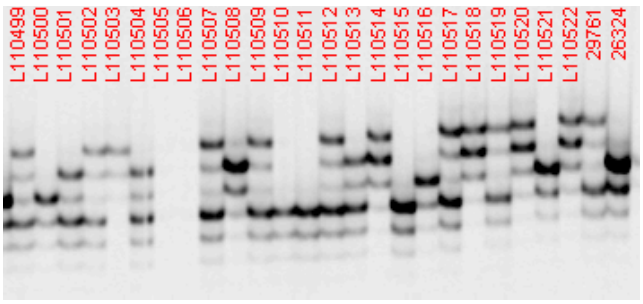
# Pine marten CSI:

# Gaining knowledge from tongues and faeces

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Tim Hofmeester

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Fons Debets (Genetics)

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# Preface

As a biology student, specializing in animal ecology, this thesis research was an ideal opportunity to be able to gain more knowledge about genetics and the use of genetic techniques in the field of animal ecology. I have learned a lot in this respect and I think I am now able to combine my knowledge by designing genetic based methods to gain more insight into the ecology of animals.
At first, my report was only going to incorporate the part of my research on the Dutch pine marten genetics, which is now Chapter 2. But as I have spent a lot of time in the lab, working on the non-invasive genetics study, I decided to also add this part of my research to this report. The analysis of Chapter 1 is however not very extensive, as I spend most of my time analysing the data for Chapter 2.
The working environment at Alterra gave me the opportunity to think about my ideas, optimise my results and get the best out of my research. Therefore, I would like to thank Jan Bovenschen, Hans Peter Koelewijn, Dennis Lammertsma, and Ivo Laros for helping me during the study, both by discussing my ideas and helping me to get a grip on the ins and outs of working in a molecular lab. Furthermore, I would like to thank my supervisors Fons Debets and Hugh Jansman for supporting me during my research and making this thesis research possible.

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# Summary

During this Master thesis, two different aspects of pine marten genetics were investigated. A lab protocol for the extraction of DNA from pine marten faeces was optimized and used to investigate the pine marten population in the Wieden-Weerribben, a lowland peat marsh in the Netherlands. The faecal samples were analysed using ten microsatellite loci, including two loci which could distinguish pine marten scats from the scats of the very similar and sympatric stone marten. Furthermore, a locus situated on the Y chromosome was used to determine the sex of the animals. A minimum of 21 individuals were found, of which 8 males, 12 females and 1 with unknown sex, due to problems with the amplification of the sex primer. From each individual 1 – 6 scats were found, resulting in an average number of observations per animal of 1.33, which was to low to make an estimation of the total population size. The minimum population size found did however corresponding to the amount of animals estimated from a camera trap study. Due to the lack of selection in the field, the success rate was very low (49%). It was, however, shown that fresh scats have a higher success rate and therefore it is recommended to collect only fresh scats for future studies.
As a second part of this study, both faecal and tissue samples of 270 pine martens were used to investigate the population and landscape genetics of pine martens in the Netherlands. Using eight microsatellite loci in the software packages STRUCTURE and GENELAND, the population structure and landscape genetics were analysed. The Dutch pine marten population was found to have a rather high genetic variation ($H_e$ = 0.64) and a rather low genetic structure. GENELAND, however, found four different clusters, separated by barriers to gene flow. These barriers were correlated to lack of suitable habitat, highways and waterways. This shows that the Dutch population still has a rather high genetic variation, but recent barriers start to form problems for gene flow. This could be countered by taking mitigating measures, to overcome the barriers.

# Chapter 1: 'Tree otters' in the mire: Using non-invasive genetics to investigate the population status of a pine marten population in a lowland peat marsh

**Abstract**
The pine marten is an elusive animal which is difficult to study. Therefore, non-invasive genetic methods were used to study the pine marten population in the Wieden-Weerribben area in the Netherlands. Scats were collected and analysed with the use of 10 microsatellite loci, and a locus situated on the Y chromosome was used to determine the sex of the animals. A total of 21 animals were found, of which 8 males, 12 females and 1 unknown sex due to failure of amplification of the sex primer. However, due to the low average amount of records per individual (1.33), the total population size could not be estimated. The expected heterozygosity of these 21 animals was rather high ($H_e$ = 0.57), indicating that there is gene flow between the Wieden-Weerribben population and other Dutch pine marten populations. Furthermore, it was found that fresh scats had a higher success ratio than old scats and that scats containing mostly berries had a higher success ratio than scats containing either mostly hair or mostly feathers. Therefore, in future studies, it is recommended to collect only fresh scats, preferably containing berries, to have a high success ratio, while keeping the costs and efforts as low as possible.

**Keywords:** *faecal samples, genetic variation, population size, success ratio*

## Introduction

The European pine marten (*Martes martes*) occurs mainly in forests (Baltrunaite 2010; Brainerd& Rolstad 2002), although populations have been found in more open, but dry, areas (Clevenger 1994; Pereboom et al. 2008). Therefore, the pine marten population in the Wieden-Weerribben, a lowland peat marsh in the region of Northwest Overijssel in the Netherlands, is very interesting, as this region consists mostly of wetlands, reed beds and grasslands, with only small occasional wet forests (Tuitert *et al.* 2009).

The presence of pine martens in this area has been known since the late 19$^{th}$ century, although they have always been rare (Bode *et al.* 1999; IJsseling& Scheygrond 1950). Recently, the population has increased, pine martens are now being found throughout the area, and reproduction has been recorded for the last couple of years (Tuitert *et al.* 2009). It is however not known how large this population is and if this population is viable.

A good method to study illusive carnivore populations is the use of non-invasive population genetics (Piggott& Taylor 2003). In this way information about the number of animals, their relatedness and the genetic variation in the population can be studied without even seeing the animals. For this method, hair, feathers and faeces can be used (Piggott& Taylor 2003). In Ireland, hair and faeces have been used to great success to study the genetic status of the pine marten population and hair was found to be the most successful source of DNA (Mullins *et al.* 2010). In the Netherlands, however, faecal samples are used to monitor the otter population in the Wieden-Weerribben area (Koelewijn *et al.* 2010). Therefore, scats were collected to investigate the population size and genetic variation of the pine martens in the Wieden-Weerribben. These scats were investigated using 10 microsatellite loci to identify individuals and to calculate the expected heterozygosity of the population.

## Methods

*Genetic analysis*

Faecal samples were collected from April 2009 until May 2010. Samples were stored in 96% ethanol and after arrival in the lab at -21°C. DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN). Ten microsatellite loci were used (Gg454, Ma-1, Ma-2, Ma-4, Mel-1, Mel-6, Mel-10, Mvis020, Mvi-57 and Ma-18) and the sex was determined using the DBY7Ggu locus, located on the Y chromosome (Bijlsma *et al.* 2000; Davis& Strobeck 1998; Domingo-Roura 2002; Fleming *et al.* 1999; Hedmark *et al.* 2004; Kyle& Strobeck 2003; O'Connell *et al.* 1996; Walker *et al.* 2001; Wisely *et al.* 2004).

PCR reactions were performed using a total volume of 10µl of which 2µl was DNA extract. For all microsatellite loci except Ma-2 the PCR-mix contained 0.3 Units of *Taq* (Invitrogen *Taq* DNA polymerase), amounts of PCR buffer and W-1 according to the Invitrogen protocol, 100nM of both primers, 200µM of each dNTP, 3mM $MgCl_2$ and 320 µg/ml BSA. Forward primers were labelled with either an IRD-700 or an IRD-800. The PCR programme used was 94°C for 2 min, 36 cycles at 94°C for 30s, $T_a$ for 30s, 72°C for 1 min and a final extension step of 20 min at 72°C. For most primers $T_a$ was 56°C, except for Ma-1, Mvi-57 and Mel-1 ($T_a$ = 60°C) and Ma-4 and Mvis020 ($T_a$ = 64°C).

As DBY7Ggu only results in one single band for males and no amplification for females, as it is positioned on the Y chromosome, a positive control was used, in the form of the Ma-2 primer, to test for any problems during the PCR. For this PCR, the PCR-mix contained 0.6 Units of *Taq*, PCR buffer according to the

Invitrogen protocol, 250nM of the DBY7Ggu primer, 100nM of the Ma-2 primer, 200µM of each dNTP, 2mM $MgCl_2$ and 800µg/ml BSA. Both forward primers were labelled with an IRD-800. The PCR programme used was 95°C for 3 min, 45 cycles at 94°C for 30s, 52°C for 30s, 72°C for 1 min and a final extension step of 5 min at 72°C.
PCR products were genotyped on a 6.5% polyacrylamide gel containing 7M Urea and 0.8x TBE on a Li-Cor 4300 platform.
For the typing of the samples, the protocol of Koelewijn *et al.* (2010) was used with the following exceptions. All faecal samples were first screened using the primers Mel-10 and Ma-18 as these primers can be used to distinguish pine and stone marten (*Martes foina*; Pilot *et al.* 2007). Each sample was amplified three times for each primer, and if the sample showed amplification results in any of the six amplifications, it went through to be analyzed using the other eight microsatellite loci and the sex primer. If the sample showed amplification results suggesting that the sample came from a stone marten, the sample was not included in the rest of the analysis.
As there was only budget for three amplifications per locus, the samples that could not be genotypes by only using three amplifications were not genotyped for that locus. If a sample had a heterozygous result for all three amplifications of a locus, the sample was considered a heterozygote, if the sample was heterozygous for two amplifications, but homozygous for one amplification, the sample was also considered a heterozygote. Furthermore if a sample was heterozygous for one amplification, but homozygous for one allele in one amplification and homozygous for the other allele in the third amplification, the sample was also considered a heterozygote. If the sample was homozygous for all three amplifications, the sample was considered a homozygote.
The results of all three amplifications of all loci were given a quality index (QI) using the protocol suggested by Miquel *et al.* (2006) and all samples with a QI lower than 0.5 were not included into the final analysis.

*Statistical analysis*
The software package GenAlEx 6.4 (Peakall& Smouse 2006) was used to compute the Probability of Identity (PI) and the conservative $PI_{Sibs}$ as an estimation of the amount of loci needed to be able to distinguish individuals in a population (Taberlet& Luikart 1999). The PI was also used to estimate the amount of individuals in the population. For the 10 loci used, the PI was $4.7 * 10^{-7}$ and the $PI_{Sibs}$ was $1.5 * 10^{-3}$. A minimal $PI_{Sibs}$ of 0.01 was used when selecting for the number of loci needed to distinguish individuals, which resulted in 7 loci needed, allowing 3 mismatches when appointing samples to different individuals. Furthermore GenAlEx 6.4 was used to calculate the observed and expected heterozygosity ($H_o$ and $H_e$).
For each sample a success ratio was calculated by dividing the number of successful amplifications by the total number of amplifications. Next to that a QI was calculated for each locus (Miquel *et al.* 2006). The Spearman's rank correlation coefficient was used to test for correlations between the QI and the number of base pairs in a locus, and the success ratio of the screening and the final success ratio.

Furthermore, 100 pine marten scats collected in the same period throughout the Netherlands were used to test for factors influencing the success ratio of amplifications from scats. A Kruskal-Wallis test was used to check for differences in success ratio between different main diet components of the scats. Furthermore, a Mann-Whitney U test was used to check for a possible difference in success ratio between old (dried and hard) scats and fresh scats. For these analyses the R software package (R Development Core Team 2011) was used.

## Results

In total 28 of the 57 faecal samples collected were genotyped successfully, resulting in a success rate of 49%. These 28 samples were appointed to 21 individuals of which eight were males, twelve females and one unsexed individual due to a failed amplification of DBY7Ggu. The number of scats per genotype was 1-6, with a mean of 1.33 observations per individual. The mean observed heterozygosity over all loci was 0.639 and the mean $H_e$ was 0.57.
A clear correlation was found between the quality index of a locus and the length of that locus, with larger loci having a lower quality index (Spearman's rank: r= -0.683, p = 0.03, n = 10; fig. 1). A clear correlation was also found between the success ratio of the screening with the loci Mel-10 and Ma-18 and the total success ratio of a sample (Spearman rank: r= 0.954, p< 0.0001, n= 67; fig. 2).

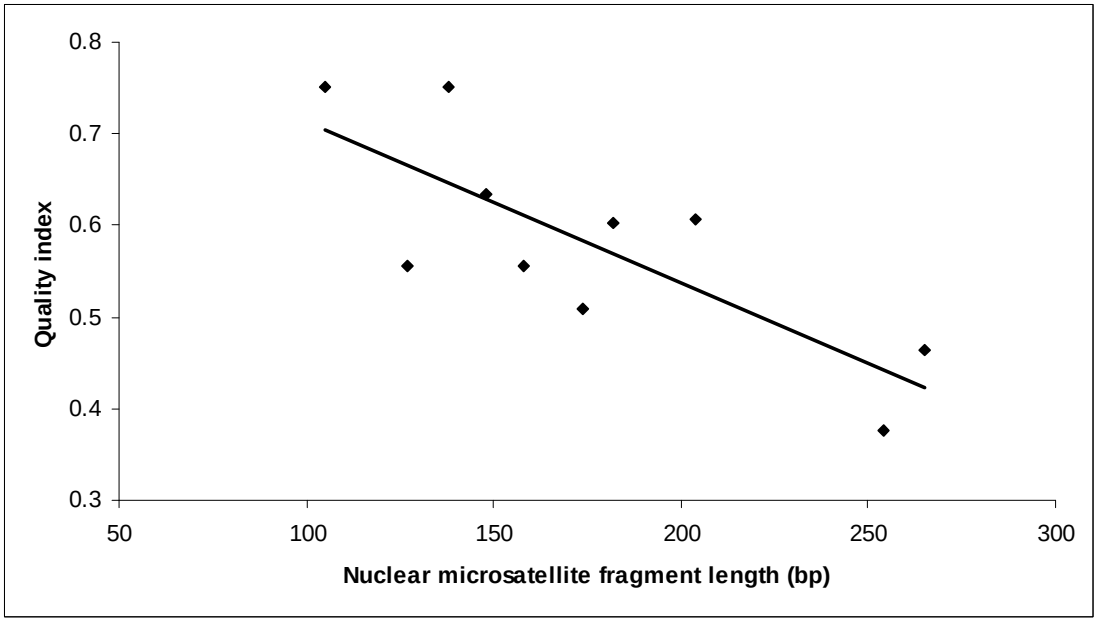



Figure 1. Correlation between quality index and nuclear microsatellite fragment length (in base pairs). Larger loci have a lower quality index, indicating that smaller loci give better results in studies using low quality DNA.

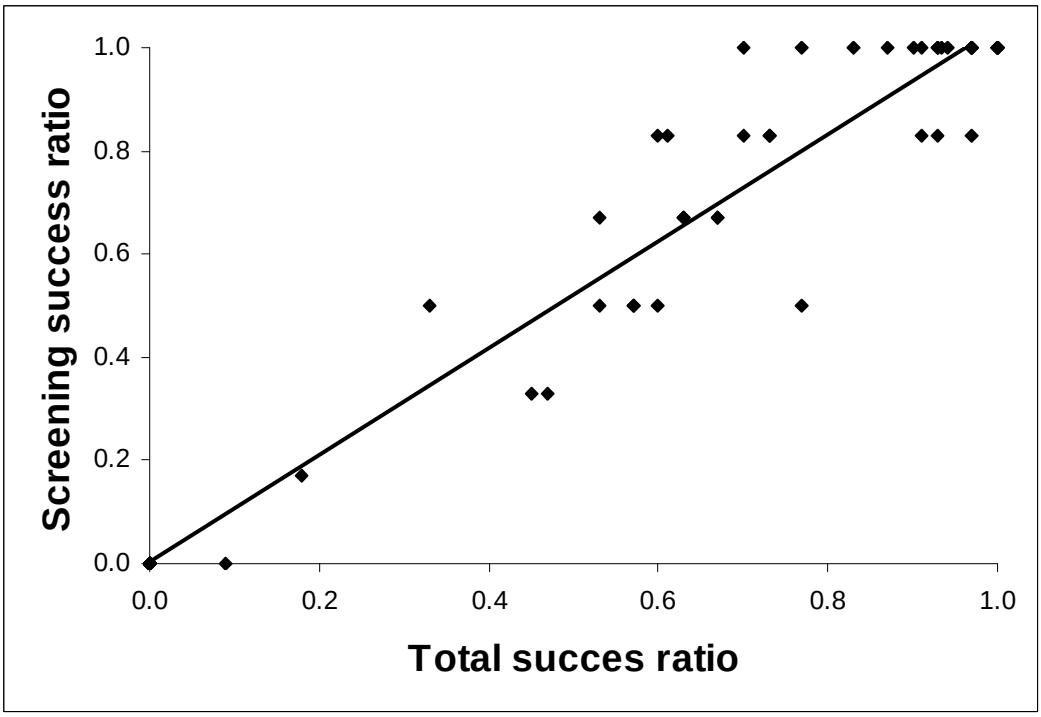



Figure 2. Correlation between screening success ratio and total success ratio. This shows that Mel-10 and Ma-18 are good loci to screen with and that selection for good quality samples can already take place after screening.

Not all scats could be appointed to one of the main diet components, resulting in 55 scats divided over mostly hair (n=22), mostly feather (n=19) and mostly berries (n=14). The success ratio of scats consisting mostly of berries was significantly higher than the success ratio of scats consisting mostly of hairs or feathers (Kruskal-Wallis: $\chi 2 = 11.415$, p. = 0.003; table 1). From the 100 scats, 35 were classified as old. It was found that old scats had a lower success ratio than fresh scats (Mann-Whitney U: $p < 0.001$; table 2).

Table 1: Influence of the main prey item in the scat and success ratio. The differences between the different diets (indicated by letters) were calculated by applying a Bonferroni adjustment to a pair wise Mann-Whitney U test.

| Main prey item in scat | Number | Success ratio |
|---|---|---|
| Hair | 22 | 0.45[a] |
| Feather | 19 | 0.38[a] |
| Berries | 14 | 0.78[b] |

Table 2: Influence of the age of the scat on the success ratio. The difference was calculated by performing a Mann-Whitney U test.

| Age of scat | Number | Success ratio |
|---|---|---|
| Old (hard and dried) | 35 | 0.31 |
| Fresh | 65 | 0.62 |

## Discussion

In this study, a minimal population size of 21 pine martens was found in the Wieden-Weerribben. This minimum corresponds well to the 22-27 animals estimated by Tuitert et al. (2009), showing that non-invasive genetics and a camera trap study both give the same estimate for population size. The average number of observations per animal of 1.33 was however too low to give a good estimation of the population size, resulting in only a minimum number of animals (Miller *et al.* 2005).
The success rate of 49% of the samples genotyped is relatively low compared to other studies using faecal DNA (Broquet *et al.* 2007), although it is roughly identical to the success rates found in otter spraints (Hájková *et al.* 2009; Koelewijn *et al.* 2010). This is most probably due to the lack of selection when collecting the samples in the field. The difference in success ratio between fresh and old scats shows that it pays of to select for only fresh scats in the field. This can be improved further by specific selection for scats containing mostly berries. Furthermore, the success rate could be low due to possible misidentification between marten and fox (*Vulpes vulpes*) scats (Davison *et al.* 2002). In this study, no primers were used which could identify fox scats, so scats with an overall success ratio of zero could actually have belonged to foxes.
The screening with the loci Ma-18 and Mel-10 proved to be successful. In future studies, the selection of good quality samples can already occur after screening, to save time and money. A screening success ratio of 0.67 would be a good minimum for analysis of the other 8 loci. The used primers turned out to work well for the low quality DNA obtained from the scats, although a selection for loci with small microsatellite fragment lengths could further improve the success rate, which was also found by Broquet *et al.* (2007).
The pine martens in the Wieden-Weerribben area seem to have a rather large genetic variation as the found $H_e$ of 0.57 is roughly the same as the average $H_e$ (0.58) found in European populations (Kyle *et al.* 2003) indicating that the pine marten population of the Wieden-Weerribben is genetically connected to other Dutch populations or had a founder population with a very high genetic variation. The average $H_e$ found is however lower than the average $H_e$ found in the total Dutch pine marten population (0.64; Chapter 2), the $H_e$ of 0.63 found in Canadian pine martens (*M. Americana*; Kyle& Strobeck 2003) and the $H_e$ of 0.62 found in Canadian fishers (*M. pennanti*; Kyle *et al.* 2001), which is most likely due to a founder effect (Barton& Charlesworth 1984). When comparing the genotypes found in this study to a dataset with genotypes of pine martens from the whole of the Netherlands, it becomes clear that the pine martens from the Wieden-Weerribben are clustered into the same population as the animals from South-West Drenthe and the Noordoostpolder (Chapter 2) showing that the Wieden-Weerribben population is indeed not genetically isolated from other Dutch populations.
This study shows that it is possible to obtain consensus genotypes from pine marten scats, which can be used for studies on population parameters. It is however important to collect fresh scats in a short period of time to be able to make an estimation of the true population size and to minimize costs and effort.

# Chapter 2: Landscape genetic structure and genetic diversity of the Dutch pine marten population

**Abstract**

The European pine marten is a specialized woodland carnivore living in large parts of Europe. In the Netherlands, the pine marten population has been declining from 1946 until 1988, after which the population has started to increase. Therefore, the genetic status and landscape genetics of the Dutch population were studied, using eight microsatellite loci on DNA samples collected from dead animals and scats. Allele frequencies, heterozygosity and pair-wise $F_{st}$ values were used to study the genetic variation and the population structure was investigated with the use of the software programs STRUCTURE and GENELAND. When using different models, the Dutch pine marten population was found to consist of three or four subpopulations. These subpopulations had a rather large genetic variation compared to other European pine marten populations, with an average expected heterozygosity of 0.64. Furthermore, they had low pair-wise $F_{st}$ values, ranging from 0.009 to 0.026. At the same time, barriers to gene flow were found between the different subpopulations. These barriers were related to areas without suitable habitat, highways and rivers and, as the genetic variation is still large, these barriers are thought to be recent. Therefore, it is suggested to build vegetated wildlife crossings to increase the gene flow between the different subpopulations and monitor these crossings with the use of telemetry and non-invasive genetics.

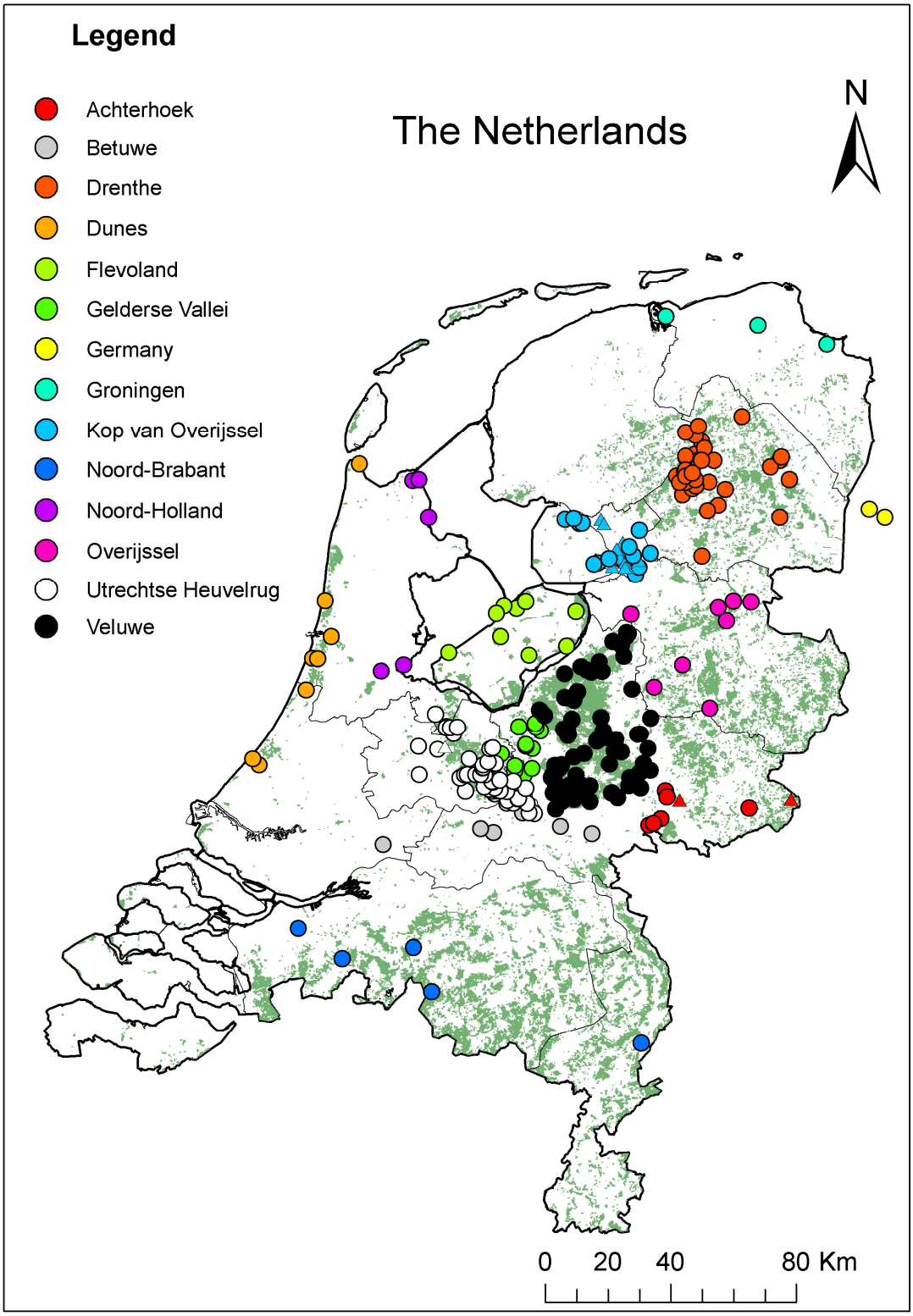
**Keywords:** *barriers, dispersal, gene flow, landscape genetics, population structure*

## Introduction

The European pine marten (*Martes martes*) is a specialized woodland mesocarnivore of the *Mustelidae* family, living in large parts of Europe, from the Mediterranean to the taigas of Scandinavia and Russia (Proulx *et al.* 2004; Stubbe 1993). It occurs mainly in forests (Baltrunaite 2010; Brainerd& Rolstad 2002), but has also been reported from more open habitats (Clevenger 1994; Pereboom et al. 2008) and even lowland marshland (Tuitert et al. 2009). In the last few decades, many of its populations have suffered from habitat loss and persecution and therefore conservation measures have been taken to aid the species in several countries such as Britain, Ireland, Belgium and Denmark (Proulx et al. 2004).
As a consequence, the habitat loss and persecution may have resulted in fragmented populations, with decreased levels of gene flow, causing loss of genetic variation (Lande& Shannon 1996). Recent population genetics studies show that most of the Western-European pine marten populations indeed have a higher level of genetic structure and a lower genetic variation than the closely related American pine marten (*Martes Americana*; Kyle et al. 2003).
The Dutch pine marten population is rather small with an estimated 400 animals, of which the majority is found in three large subpopulations, Veluwe, Utrechtse Heuvelrug and Drents-Friese Wold (Tuitert et al. 2009). The genetic status of the Dutch pine marten population is unknown, apart from ten Dutch pine martens which were incorporated in the study of Kyle et al. (2003). It is also not known if the Dutch population is isolated from other populations. In the South, it seems isolated as pine martens are very rare in Flanders (Van den Berge et al. 2000) and in the East there seem to be populations of moderate size in Western Germany, which could cause an influx for the Dutch population (Zekhuis 2011).
The pine marten population in the Netherlands has declined from 1946 until 1988 (Müskens *et al.* 2000). This decrease could have caused loss of genetic variation and an increased genetic structure in the pine marten population, which can cause problems as the population is expanding again (Barton& Charlesworth 1984; Broekhuizen *et al.* 1990). Furthermore, in the last decades, barriers such as urban areas and roads have increased (Balkenhol& Waits 2009). These barriers together with more natural barriers such as waterways and areas without suitable habitat can cause barrier to gene flow, between the existing expanding subpopulations (Riley et al. 2006; Short Bull et al. 2011). For this purpose, landscape genetics can be used to understand the effect of the landscape on gene flow, as prior knowledge about the population structure is not needed (Manel *et al.* 2003).
Next to that, the increase has led to pine martens re-appearing in areas where they were thought to have disappeared and appearing in areas where they were never found before such as the dunes and Noord-Holland (Broekhuizen et al. 1990; Müskens et al. 2000). This expansion into new areas, with less suitable habitat, could be caused by the entire suitable habitat in the old areas being populated by pine martens, forcing young animals to disperse, which raises questions about the origin of the pine martens found in the new areas.
Therefore, the genetic status of the Dutch pine marten population was studied. Eight microsatellite loci were used to study genetic variation and Bayesian clustering methods were used to define genetic structure as well as the landscape genetics, by defining genetic discontinuities between the different subpopulations. Land use maps of the Netherlands were used to relate these discontinuities with different landscape features and management

recommendations were made based on these findings. Furthermore, these Bayesian clustering methods were used to investigate the origin of the pine martens found in the dunes and Noord-Holland.

Figure 3: Map of the Netherlands with indication of the sites where samples were collected. These sites were arbitrarily chosen as labels for the STRUCTURE output. Tissue samples are illustrated as circles, while faecal samples are illustrated as triangles. Suitable forest habitat for pine martens is illustrated in dark green.



## Methods

*Sample collection*
Genotypes of 270 individuals were used in this study. These genotypes were derived from 251 tissue samples collected from traffic victims and other dead found animals and 66 faecal samples collected in the field. The traffic victims and other dead animals were all collected between 1992 and 2010, with the exception of one individual from Noord-Brabant, which was collected in 1985. The dead animals were collected in various parts of the Netherlands, with the exception of two individuals, which were found just over the border in Germany (fig. 3).
Although the period over which the samples were collected is rather large, it is assumed that the genetic composition of the Dutch pine marten population did not change dramatically over this period and therefore, the samples from the whole period were combined. Especially as most of the 127 samples collected before 2000 were collected in the Veluwe and Utrecht areas, which are thought to be the most stable subpopulations. For example, the animals found in the Utrecht area were analysed for two different periods, from 1992 until 1999 (P1; n=27) and from 2000 until 2010 (P2; n=22). The expected heterozygosities were calculated for these periods, where P1 had a $H_e$ of 0.63 and P2 had a $H_e$ of 0.61. This difference was thought to be small enough to be able to combine the samples over both periods.
The other 19 genotypes were obtained from the 66 faecal samples, collected in the Wieden-Weerribben area, except two samples from the Achterhoek and one sample from Utrecht, in 2009 and 2010 (fig. 3). All tissue and faecal samples were stored in 96% Ethanol at -20°C after collection.

*DNA extraction and microsatellite analysis*
Nuclear DNA was extracted from the faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN) and from tissue samples using the DNeasy Blood & Tissue kit (QIAGEN). Eight microsatellite loci (Gg454, Ma 1, Ma 2, Ma 4, Mel 1, Mel 6, Mel 10, and Mvi57) were used (Bijlsma *et al.* 2000; Davis& Strobeck 1998; Domingo-Roura 2002; Kyle& Strobeck 2003; O'Connell *et al.* 1996; Walker *et al.* 2001). At first a ninth microsatellite locus was used (Mvis020; Fleming *et al.* 1999). This locus was discarded because the average $F_{is}$ value of 0.692 was thought to be too large. This large $F_{is}$ value was mostly caused by a large number of homozygous samples, which was thought to be due to null alleles, as all other loci had far lower $F_{is}$ values.
PCR reactions were performed using a total volume of 10µl of which 2µl was DNA extract. For all microsatellite loci the PCR-mix contained 0.3 Units of *Taq* (Invitrogen *Taq* DNA polymerase), amounts of PCR buffer and W-1 according to the Invitrogen protocol, 100nM of both primers, 200µM of each dNTP, 3mM $MgCl_2$ and 320 µg/ml BSA. Forward primers were labelled with either an IRD-700 or an IRD-800.
Because of the lower DNA quality, faecal samples were amplified three times and only samples which showed the same genotype in all three amplifications were used in the analysis. All tissue samples were amplified once. The PCR programme used was 94°C for 2 min, 36 cycles at 94°C for 30s, $T_a$ for 30s, 72°C for 1 min and a final extension step of 20 min at 72°C for faecal samples, and 94°C for 2 min, 30 cycles at 94°C for 30s, $T_a$ for 30s, 72°C for 30s and a final extension step of 1 min at 72°C for tissue samples. For most primers $T_a$ was 56°C, except for Ma-1, Mvi-57 and Mel-1 ($T_a$ = 60°C) and Ma-4 ($T_a$ = 64°C). The PCR products

were separated on a 6.5% polyacrylamide gel containing 7M urea and 0.8 x TBE on a Li-Cor 4300 platform.

*Analysis of genetic structure*
To investigate genetic structure in the Dutch pine marten population, without taking the spatial distribution of the samples into account, the software STRUCTURE 2.3.3 was used. This program uses a Bayesian model to estimate the amount of genetic clusters (or populations) which are assumed to be in Hardy-Weinberg Equilibrium (HWE) and have linkage equilibrium (Falush *et al.* 2003; Pritchard *et al.* 2000). In this program, the number of clusters (K) is fixed for each simulation, so multiple simulations with different values of K have to be run.
To estimate the most likely probable number of clusters, an initial two replicates for each K value, with K values ranging from one to ten were performed using the admixture and correlated allele frequency models (Falush et al. 2003). Burn-in periods of 50,000 steps were followed by 100,000 Monte Carlo iterations to obtain the posterior probability of the data (LnPD). After these initial short runs, longer runs were performed to get better estimates of the LnPD for the clusters with the highest LnPDs. These long runs existed of a burn-in period of 50,000 steps followed by 450,000 Monte Carlo iterations, using both the admixture and correlated allele frequency models. For these runs, five replicates were conducted for K values ranged from one to six populations. Afterwards, the most likely number of clusters (or populations) was calculated by calculating ΔK for each K value used, and selecting the modal value of the different ΔK values as the true K (Evanno et al. 2005). For the true K found and for lower K values, the coefficients of individual membership ($q_i$) of the five runs were combined to calculate an average for each sample using the software CLUMPP 1.1.2 (Jakobsson& Rosenberg 2007). To visualize the genetic structure of the different sample populations over the clusters, DISTRUCT 1.1 was used to plot the average $q_i$ values per individual (Rosenberg 2004).

*Analysis of landscape genetics*
To analyse the influence of the landscape on the genetic structure of the Dutch pine marten population, by taking the spatial distribution of the samples into account, the software GENELAND 3.2.4 was used to detect the locations of possible genetic discontinuities (Guillot et al. 2005b). This program also uses a Bayesian model to estimate the amount of genetic clusters (or populations) which are assumed to be in HWE and have linkage equilibrium, but it takes the spatial distribution of the samples into account (Guillot et al. 2005a). An initial ten runs of 500,000 iterations with a thinning of 500, with the spatial, null allele and correlated allele frequency models were performed to estimate the optimal number of clusters (K). The number of clusters was set to vary between 1 and 15 to make sure that the maximal value was far higher than the number of expected clusters. Furthermore, the uncertainty of coordinates was set to 1km, as the minimal spatial resolution of the data was 1km.
The optimal K was chosen by selecting the K with the highest average log posterior probability (ALPP) after a burn-in of 50.000 steps. After obtaining an optimal K, another ten runs with the same settings, but with a fixed K at the optimal number were performed to get the best assignment of individuals to the inferred clusters as was described by Guillot et al. (2005a). The five runs with the highest average posterior probability were used to assign the different individuals to the different clusters. A cluster analysis was performed using the

program PC-ORD, with a Euclidean distance measure and the nearest neighbour linkage method. This was done, because due to the modelling approach, every outcome will be slightly different, depending on chance, and clustering the five best runs will insure that the outcome will be more robust. A clustering program was used to cluster the GENELAND outputs as objectively as possible into different subpopulations. These clusters were also used to assign the origin of the animals found in the dunes, Noord-Holland, the areas bordering Germany and the Kop van Overijssel. The genetic discontinuities between the subpopulations found by GENELAND were compared with different landscape features by plotting the different samples with their assigned population on a map of the Netherlands, together with the main roads, the main waterways and the suitable forest habitat for pine martens. This was done using the ArcGIS 9.3.1 software. By comparing the genetic discontinuities with landscape features, possible barriers to gene flow between the different subpopulation could be detected. Furthermore, two different age classes (adult and juvenile/sub-adult) were plotted together with the landscape features to investigate the relation between genetic discontinuities and age.

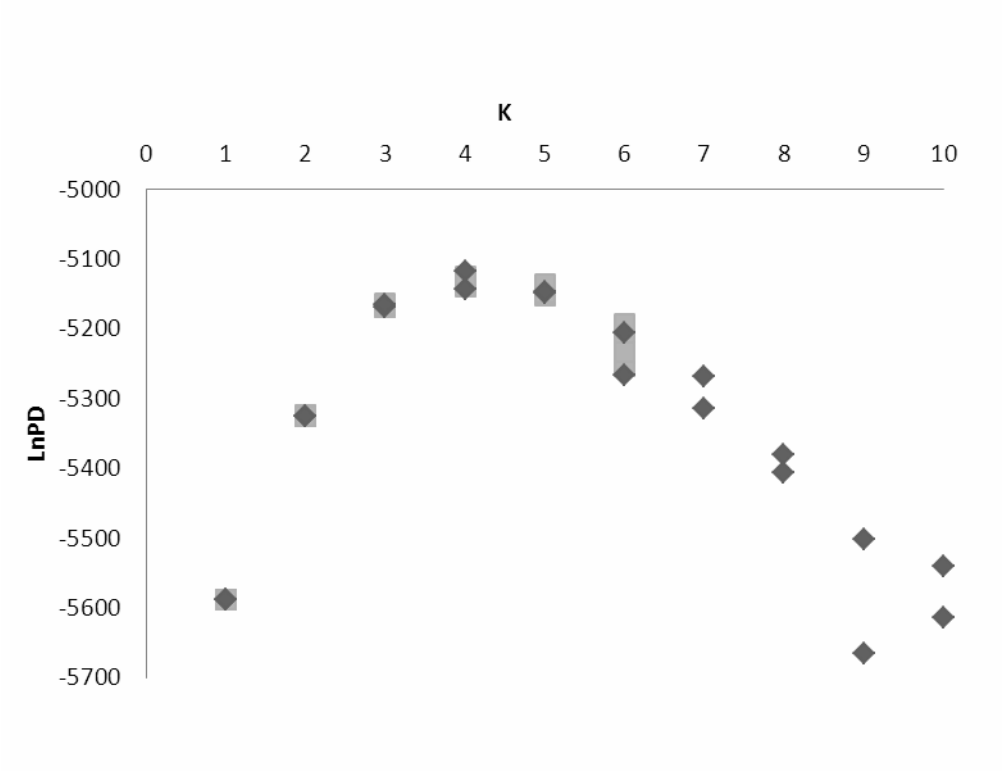
*Analyses of microsatellite variation*
The total dataset was analysed for microsatellite variation within and between the subpopulations found by GENELAND, using the GenAlEx 6.4 software package (Peakall& Smouse 2006). Both observed and effective average number of alleles per locus ($A_o$ and $A_e$) and average observed and expected heterozygosity ($H_o$ and $H_e$) were calculated per subpopulation. Furthermore, pair-wise $F_{st}$ values were calculated for all combinations of subpopulations, to investigate the genetic differentiation between the subpopulations. A Principal Component Analysis (PCA) was conducted to visualize possible differentiation between subpopulations.

## Results

*Genetic structure*
The STRUCTURE analysis showed that the Dutch pine marten population can be divided into three subpopulations (K=3). Although, the LnPD values showed a clear maximum at K=4 (fig. 4a), the modal ΔK was found to be 3 (fig. 4b). A clear distinction between a northern and a southern cluster was found using the $q_i$ values for K=2, with the most animals found in Drenthe, Flevoland and Kop van Overijssel being assigned to the northern cluster (fig. 5a; orange). For K=3, the three most commonly divided subpopulations (Drenthe, Veluwe and Utrechtse Heuvelrug) can be mostly distinguished, although there is some overlap (fig. 5b).

Figure 4: a) Average Ln of the posterior probability of the data (LnPD) values as a function of K for all STUCTURE runs. Diamonds illustrate LnPD values of the short runs with a burn-in period of 50.000 steps and a length of 150.000 steps and squares illustrate LnPD values of the long runs with a burn-in period of 50.000 steps and a length of 500.000 steps showing a maximum at a K of four. b) ΔK as a function of K, showing the modal value of ΔK, and therefore the most probable real amount of populations, to be three.

a)




b)

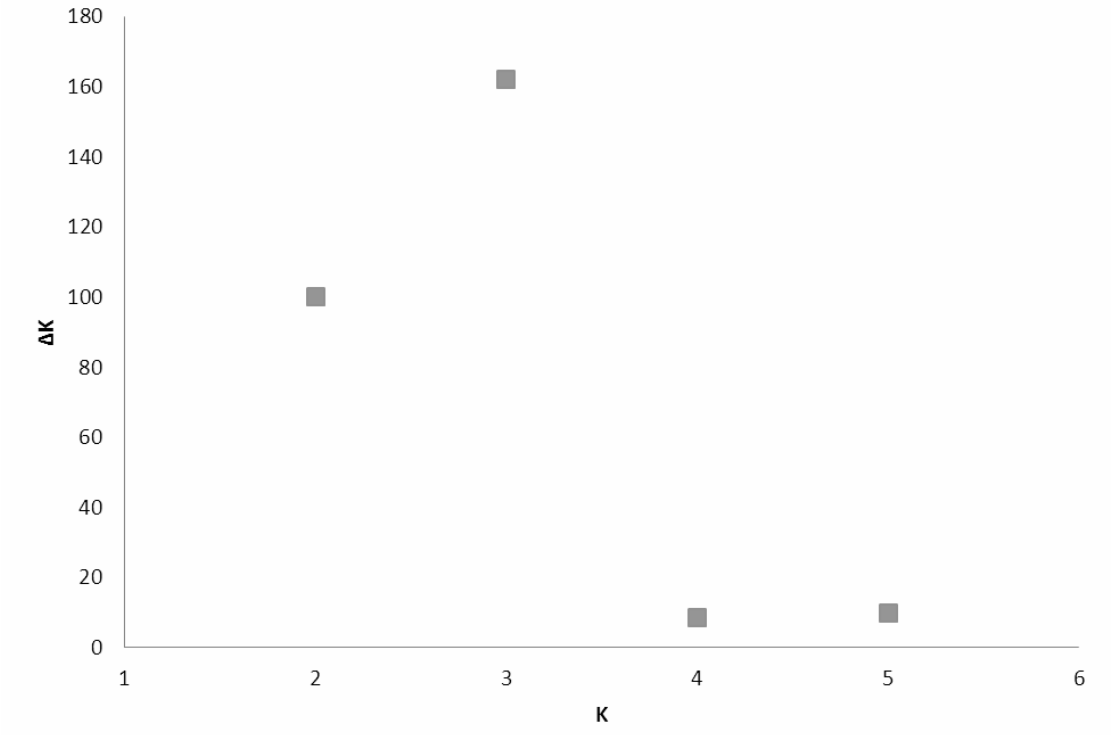
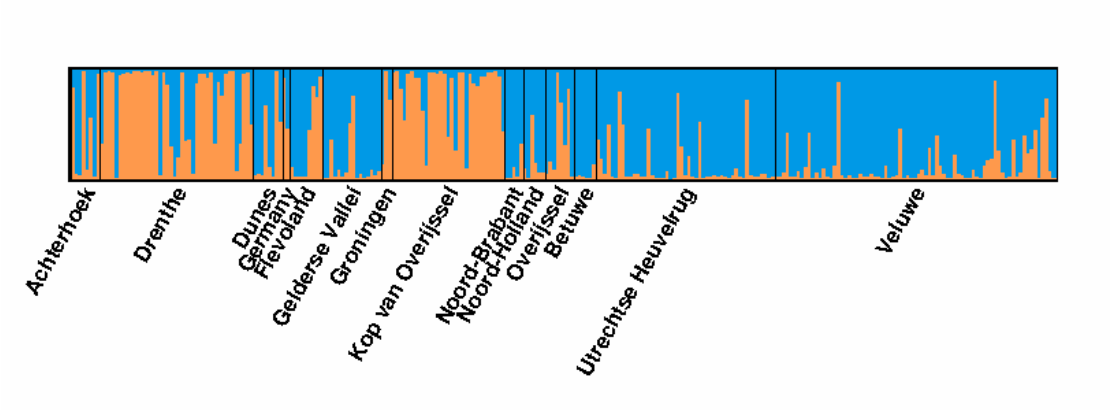
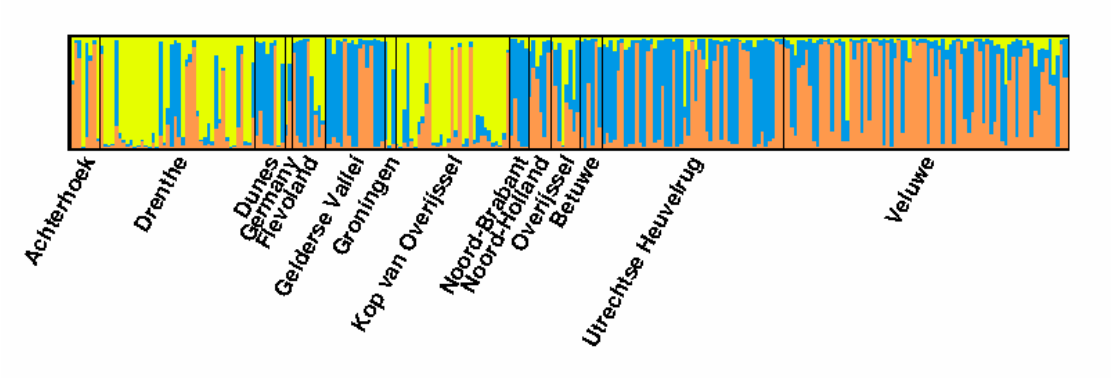


Figure 5: The average coefficients of individual membership ($q_i$) values of the different samples for the best five STRUCTURE runs for the different clusters with a) K=2 and b) K=3. The given names stand for the areas in which the samples were collected. For K=2, a clear distinction between an orange northern cluster (Drenthe, Kop van Overijssel, half of Overijssel and Flevoland) and a blue southern cluster is shown. For K=3, the southern cluster is further divided into the Veluwe and Utrechtse Heuvelrug subpopulations (orange and blue respectively), with the Betuwe, Noord-Brabant, most of the Dunes and half of the Gelderse Vallei samples being assigned to the Utrechtse Heuvelrug cluster. The Northern cluster is still visible in yellow.

a)




b)



*Landscape genetics*

The initial GENELAND runs resulted in multiple runs with a K of 7 or 8, but with the runs with K=8 having a higher ALPP (table 3). Therefore 10 runs were performed with a fixed K=8 and with a fixed K=7 to determine which of the two gave the best results. The five best runs with K=8 resulted in an average ALPP of -7279.16, while the five best runs with K=7 resulted in an average ALPP of -7580.98. Therefore K=8 was selected for further analysis. The ten runs with K=8 resulted in ten possible distributions of the samples over 8 populations. Of these ten distributions, the five runs with the highest ALPPs were used in a cluster analysis resulting in six clusters, which explained a total variation of 50%. The six clusters consisted of 1) a Veluwe cluster, 2) a Utrecht cluster, 3) a Drenthe cluster, 4) a Dutch-German border cluster, 5) an Amsterdam-Flevoland cluster and, 6) a cluster with one individual from Drenthe. When looking at the GENELAND output, it was found that there were two 'ghost populations', populations without any assigned samples, in the best run, supporting the cluster analysis of six clusters.

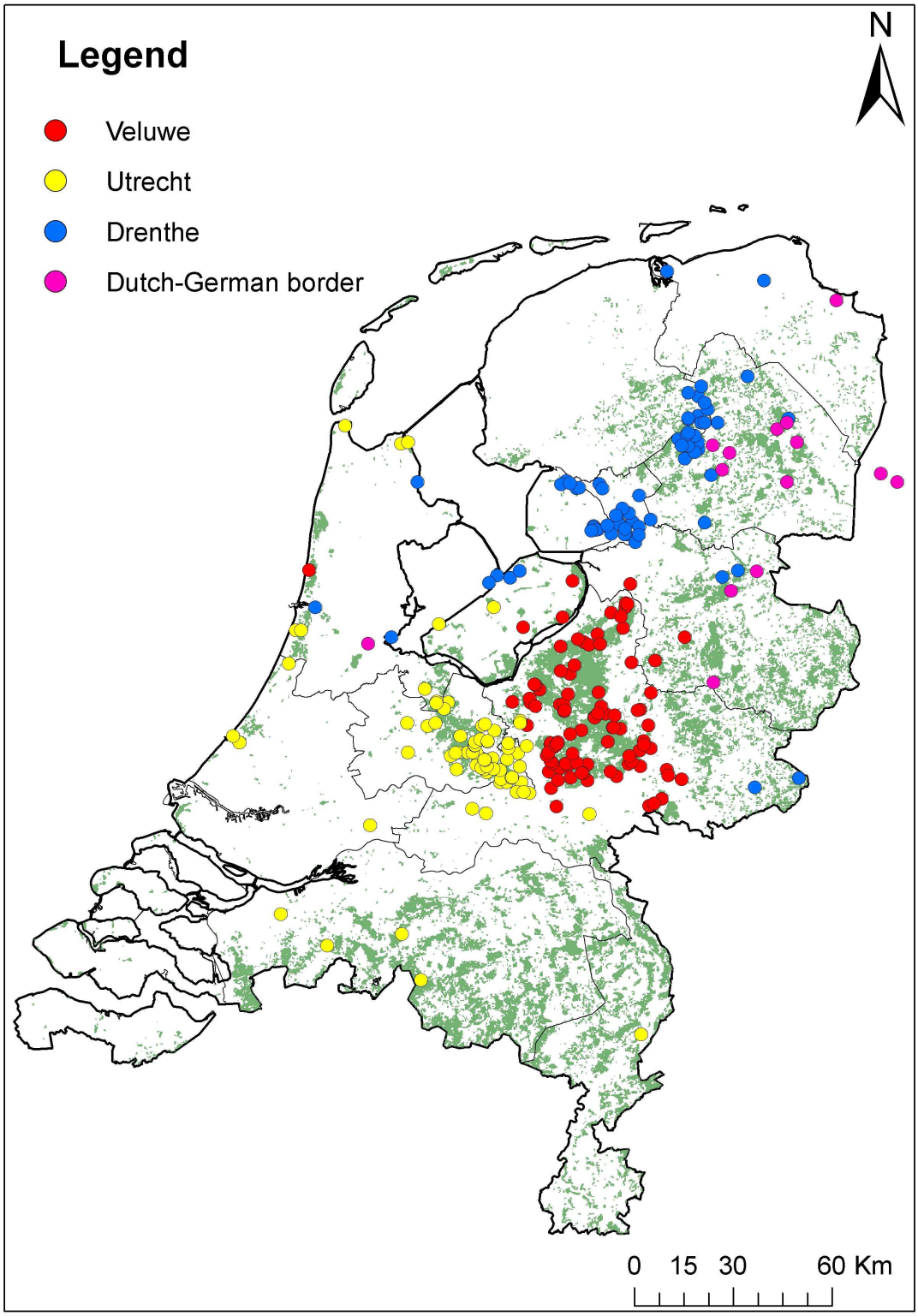
Table 3: Outcome of the first 10 GENELAND runs of 500,000 iterations with a thinning of 500, with the spatial, null allele and correlated allele frequency models, were the number of clusters was set to vary between 1 and 15 and the uncertainty of coordinates was set to 1km. The ALPP was calculated after a burn-in period of 50.000 iterations.

| Run number | K | Average log posterior probability (ALPP) |
|---|---|---|
| 2 | 8 | -7301.39 |
| 4 | 8 | -7371.37 |
| 3 | 7 | -7398.50 |
| 7 | 8 | -7403.19 |
| 10 | 7 | -7608.78 |
| 8 | 8 | -7619.04 |
| 6 | 7 | -7734.64 |
| 9 | 7 | -7781.16 |
| 5 | 6 | -7810.75 |
| 1 | 6 | -7819.03 |

As cluster 5) only consisted of four animals and cluster 6) of one animal, these clusters were assigned to one of the other clusters as it was assumed that a population cannot consist of only four animals. The Amsterdam-Flevoland cluster was clustered apart from the rest in four of the five GENELAND runs, but because there was no apparent genetic support for this small cluster, it was further investigated using the assignment test in GenAlEx 6.4. This assignment resulted in all four samples being assigned to one of the other four clusters. Next to that, the single animal which was clustered individually, was assigned to the Dutch-German border population in the same assignment test, resulting in four subpopulations.

These four subpopulations were plotted onto the map of the Netherlands to visualize the GENELAND output (fig. 6). From the GENELAND output it becomes clear that the animals from the dunes area originated from the three main subpopulations Veluwe, Utrecht and Drenthe, the animals from Noord-Holland mostly originated from Drenthe and Utrecht, with the exception of one animal found in the middle of Amsterdam, which originated from the Dutch-German border subpopulation.

Figure 6: Map of the Netherlands with the four subpopulations found with GENELAND represented by different colours. Suitable forest habitat for pine martens is illustrated in dark green.



When comparing the genetic discontinuities found between the different subpopulations, with the land use maps, it becomes clear that all barriers to gene flow are related to landscape features such as different main roads, rivers or lack of suitable forest habitat (fig. 7, 8 and 9). The genetic discontinuity between the Utrecht and Veluwe subpopulations seems to be related to the highways A1 and A30, together with a 6km wide area without suitable habitat (fig. 7). However, it seems that the corridor with patches of suitable habitat between the two populations is mostly used by animals from the Utrecht subpopulation to get to the Veluwe subpopulation, as only animals from the Utrecht subpopulation were found dead on the A30.
The genetic discontinuity between the Veluwe and Dutch-German border subpopulations, seems to be related to the river IJssel and the lack of suitable habitat, although in the Southern part there is a small part of the Veluwe subpopulation, which has crosses the river IJssel and settled in a forest habitat on the other side of the river (fig. 8). The genetic discontinuity between the Veluwe and Drenthe subpopulations also seems to be related to the river IJssel and the lack of suitable habitat (fig. 8) and the genetic discontinuity between the Drenthe and Dutch-German border subpopulations seems to be related to the highway A28, although some individuals from both subpopulations have been found on the other side of the highway (fig. 9). Furthermore, the genetic discontinuity between the Veluwe and the Utrecht populations was compared to the age classes of the used samples, showing that there were no adult animals found near the border between the two populations (fig. 10).

Figure 7: Detailed map of the border between the Veluwe and Utrecht subpopulations. Possible barriers in the form of waterways, main roads and railways are illustrated, as is the, for pine martens, suitable forest habitat. A clear discontinuity between the two subpopulations was found, which is related to the highways A1 and A30 and the lack of suitable habitat south of the A30, creating a northwest-southeast oriented boundary.

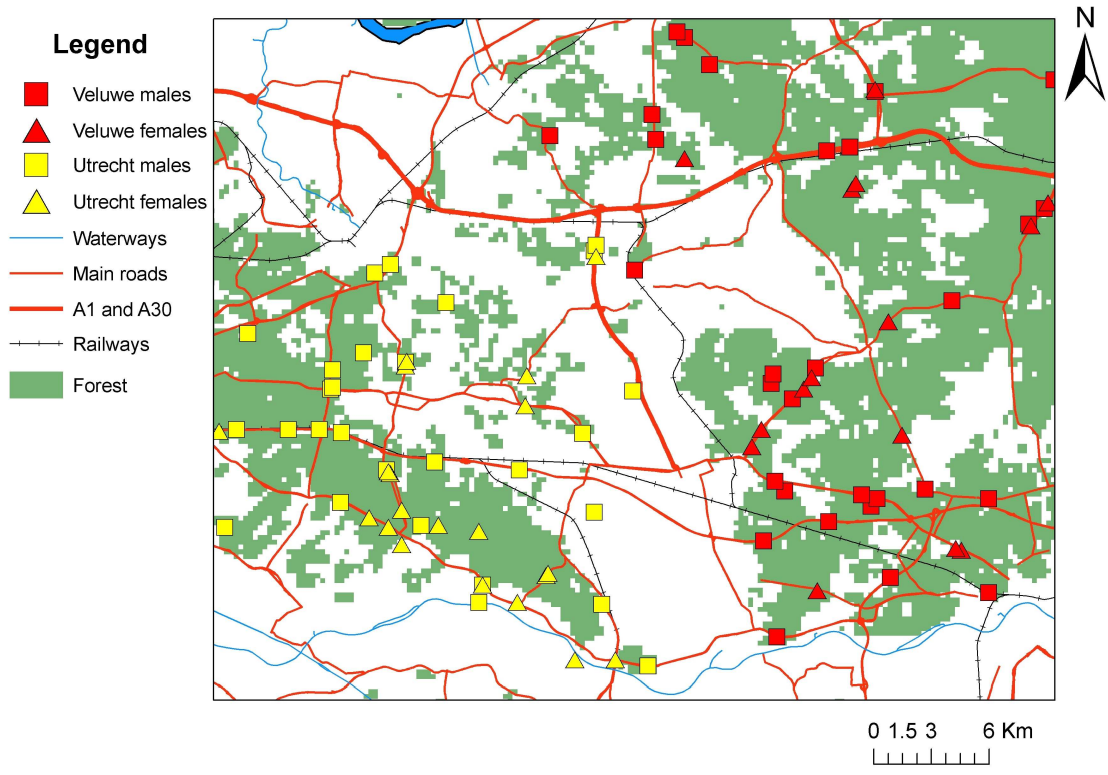


Figure 8: Detailed map of the border between the Veluwe, Drenthe and Dutch-German border subpopulations. Possible barriers in the form of waterways, main roads and railways are illustrated, as is the, for pine martens, suitable forest habitat. A clear discontinuity between the Veluwe and Dutch-German border subpopulations was found which seems to be related to the river IJssel. Next to that, the discontinuity between the Veluwe and Drenthe subpopulations seems to be related to the same river and the highway A28, in combination with the lack of suitable forest habitat in a broad area between the two subpopulations.

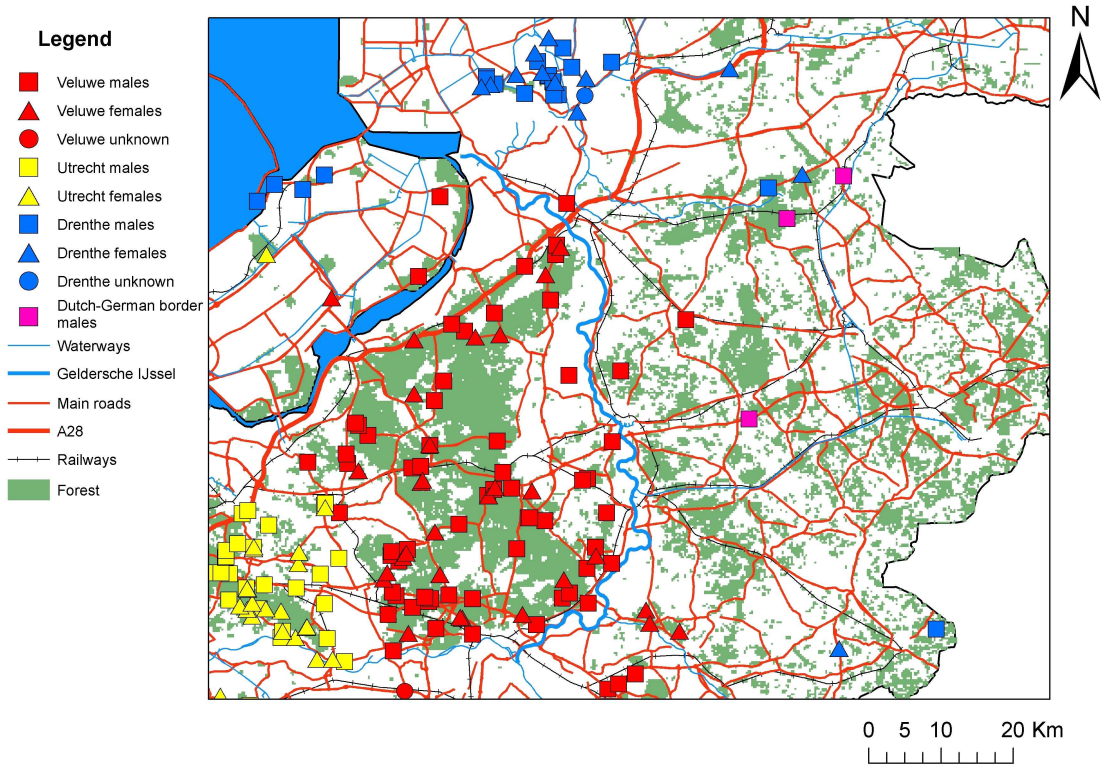



Figure 9: Detailed map of the border between the Drenthe and Dutch-German border populations. Possible barriers in the form of waterways, main roads and railways are illustrated, as is the, for pine martens, suitable forest habitat. A clear discontinuity between the two subpopulations was found, which seems to be related to the highway A28, although some animals of both subpopulations have been found on the other side of the highway.

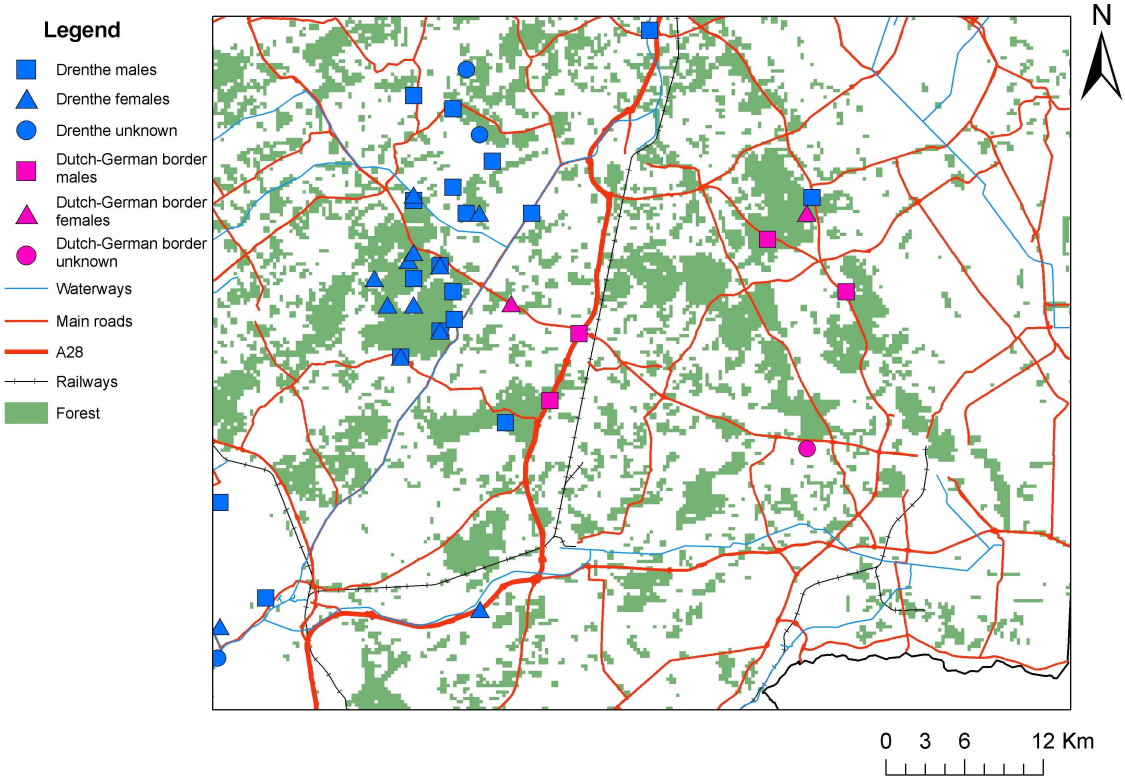
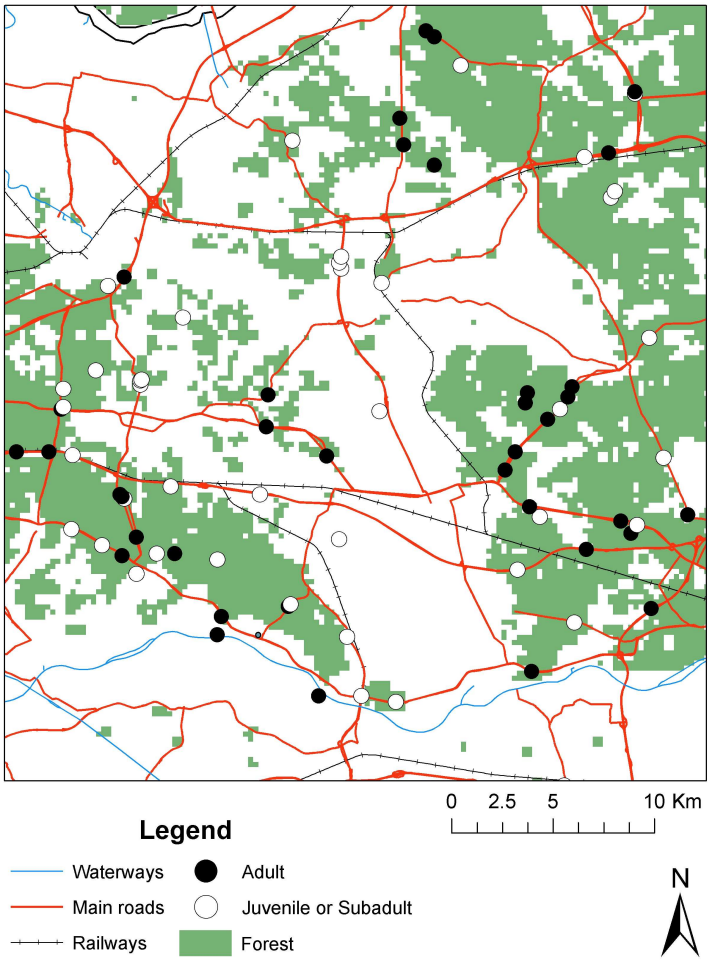


Figure 10: Detailed map of the border between the Veluwe and Utrecht subpopulations. Possible barriers in the form of waterways, main roads and railways are illustrated, as is the, for pine martens, suitable forest habitat. See figure 7 for the division of the two subpopulations. Due to the lack of adult animals found dead near the boundary, the main factor causing this boundary is thought to be the lack of suitable habitat, were juvenile or sub-adult dispersers suffer from the added barrier effect of the road.




*Microsatellite variation*

All investigated loci were polymorphic and the different subpopulations found by GENELAND showed an average allele number per locus of $A_o$=4.4, ranging from $A_o$=4.0 for the Dutch-German border subpopulation to $A_o$=4.9 for the Veluwe subpopulation (table 4). The average effective allele number per locus was $A_e$=3.2, with the lowest value for the Drenthe and Dutch-German border subpopulations ($A_e$=3.0) and the highest value for the Veluwe subpopulation ($A_e$=3.4). The samples showed an average observed heterozygosity of $H_o$=0.63, with the values ranging from $H_o$=0.58 (Dutch-German border subpopulation) to $H_o$=0.65 (Utrecht subpopulation) and an average expected heterozygosity of $H_e$=0.64 with the Drenthe and Dutch-German border subpopulations having the lowest value ($H_e$=0.62) and the Utrecht subpopulation having the highest value ($H_e$=0.67).

Table 4: Genetic diversity of the four subpopulations found by GENELAND and of the overall Dutch population based on eight microsatellite loci. All values given, except for n, are averages over the whole (sub)population.

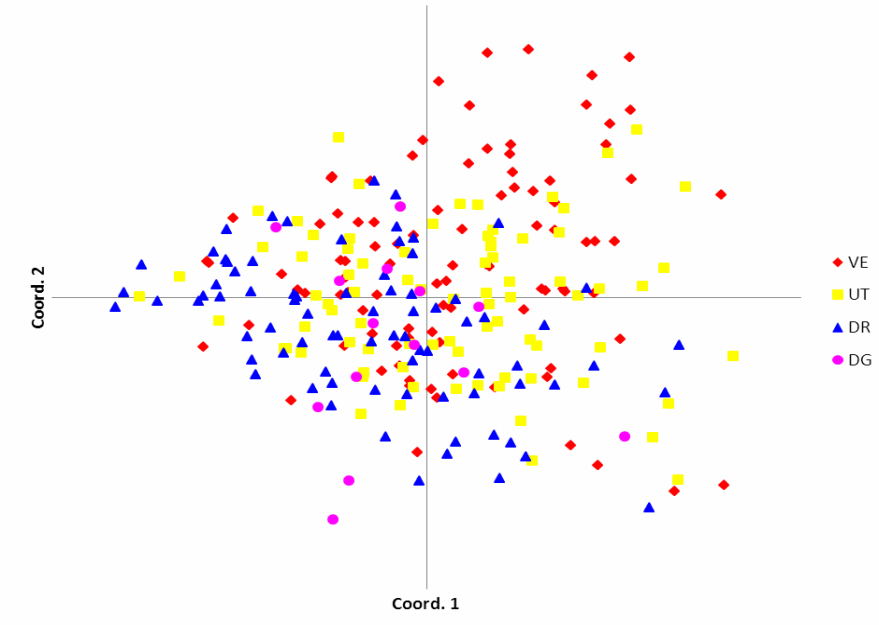
| Population | Abbrev. | n | $A_o$ | $A_e$ | $H_o$ | $H_e$ |
|---|---|---|---|---|---|---|
| Veluwe | VE | 96 | 4.9 | 3.4 | 0.64 | 0.65 |
| Utrecht | UT | 81 | 4.8 | 3.3 | 0.65 | 0.67 |
| Drenthe | DR | 79 | 4.3 | 3.0 | 0.63 | 0.62 |
| Dutch-German border | DG | 14 | 4.0 | 3.0 | 0.58 | 0.62 |
| **Total** | **NL** | **270** | **4.4** | **3.2** | **0.63** | **0.64** |

Table 5: Pair-wise $F_{st}$ values between the four subpopulations found by GENELAND. The low values indicate only a slight differentiation between the subpopulations.

| | Veluwe | Utrecht | Drenthe | Dutch-German border |
|---|---|---|---|---|
| Veluwe | 0.000 | | | |
| Utrecht | 0.010 | 0.000 | | |
| Drenthe | 0.019 | 0.014 | 0.000 | |
| Dutch-German border | 0.026 | 0.024 | 0.009 | 0.000 |

The PCA showed no distinct clustering of subpopulations (fig. 11) which is supported by the pair-wise $F_{st}$ values for the different subpopulations, which are very low (between 0.009 and 0.026; table 5). Although the $F_{st}$ values are very low, it is apparent that the Drenthe and Dutch-German border subpopulations are the most closely related (pair-wise $F_{st}$ value = 0.009). After that, the Veluwe and Utrecht subpopulations are second most closely related (pair-wise $F_{st}$ value = 0.010). And the Drenthe and Dutch-German border subpopulations are less related to the Veluwe and Utrecht subpopulations (pair-wise $F_{st}$ values = 0.014-0.026).

Figure 11: Principal Component Analysis (PCA) of the subpopulations found with GENELAND. It shows that the subpopulations are genetically very identical and no strong differentiation has taken place. The variation explained by the first two axes is 44%.



## Discussion

*Genetic variation*
In this study, it was found that, the Dutch pine marten population was divided into three to four subpopulations. These have a relatively high genetic variation (average $H_e$= 0.64) compared to other Western-European pine marten populations ($H_e$= 0.34-0.66; Kyle *et al.* 2003), although it is slightly lower than the genetic variation found in Danish pine marten populations ($H_e$= 0.67-0.72; Pertoldi *et al.* 2008).
Together with the low pair-wise $F_{st}$ values found between the subpopulations, this shows that there is still a rather large genetic variation in the Dutch population. The relatively high $H_e$ and low pair-wise $F_{st}$ values could, however, also be caused by incidental dispersers between further isolated subpopulations as was suggested for American pine martens (Broquet et al. 2006). Therefore, these parameters alone are not enough to properly investigate the genetic status of a population.

*Population structure*
The STRUCTURE analysis showed three genetic clusters. Only one of these clusters was mostly restricted to one geographical area, namely the cluster with animals from Drenthe and Kop van Overijssel. The other two clusters showed far more overlap between the geographical areas, especially between the Utrecht and Veluwe areas, which are considered to support the largest subpopulations in the Netherlands (Müskens et al. 2000). Taking these data into account, it appears that there are two subpopulation in the Netherlands, namely a northern subpopulation and a southern subpopulation, which consists of two genetic clusters, which are not geographically divided.
The population structure was, however, further divided into four clusters by the GENELAND analysis. The combination of distinct genetic discontinuities found by GENELAND and low pair-wise $F_{st}$ values shows evidence for a recent isolation of the different subpopulations. Which illustrates that pair-wise $F_{st}$ values cannot be used to detect recent barriers to gene flow (Landguth et al. 2010). Furthermore, it shows that geographical data can be used to detect even more subtle substructure in a population, resulting in earlier detection of barriers to gene flow. Such a difference between GENELAND and STRUCTURE outputs was also found in a young row deer (*Capreolus capreolus*) population with low genetic differentiation, where GENELAND showed population structure in the population and, STRUCTURE found none (Coulon et al. 2006). This indicates that GENELAND detects genetic structure even when there is so little differentiation that other methods cannot detect it, making GENELAND a reliable method of finding recent genetic structure.
That GENELAND perhaps shows genetic structure where there is none was shown by the animals found near the Dutch-German border in the Achterhoek. These two samples were assigned to the Drenthe subpopulation by GENELAND, although they were found far away from the main part of this subpopulation. This could indicate that the differentiation made by GENELAND between the Drenthe and Dutch-German border subpopulations is not consistent and that these subpopulations should be clustered together resulting in three subpopulations, which is the same amount as found by STRUCTURE. This, together with the findings of different 'ghost populations', shows that although GENELAND can detect very small differences in genetic structure, the output should be considered with great care.

*Landscape genetics*
The subpopulations found by GENELAND all had genetic discontinuities which were related to landscape features. Most of these barriers to gene flow were caused by a combination of areas without suitable habitat, highways and waterways, which are often found as barriers to gene flow (Coulon *et al.* 2006; Short Bull *et al.* 2011). What was apparent is that for the Veluwe subpopulation, it was found that the river IJssel was related to a barrier to gene flow, while the large shallow lakes between Gelderland and Flevoland were not.
That landscape features caused discontinuities in gene flow suggests that although pine martens can disperse over vast distances, this is not enough for maintaining high levels of gene flow between the subpopulations. A similar situation was detected in North America, where a highway formed a barrier to gene flow between two populations of bobcats (*Lynx rufus*) and coyotes (*Canis latrans*) although telemetry data showed that individual animals crossed the road on multiple occasions (Riley et al. 2006). Riley et al. (2006) concluded that the restricted gene flow was related to the highway forming an artificial territorial boundary, which formed both a social and behavioural barrier for possible migrants from the other side of the road.
The same could be true for pine marten territories next to roads and rivers or at the edges of large forested areas, where dispersing animals encounter occupied territories, causing them to move on, making the chance of getting killed by traffic even larger and lowering the gene flow between adjacent subpopulations. This could explain the genetic discontinuities found between the different subpopulations, which is supported by the results found for the Veluwe and Utrecht populations, were mostly juvenile and sub-adult animals were found near the border, indicating that animals do disperse from one subpopulation towards the other, but that they do not contribute to the gene pool on the other side. Furthermore, it explains long distance dispersers, which disperse over fast distances without suitable habitat to end up in new areas with suitable habitat, such as the pine martens that are now found in the dunes and Noord-Holland.
As road casualties were used to study the landscape genetics of the pine martens in the Netherlands, this could cause problems as the coordinates where the animals were found do not have to correspond to the coordinates of the area where the animals lived. However, apart from the long distance dispersers found in the dunes, Noord-Holland and Flevoland, most animals are believed to have died near the area where they were born. This is supported by the fact that in most areas both males and females were found, as females disperse over smaller distances than males (Broekhuizen& Müskens 2000). Furthermore, for the main subpopulations found, most animals were found near the centre of the subpopulation, in suitable habitat, showing that the animals most probably died near their own or their parents' territory. The analysis was made even more robust by giving the coordinates an uncertainty of 1km in the GENELAND model.

*Origin of animals in the dunes and Noord-Holland*
As for the origin of the pine martens found in the dunes and Noord-Holland, it was found that they originated from one of the three largest populations (Veluwe, Utrecht, and Drenthe). This shows that pine martens can disperse over long distances, overcoming barriers such as highways, large canals and large areas without suitable forest habitat. One of the traffic victims used in this study was actually found on the Houtribdijk, a 27km long dike, with water on both

sides between Lelystad and Enkhuizen, showing that pine martens can cross any kind of terrain when dispersing.
The only exception was one individual found in Amsterdam, which was assigned to the Dutch-German border subpopulation. This animal could, of course, have gotten to Amsterdam by natural means, as it was shown that pine martens can disperse over large distances. It is, however, also possible that this animal got to Amsterdam either by car or by boat from the eastern part of the Netherlands or from Germany.

*Recommendations for the future*
The limited gene flow found between the subpopulations could cause problems for the Dutch pine marten population in the future as the estimated population size of 400 individuals is not very large (Tuitert et al. 2009). Especially, as in American pine martens, a simulation study showed that a population with 75-125 reproducing females, was needed to have a viable population in the long term (Schneider& Yodzis 1994). This shows that in order to have a sustainable population, there should be at least a certain amount of gene flow between the different subpopulations. To reach this, vegetated wildlife crossings could be used to increase the gene flow between populations on both sides of a barrier, as was suggested by Riley et al. (2006). That this can work in the Netherlands, was shown for the Dutch badger (*Meles meles*) population, for which little genetic structure was found, which was suggested to be related to the high amount of underpasses designed for badgers (Van de Zande et al. 2007). Therefore, it is suggested that creating wildlife crossings on the A30 and A28 could increase the gene flow between the different pine marten subpopulations, increasing the viability in the long term of the Dutch pine marten population. These crossings have to be placed were suitable habitat crosses the highway, as these were found to be the areas with the largest amount of road kills in a population of the sympatric stone marten (*Martes foina*; Grilo *et al.* 2009). Furthermore, a forested corridor between the Drenthe and Veluwe subpopulations could be created. In this way all subpopulations will be connected with each other and Germany, creating a meta-population. It would be advisable to monitor these mitigating measures both with telemetry and with genetics, to be able to study the effect of the mitigating measures on the habitat use and gene flow of the pine martens (Corlatti *et al.* 2009). For this purpose, it would be advisable to use non-invasive methods such as faecal or hair samples (Clevenger& Sawaya 2010; Mullins *et al.* 2010) as these can show the genetic variation of the current pine martens and possible changes after implementation of the measures.

*Conclusion*
This study showed that the Dutch pine marten population has a rather large genetic variation, although landscape features are causing recent barriers to gene flow. These barriers are mostly related to highways, rivers and areas without suitable forest habitat. Therefore, mitigating measures such as wildlife crossings could be used to counteract the barriers caused by roads, hopefully creating enough gene flow for a viable population in the long term.

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