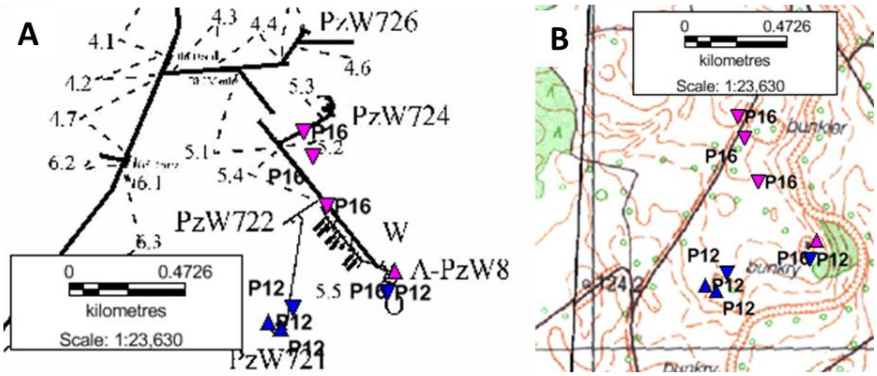


Figure 13. Location of scats from P12, a male pine marten ▲ and P16 a female pine marten, ▲ A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

Figure 13 shows the location of scats collected from P16 and P12, a female and male pine marten respectively. Both individuals were detected in, and above section 5. P12 was found above ground in April 2011, both above and below ground in December 2011 and again below ground in May 2013. The timeframe here again suggests a resident animal. All P12 scats were collected either in and around, or directly below A-PzW8 or PZW 721 suggesting these two bunkers as access points. P16 was detected 3 times below ground in September 2013 and once again, above ground in May 2014 suggesting a resident animal. As with P12, P16 may have been gaining access to the system via A-PzW8 but also through PzW724, 0.9 km further north. The results suggest at least one male and one female pine marten were present in section 5 during this project.

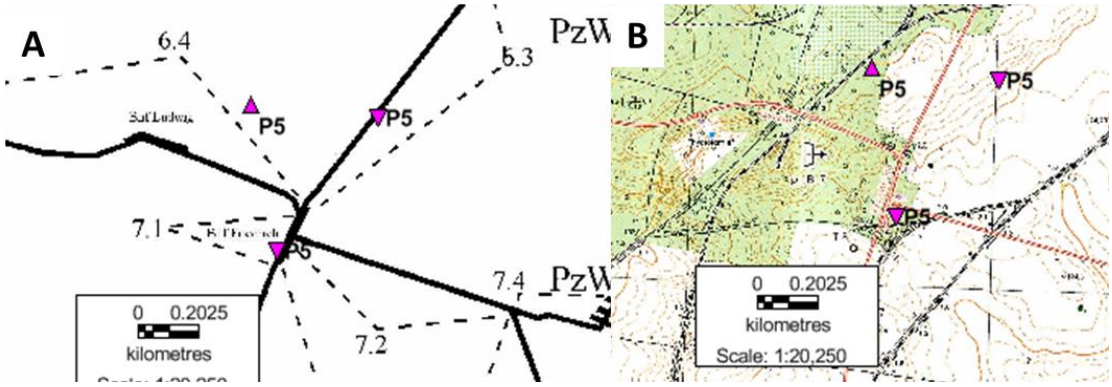


Figure 14. Location of scats from P5, a female pine marten ▲ ; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

P5, a female pine marten was detected a total of 3 times (Figure 14). It was detected above ground over section 6 in October 2012, below ground in section 7 during the January 2013 survey and below ground in May 2014. The closest bunker to where this individual was detected below ground in section 6.3 is PzW 720 which is nearly 1 km away in section 7. This data would appear to contradict the earlier suggestion that marten are leaving their scats close to access points. However section 6.4 comprises a road that stretches from the tunnel floor up to the surface. On the surface, the remains of what appear to be either air vents or chimneys are evident. Whatever their initial purpose these structures may be providing an alternative to the human access points for marten to gain entry to section 6.4.

Figure 15 shows the location of scats from P10 and P15. P10 is a female pine marten and was detected twice above ground during the July 2011 survey and twice below ground in section

8 18 months later during the January 2013 survey. The below ground scats had been deposited less than 1 km away from where the above ground scat was collected in July 2011 suggesting this animal was a resident female in this area during this time. P15 is a male pine marten and was detected twice above ground; in April 2011 and in May 2013, and once below ground during the September 2013 survey again suggesting this was a resident animal. At least 1 female and 1 male pine marten were present in section 8 of the system during this time.

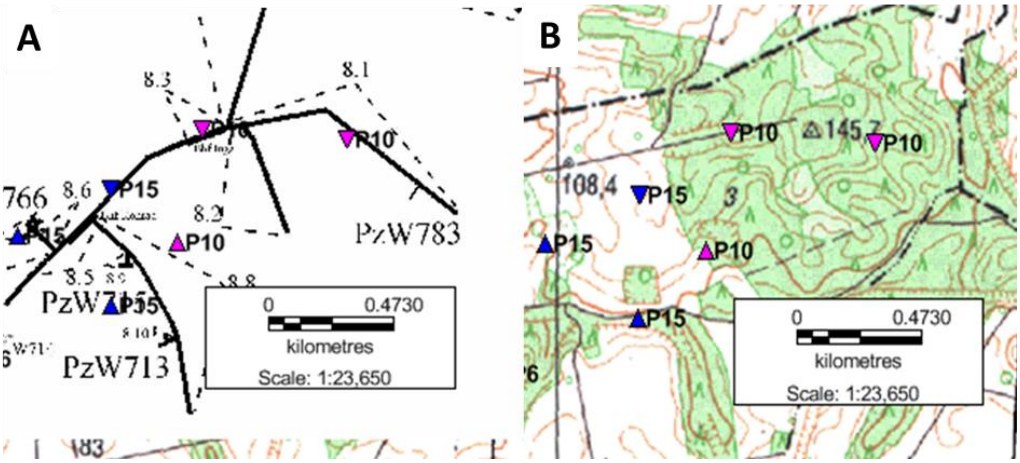


Figure 15. Location of scats from P15, a male pine marten ▲ and P10 a female pine marten, ▲ A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

P6 is a female pine marten and the location of scats from P6 can be seen in Figure 16. All scats from this animal were detected below ground within a 0.8 km stretch of the tunnels. It was first detected in September 2012 and again in October 2012 and there were 3 scats from P6 collected during the May 2013 survey. This suggests P6 was a resident around section 9 of the system for this time period. From Figure 2.51 it can be seen that there are multiple access points in section 9 with P6 scats being detected close to PzT 3 and PzW714 suggesting these may be the martens access route.

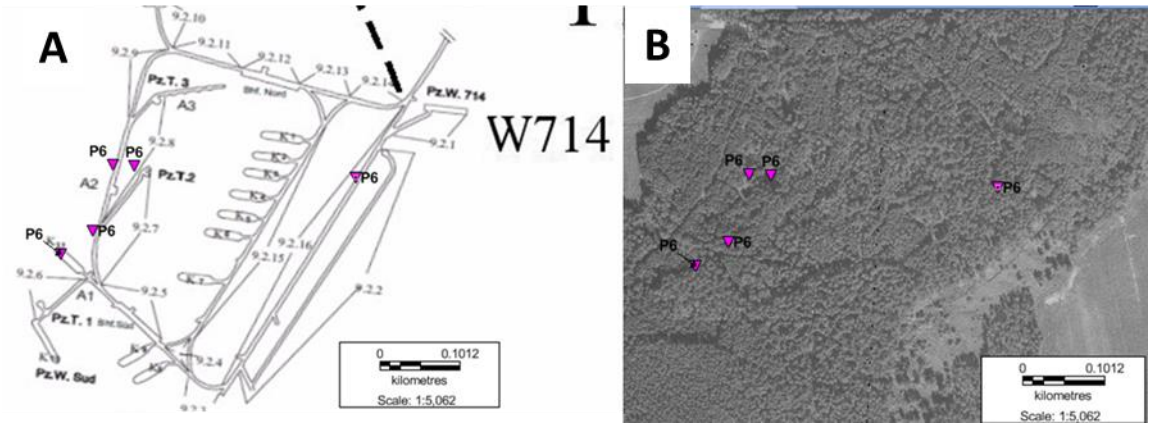


Figure 16. Location of scats from P6, a female pine marten ▲ ; A= the location of scats on a map of the tunnels, B= location of scats with respect to the above ground area.

APPENDIX V- ACCESSION NUMBERS FOR mtDNA BAT SEQUENCES RETRIEVED FROM GENBANK

Myotis myotis					
GU817341.1	EU374609.1	EU374588.1	AY699870.1	AF368763.1	KF218472.1
GU817340.1	EU374608.1	EU374587.1	AY699869.1	AF368762.1	KF218470.1
GU817339.1	EU374607.1	EU374586.1	AY699868.1	KF218384.1	DQ915043.1
EU374626.1	EU374606.1	EU374585.1	AY699867.1	KF218383.1	DQ120800.1
EU374625.1	EU374605.1	EU374584.1	AY699866.1	KF218380.1	AF401443.1
EU374624.1	EU374604.1	EU374583.1	AY699865.1	KF218379.1	AF401442.1
EU374623.1	EU374603.1	AF368777.1	AY699864.1	JX442114.1	AF401441.1
EU374622.1	EU374602.1	AF368776.1	AY699863.1	JX442113.1	AF401440.1
EU374621.1	EU374601.1	AF368775.1	AY699862.1	JX442105.1	AY033986.1
EU374620.1	EU374600.1	AF368774.1	AY699861.1	JX442103.1	AY699874.1
EU374619.1	EU374599.1	AF368773.1	AY699860.1	JX442102.1	AY699873.1
EU374618.1	EU374598.1	AF368772.1	AY699859.1	JX442099.1	AY699872.1
EU374617.1	EU374597.1	AF368771.1	AY699858.1	AF246246.1	AY699871.1
EU374616.1	EU374595.1	AF368770.1	AY699857.1	AF246245.1	
EU374615.1	EU374594.1	AF368769.1	AY699856.1	AF246244.1	
EU374614.1	EU374593.1	AF368768.1	KJ948289.1	AF246243.1	
EU374613.1	EU374592.1	AF368767.1	KJ948288.1	AF246242.1	
EU374612.1	EU374591.1	AF368766.1	KJ948287.1	AF246241.1	
EU374611.1	EU374590.1	AF368765.1	KJ948286.1	KF218474.1	
EU374610.1	EU374589.1	AF368764.1	KJ948285.1	KF218473.1	

Myotis daubentonii					
AY699872.1	HQ657339.1	DQ120897.1	JF442957.1	EU153107.1	JF442973.1
KF218460.1	HQ657338.1	DQ120896.1	JF442956.1	EU153106.1	JF442972.1
DQ915046.1	HQ657337.1	EU153123.1	JF442955.1	EU153105.1	JF442970.1
HQ657356.1	HQ657336.1	EU153122.1	JF442954.1	EU153104.1	JF442967.1
HQ657355.1	HQ657335.1	EU153121.1	JF442952.1	EU153103.1	JF442966.1
HQ657354.1	HQ657334.1	EU153120.1	JF442951.1	EU153102.1	JF442965.1
HQ657353.1	HQ657333.1	EU153119.1	JF442950.1	AB106590.1	JF442964.1
HQ657352.1	HQ657332.1	EU153118.1	JF442949.1	AB106589.1	JF442963.1
HQ657351.1	HQ657331.1	EU153117.1	JF442948.1	FR856683.1	JF442962.1
HQ657350.1	HQ657330.1	EU153116.1	JF442947.1	FR856682.1	JF442961.1
HQ657349.1	HQ657329.1	EU153115.1	JF442946.1	FR856681.1	JF442960.1
HQ657348.1	HQ657328.1	EU153114.1	JF442945.1	FR856680.1	JF442959.1
HQ657347.1	AY033985.1	EU153113.1	JF442944.1	FR856679.1	JF442958.1
HQ657346.1	FN643239.1	EU153112.1	JF442943.1	JX008068.1	
HQ657345.1	AB106568.1	EU153111.1	JF442941.1	JF442971.1	
HQ657344.1	AB079824.1	EU153110.1	JF442940.1	JF442969.1	
HQ657343.1	AY665137.1	EU153109.1	JF442939.1	JF442968.1	
HQ657342.1	AF376862.1	EU153108.1	JF442938.1	JF442953.1	
HQ657341.1	AF376847.1	EU153125.1	JF442937.1	JF442942.1	
HQ657340.1	DQ120898.1	EU153124.1	GU270554.1	JF442974.1	

Myotis brandtii		*Myotis mystacinus*	
HQ529621.1	AY699869.1	AB106583.1	JX645306.1
HQ529620.1	AY699868.1	KF218482.1	JX645305.1
HQ529619.1	JX645287.1	KF218481.1	JX645304.1
HQ529618.1	JX645286.1	KF218480.1	JX645303.1
HQ529617.1	JX645285.1	KF218479.1	JX645302.1
HQ529616.1	JX645284.1	KF218478.1	JX645301.1
HQ529615.1	JX645283.1	AY699865.1	JX645300.1
HQ529612.1	JX645282.1	AY699864.1	JX645299.1
HQ529611.1	JX645281.1	AY699863.1	JX645298.1
HQ529610.1	JX645280.1	KF218477.1	JX645297.1
DQ915035.1	JX645279.1	KF218476.1	JX645296.1
AY027858.1	JX645278.1	KF218475.1	JX645295.1
AY027857.1	JX645277.1	JX645319.1	JX645294.1
AY027856.1	JX645276.1	JX645318.1	JX645293.1
AY027855.1	JX645275.1	JX645317.1	JX645292.1
AY027854.1	JX645274.1	JX645316.1	JX645291.1
AY027853.1	JX645273.1	JX645315.1	JX645290.1
AY027852.1	JX645272.1	JX645314.1	JX645289.1
AY027851.1	JX645271.1	JX645313.1	JX645288.1
KJ948306.1	JX645270.1	JX645312.1	EU360611.1
KJ948305.1	JX645269.1	JX645311.1	EU360610.1
HQ529622.1	JX645268.1	JX645310.1	EU360609.1
HQ529614.1	JX645267.1	JX645309.1	EU360608.1
HQ529613.1	HQ529624.1	JX645308.1	HQ529609.1
HM046628.1	HQ529623.1	JX645307.1	HQ529608.1

Myotis bechsteinii		*Myotis nattereri*		*Myotis dasycneme*	
KF218454.1	EU531450.1	FR856731.1	FR856717.1	AF376846.1	JF442932.1
DQ915059.1	EU531449.1	FR856730.1	FR856716.1	AF401454.1	JF442931.1
DQ915058.1	EU531448.1	FR856729.1	FR856715.1	AF401453.1	JF442930.1
DQ915057.1	EU531447.1	FR856728.1	FR856714.1	AY033977.1	JF442929.1
AF401462.1	EU531446.1	FR856727.1	FR856713.1	JF442935.1	DQ915048.1
AF401461.1	EU531445.1	FR856726.1	FR856712.1	JF442936.1	DQ915047.1
AF401460.1	EU531444.1	FR856725.1	FR856711.1	JF442934.1	AM265652.1
AF401459.1	EU531443.1	FR856724.1	JX008073.1	JF442933.1	
AY033978.2	EU531442.1	FR856723.1	JX008072.1		
EU531456.1	EU531441.1	FR856722.1	JF443018.1		
EU531455.1	EU531440.1	FR856721.1	JF443017.1		
EU531454.1	EU531439.1	FR856720.1	JF443016.1		
EU531453.1	EU531438.1	FR856719.1	JF443015.1		
EU531452.1	EU531437.1	FR856718.1	GU270561.1		
EU531451.1	EU531436.1				

Plecotus auritus				
AY699874.1	DQ915078.1	AF401369.2	AF516273.1	FR856803.1
KF218516.1	DQ915077.1	AF401373.1	AB079817.1	FR856802.1
KF218515.1	DQ915076.1	AF401372.1	FR856810.1	JF443100.1
KF218514.1	DQ915075.1	AF401370.1	FR856809.1	JF443099.1
NC_015484.1	DQ915074.1	AF401368.1	FR856808.1	JF443096.1
HM164052.1	DQ915073.1	AY328906.1	FR856807.1	JF443095.1
DQ915081.1	DQ915072.1	AY131291.1	FR856806.1	JF443101.1
DQ915080.1	AF401374.2	AF516277.1	FR856805.1	JF443098.1
DQ915079.1	AF401371.2	AF516276.1	FR856804.1	JF443097.1

Eptesicus sp				
JX008042.1	KF218438.1	EU786975.1	FR848493.1	EU786981.1
JX008041.1	KF218437.1	EU786972.1	FR848492.1	EU786980.1
JX008040.1	FR848495.1	EU786971.1	DQ915025.1	EU786979.1
JX008039.1	FR848494.1	EU786970.1	AF401475.1	EU786978.1
JF442802.1	DQ915028.1	EU786969.1	AF401474.1	EU786977.1
JF442800.1	DQ915024.1	EU786968.1	AF401473.1	EU786976.1
JF442799.1	DQ915023.1	EU786967.1	AY033987.1	
JF442822.1	DQ120804.1	EU786966.1	AB079822.1	
JF442821.1	DQ120803.1	NC_022474.1	EU786999.1	
JF442819.1	AF401472.1	KF019094.1	EU786998.1	
JF442818.1	AF401471.1	KF019093.1	EU786997.1	
JF442817.1	AY033950.1	KF019080.1	EU786996.1	
JF442810.1	JF442812.1	KF019079.1	EU786995.1	
JF442809.1	JF442811.1	KF019078.1	EU786994.1	
JF442808.1	FR856642.1	KF019077.1	EU786987.1	
JF442807.1	FR856641.1	KF019076.1	EU786986.1	
JF442806.1	JX008047.1	KF111725.1	EU786985.1	
JF442805.1	JX008045.1	KF218441.1	EU786984.1	
JF442804.1	JX008044.1	KF218440.1	EU786983.1	
JF442798.1	JX008043.1	KF218439.1	EU786982.1	

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