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# Conservation Genetics of Scandinavian Wolverines

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**Abstract**
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In this thesis, genetic methods for individual identification and sex determination of wolverines from non-invasive samples were developed and applied in genetic monitoring of Scandinavian wolverine populations. Paternity and mating system of wolverines were studied by combining genetic analysis with telemetry data. Moreover, the possibility to obtain DNA from claws left on tanned carnivore hides was investigated.

Non-invasive genetic sampling was effective in revealing important population parameters. For the subpopulation in southern Norway, a population size of approximately 90 individuals, an equal sex ratio and similar levels of genetic diversity as in the main Scandinavian population were revealed. Genetic erosion in this small population has likely been counteracted by immigration of individuals from the main population since its re-establishment around 1970.

During the 1990s, two areas in east-central Sweden were colonised by wolverines. In a survey comprising 400 non-invasive samples collected during five winters, a total of 22 wolverines were detected. Genetic data suggest that inbreeding has occurred in both areas and that the two populations were founded by as few as 2-4 individuals. These findings suggest that gene flow from the main population is crucial for their survival even in a short time perspective. The detection of occasional stray individuals from the main population shows that this is indeed feasible.

Paternity analysis of 145 wolverine offspring in northern Sweden and southern Norway confirmed a polygamous mating system in wolverines. Breeding pair formation was generally consistent with the territories held by males and females, i.e. breeding pairs had overlapping territories. In the majority of litters, siblings were assigned the same father, thus indicating that multiple paternity is rare.

Tanning is a common form of preservation of mammalian specimens that normally precludes genetic analysis. Nevertheless, I demonstrate the possibility to successfully extract and amplify DNA from claws left on tanned carnivore hides.

*Keywords:* carnivores, conservation, genetic monitoring, Gulo gulo, molecular sexing, non-invasive genetic techniques, parentage analysis, tanning

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# List of papers

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V Hedmark E, Persson J, Segerström P, Landa A, Ellegren H. Paternity and mating system in wolverines. Manuscript.

VI Hedmark E, Ellegren H. DNA-based monitoring of two newly founded Scandinavian wolverine populations. Manuscript.

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*Front cover:* Hans Andersson

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# Abbreviations

| | |
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| CI | Confidence interval |
| IUCN | International Unit for Conservation of Nature and Natural resources |
| PCR | Polymerase chain reaction |

# Introduction

Conservation genetics is a research field in which, among other things, molecular techniques are used to increase the knowledge of species and populations threatened with extinction. It comprises research on genetic processes that are typical of small populations, management, examination of taxonomic status, and application of genetic analysis in forensics and monitoring (Frankham 2002). Biodiversity is currently being lost at a rapid pace as a consequence of human activities (Kerr & Currie 1995, Novacek & Cleland 2001, Ceballos & Ehrlich 2002) and the high rate of extinction of species has made conservation genetics an essential issue of the 21st century (Hedrick 2001). Concurrent with technical advances in molecular analysis, the role of genetics as a means for more precise decisions in conservation is continuously increasing (DaSalle & Amato 2004).

According to the IUCN red list of threatened species (http://www.iucnredlist.org), about 25% of extant mammalian species are threatened with extinction. The underlying causes for decreasing populations are loss of habitat, hunting and spread of invasive species (Cardillo et al. 2004). Large-bodied species, and large carnivores in particular, tend to show several risk factors that are connected with an increased likelihood to go extinct, e.g. low population densities, slow reproductive rate, complex social systems and large home ranges (Purvis et al. 2001). In addition, large carnivores are often less tolerated by humans, e.g. because they prey on livestock; accordingly, persecution of these species is widespread (Woodroffe 2000). As conservation of species and populations is dependent on several factors that go beyond science, like social, cultural and political issues (DaSalle & Amato 2004), management often proves to be a complicated task. The complexity is evident in Scandinavian carnivores, where the persistence of even tiny populations of a limited number of species is subject to strong public conflicts.

Four large carnivore species inhabits the Scandinavian peninsula: the Eurasian lynx *Lynx lynx* (hereafter referred to as lynx); the brown bear *Ursus arctos*; the wolverine *Gulo gulo* and the grey wolf *Canis lupus*. During the last two centuries, populations of these species have shared a similar history of dramatic population declines, leading to near or complete extinction, followed by recovery and more recently, population expansion. Research described in this thesis focuses on conservation genetics of Scandinavian wolverines. However, I will start by giving an account of the recent history of all

four species, starting with an overview of their distribution in Europe, and their biology.

## Distribution and biology of Scandinavian carnivores

### Lynx

The lynx represents the largest wild felid species in Europe. It is generally thought of as a forest living species and, until recently, its distribution has been more southerly than that of the wolverine, wolf and brown bear. Today, however, the southern range has contracted and most lynx populations in western and central Europe have gone extinct (Kratochvil 1968). On the other hand, the northern range of the species has expanded during the last decades and now also includes the northernmost parts of Fennoscandia (Figure 1). Currently, the lynx is considered to be the least threatened among the four large carnivore species of northern Europe.

Lynxes are skilful hunters that primarily prey on ungulates such as roe deer and reindeer, although smaller preys also constitute an important part of the diet (Pulliainen et al. 1995, Pedersen et al. 1999, Linnell et al. 2001). The species is solitary and highly mobile, with large dispersal capability. Males generally exploit larger areas than females, and home ranges of up to 1500 $km^2$ have been reported (Linnell et al. 2001).

Mating occurs in March and one to four juveniles are born after ten weeks of gestation. Juveniles follow the mother for 9-12 months and females may reproduce each year.

### Brown bear

Historically, the brown bear had a wide Holarctic distribution, but during the last centuries its range has been vastly reduced due to habitat loss and hunting. In Europe, the highest densities are currently found in north-western Russia, although bears also are present in Finland and on the Scandinavian peninsula and in scattered populations to the south and east, e.g. in Spain, Slovakia, Poland, Ukraine and Romania (Hallanaro & Pylvänäinen 2002). The distribution of bears in Fennoscandia is shown in Figure 1.

Brown bears are solitary with a promiscuous mating system. Home ranges overlap both within and between sexes and males exploit larger areas than females (Dahle & Swenson 2003a). Dispersal is clearly male-biased as females are highly philopatric (McLellan & Hovey 2001, Proctor et al. 2004).

Although brown bears are carnivores capable of killing large prey, a major part of the diet consists of plants, berries, insects, and carcasses. Mating occurs in late spring and one to four juveniles are born inside the den during midwinter. The reproduction of brown bears is slow. Sexual maturity is not

reached before the age of four, and the interval between litters is often long because offspring reside with the mother for 1.5-3.5 years and females do not mate again until after separation (Dahle & Swenson 2003b).

## Wolverine

The wolverine is a terrestrial mustelid of the northern hemisphere. Historically, it occurred in most areas of tundra and taiga in North America and Eurasia (Banki 1994). Today, however, the distribution has contracted northwards and to higher altitudes as a result of urbanisation, deforestation and human persecution (Wilson 1982, MacDonald 2001, Hallanaro & Pylvänäinen 2002). In Europe, the wolverine occurs in north-western Russia, eastern and northern parts of Finland and in alpine areas of the Scandinavian peninsula. Its distribution in Fennoscandia is shown in Figure 1.

The wolverine is a carnivore and a scavenger. Although it is rather small (8-20 kg), it is capable of killing large prey such as reindeer. In the Nordic countries, reindeer comprise the most important food source for wolverines (Haglund 1966, Pulliainen 1968, Myhre & Myrberget 1975, Landa et al. 1997), both as prey and as carrion. Being a generalist, the wolverine also utilises a range of other food sources, e.g. birds and small mammals (Myrberget & Sørumgård 1979, Magoun 1987).

Wolverines are solitary and both males and females use large areas. Male home ranges may be as large as 1000 $km^2$ and usually overlap the ranges of a few females (Magoun 1985, Banci 1994). Mating occurs between April and August. However, because wolverines exhibit delayed implantation, the gestation period, which is 30-50 days, does not start until November or later. Juveniles are generally born in February or March and the litter size is one to four. The potential for fast population growth is limited as wolverine females have a late onset of reproduction and appear to have low productivity throughout life (Persson et al. 2006).

## Grey wolf

Historically, the grey wolf had a wide distribution that included most terrestrial habitats of the northern hemisphere. However, during the last few centuries wolves have disappeared from large parts of their former range (Young and Goldman 1944, Randi et al. 2003). Currently, the European distribution is patchy with small populations in the south and north-west, and somewhat larger populations further to the east, e.g. in north-western Russia and in Romania (Hallanaro & Pylvänäinen 2002). The distribution of wolves in Fennoscandia is shown in Figure 1.

Wolves are social and live in packs with a strict hierarchy. A typical pack consists of a family with a single breeding pair (the alpha pair) and various numbers of decedents (Mech 1999). Wolves use large areas and the dispersal

capacity is huge. There are examples of dispersal distances of up to 900 km (Fritts 1983) and records of wolves travelling 4000 km in 180 days (Merril & Mech 2000). Wolves are effective predators that primarily prey on larger ungulates such as moose and deer. The hunt is often a co-operation among members of the pack. The mating season occurs in February and March and gestation comprises nine weeks. Litters usually contain up to six offspring, but can even be larger. That wolves have the potential for rapid population growth has been documented both in Scandinavia (Wabakken et al. 2001) and in North America (Pletscher et al. 1997).

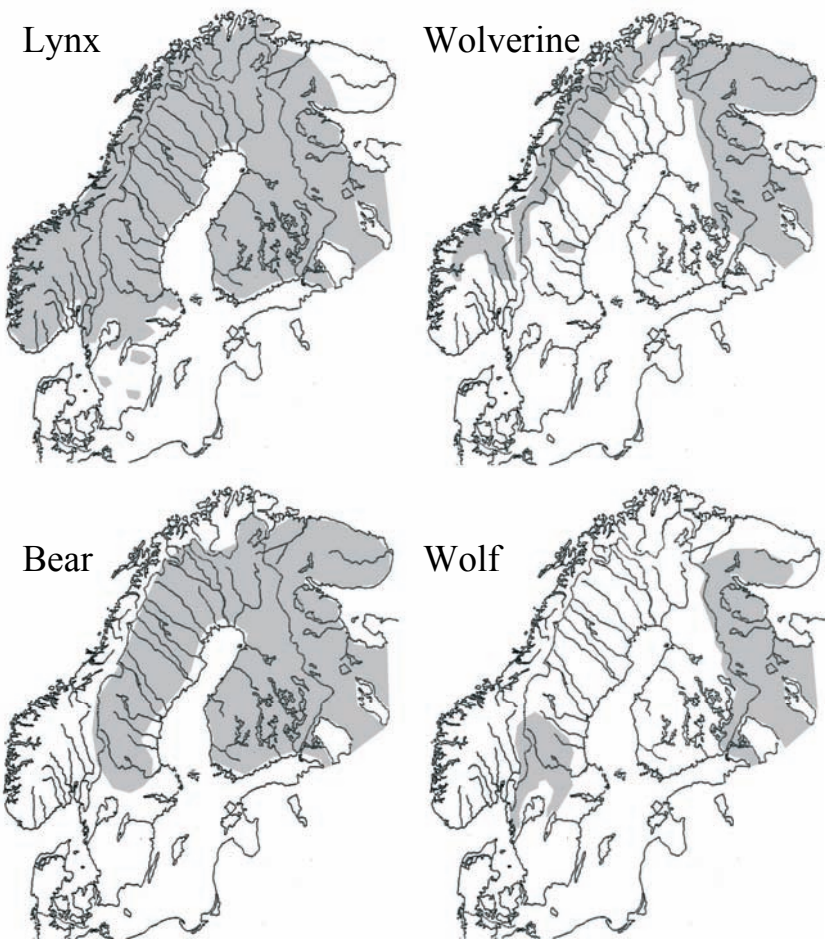


Figure 1. Approximate distribution of lynx, brown bear, wolverine and grey wolf in Fennoscandia today. Drawn according to Hallanaro & Pylvänäinen (2002) and information obtained from the Swedish Environmental Protection Agency (http://www.naturvardsverket.se/dokument /natur/rovdjur/rovdok/varfinns.htm).

# Population histories in Scandinavia

## Population decline

Previous to the 19th century, grey wolves, lynxes, brown bears and wolverines were numerous and wildly distributed on the Scandinavian peninsula (Lönnberg 1929, Mech 1970, Bjärvall 1988). However, the presence of large carnivores is often in conflict with human activities (Breitenmoser 1998). In Scandinavia, large carnivores have long been regarded as pests mainly due to their predation on livestock, primarily semi-domestic reindeer and domestic sheep (Landa & Tömmerås 1996, Sagør et al. 1997, Landa et al. 1999, Pedersen et al. 1999), but also because they compete with hunters for game species such as elk and roe deer (Boitani 2000, Linnell et al. 2001). Consequently, all four species have been subjected to human persecution and state bounties were previously provided for killed carnivores in both Sweden and Norway. During the 19th century, when hunting became very effective by the introduction of firearms, all species started to decline rapidly. In the 20th century, the lynx, brown bear and wolverine were all hunted to the brink of extinction (Lönnberg 1930, Swenson et al. 1995, Landa et al. 2000). Around 1930, only 30-100 lynxes remained (Lönnberg 1930) and the brown bear population comprised 130 individuals distributed in four different regions (Swenson et al. 1995). The wolverine population was at its lowest number in the 1960s when only about 100 individuals remained in the most remote mountainous areas along the border of Sweden and Norway (Landa et al. 2000). For the wolf the situation became even more severe and in the 1960s only a few individuals remained in the northernmost part of Sweden (Bjärvall 1988). No reproduction was recorded after 1964 and by the early 1970s the wolf was considered to be functionally extinct from the Scandinavian peninsula (Wabakken et al. 2001).

## Legal protection and population increase

**Lynx**

In Sweden, state bounties for killed lynx were removed in 1912 and full protection received in 1928. Following this, the Scandinavian population increased, although hunting was allowed again in 1943. Around 1980, the population comprised 500-1200 individuals but a few years later population surveys indicated a rapid decline (Swedish Environmental Protection Agency 2003). Consequently, the lynx was protected again in 1991 and concomitant with an increased abundance of ungulate prey (Liberg 1997), this resulted in a new, rapid population increase. In Norway, population size estimates are lacking for most of the 19th century, but hunting statistics suggests a similar trend to that seen in Sweden. Currently, the Scandinavian lynx population amounts to 1500 individuals (http://www.naturvards

verket.se/dokument/natur/rovdjur/rovdok/varfinns.htm, Brøseth et al. 2005), of which the majority (~1200) are found in Sweden.

**Brown bear**

In Sweden, bounties for killed brown bears were removed in 1893 and full protection received in 1927 (Swenson et al. 1995). In Norway, bounties were kept until 1930 and full protection not obtained until 1972. As a likely consequence, the Swedish population increased whereas the Norwegian part went extinct (Swenson et al. 1995). Today, the Swedish population amounts to 1600-2800 individuals (Kindberg et al. 2004) whereas a limited number, including 6-12 adult females, exist in Norway (Swenson et al. 2003).

**Wolverine**

The wolverine obtained full protection in Sweden in 1969 and in southern and northern Norway in 1973 and 1982, respectively. In contrast to the lynx, the wolverine population responded relatively slowly to protection. Nevertheless, southern Norway was re-colonized in the late 1970s although until recently this subpopulation has remained more or less isolated from the main population in the mountain range of Sweden and Norway (Landa et al. 2000, Flagstad et al. 2004). Currently, the Scandinavian population amounts to approximately 480 individuals in Sweden (http://www.naturvardsverket.se/dokument/natur/rovdjur/rovdok/varfinns.htm) and 330 in Norway (http://nidaros.nina.no/overvaking/jerv/Nystatus 30112005.pdf).

**Grey wolf**

Wolves received protection in Sweden in 1966 and in Norway in 1972, but were at that time already considered extinct from the Scandinavian peninsula (Wabakken et al. 2001).

Since then, however, a new wolf population has established itself in Scandinavia. This occurred as a result of a few wolves from Finland/Russia founding a new population in south-central Sweden during the 1980s (Ellegren et al. 1996, Vilà et al. 2003). The population has increased to comprise 130-150 individuals at the time of writing (Wabakken et al. 2005).

# Genetic status and conservation

## Patterns of genetic variation

In Scandinavia, populations of wolves, lynxes and wolverines show similar patterns of reduced genetic diversity. Each population is monomorphic for a single mitochondrial DNA haplotype (Ellegren et al. 1996, Walker et al. 2001, Hellborg et al. 2002) and also show low to moderate genetic variation at nuclear microsatellite markers (Ellegren 1999, Walker et al. 2001, Hell-

borg et al. 2002, Rueness et al. 2003). The wolf and the lynx population show considerably less variation compared to populations further east (Vilà et al. 2003, Hellborg et al. 2002, Rueness et al. 2003). Although the genetic diversity of Scandinavian wolverines has not been directly compared to that of eastern populations, the variation appears considerably lower than that which has been reported for both Russian and American wolverines (Kyle & Strobeck 2001, Kyle & Strobeck 2002).

The limited genetic diversity seen for these three species is likely to be caused by genetic drift and founder effects during times of population bottlenecks and re-colonization. In a broad sense, low or moderate levels of genetic variation within Scandinavian populations may be related to the fact that Scandinavia is a peninsula on the edge of the Eurasian continent. Peninsular populations may be partially isolated from the source population, and peripheral populations in general tend be more fragmented and to have lower and more fluctuating densities (Brown et al. 1995). These are all characters that result in a reduction of the genetic diversity (Frankham et al. 2002). Decreased genetic diversity towards the periphery of the distribution range has been shown for several carnivore species in other parts of the world, e.g. for brown bears (Paetkau et al. 1998), wolverines (Kyle & Strobeck 2002), Canadian lynxes (Schwartz et al. 2003) and fishers (Wisely et al. 2004).

In contrast to the other three large carnivore species of Scandinavia, the brown bear shows high levels of variation at microsatellite loci (Waits et al. 2000). In fact, the microsatellite variability observed among Scandinavian brown bears is comparable to levels observed in large North American brown bear populations (Waits et al. 2000). There may be several explanations for why brown bears have high levels of genetic variation despite experiencing equally severe population decline as lynx and wolverine. One is that the population was split into four subpopulations during the bottleneck and therefore experienced four separate events of genetic drift (Waits et al. 2000). This is likely to lead to the preservation of different genetic variants in the different subpopulations and potentially preserve more of the total variation. Moreover, two distinct lineages (one eastern and one western, Taberlet & Bouvet 1994) of brown bears exist in Scandinavia as a result of two different colonization routes into Scandinavia after the last ice age (Taberlet et al. 1995). It seems reasonable to assume that these separate lineages have increased the heterogeneity among Scandinavian bears in a way that did not take place in other carnivore species. Furthermore, brown bears have long lifespan (30-40 years) and a long generation time, which means that genetic drift becomes less effective in relation to time. This would parallel the situation among North European populations of the white-tailed eagle, an avian species with unusually long generation time, where population declines following from the heavy release of pollutants during the last century have not led to dramatic effects on genetic diversity (Hailer et al. 2006).

## The importance of genetic diversity

Both in long and short term perspectives genetic diversity is important for population viability and survival (e.g. Lande 1988, Hederick 2004, Frankham 2005). Small and peripheral populations face an increased risk of losing genetic diversity through stochastic processes and inbreeding (e.g. Keller & Waller 2002). Reduced levels of genetic diversity make populations less capable of responding to changes in the environment and thus more likely to go extinct in a longer perspective (Frankham et al. 2002). Inbreeding, on the other hand, typically has an immediate effect on individual fitness as it increases the likelihood of harmful alleles being expressed (Keller & Waller 2002). There are several studies that either indicate or confirm that inbreeding elevates the risk of extinction in wild populations (e.g. Newman & Pilson 1997, Saccheri et al. 1998, Crnokrak & Roff 1999, Spielman et al. 2004).

## Genetic exchange with eastern populations

From a conservation point of view, immigration from other populations into small or peripheral populations is desirable because immigrants are likely to carry new genetic variants and hence counteract the negative effects of genetic drift and inbreeding (Spielman & Frankham 1992). Given the small population sizes and limited genetic variation of large Scandinavian carnivores, these populations are likely to depend on eastern immigrants for long-term viability. At present the issue may be particularly critical for the wolf population as this was founded by very few individuals (Vilà et al. 2003) and shows levels of genetic variation that are comparable to captive wolves (Ellegren et al. 1996, Ellegren 1999) known to be severely effected by inbreeding (Laikre 1999). Moreover, most wolves in the current population have inbreeding coefficients corresponding to full sibling mating and the survival of pups has been shown to be negatively correlated with inbreeding coefficient (Liberg et al. 2005).

However, genetic exchange between Scandinavian populations and eastern populations is not uncomplicated. Among wolves, for example, the rate of immigration has presumably been low for long time periods, as genetic analysis of individuals present during the 19$^{th}$ century suggested a strong genetic differentiation from Finnish wolves (Flagstad et al. 2003). Gene flow through direct contact with eastern populations or through the immigration of eastern individuals are by natural means restricted to the land bridge in the north, unless migrants manage to travel over the Baltic Sea in winters when an ice bridge is formed (Linnell et al. 2005). In addition, northern Fennoscandia is the core area of reindeer herding (Figure 2) and represents the region where carnivores cause most harm and therefore where their presence is most controversial (Linnell et al. 2001).

Figure 2. The shadowed area shows the approximate region of reindeer herding in Fennoscandia.

## Conservation goals and the need of knowledge

Over the last century, public and political attitudes toward large carnivores in Scandinavia have changed from eradication to conservation. However, the conservation of carnivores is still in conflict with the interests of herders and farmers and to some extent also with the interests of hunters. In Sweden, the conflict is dominated by carnivore predation on semi-domestic reindeer, whereas in Norway it also includes predation on domestic sheep that frequently graze unattended in mountain and forest meadows. Consequently, the goal of management is to sustain viable carnivore populations but at the same time to reduce the existing conflicts. Measures to reduce the conflict generally include economic compensation to herders and farmers and control of numbers and geographic ranges of carnivores. Governmental management plans with guidelines for carnivore population size and distribution have been stated for each species in Sweden (Regeringens proposition 2000/01:57) and Norway (Miljøverndepartement 2003), (Table 1). In general the guidelines represent minimum population sizes and the number of individuals may be allowed to increase above these levels so long as they do not negatively affect other interests such as herding and farming. In Sweden, the numbers stated for wolverines and wolves represent stage-goals that, when they are reached, will be reconsidered in relation to the consequences recorded for e.g. animal owners.

To keep populations viable while restricting population sizes is undoubtedly a subtle and difficult matter. For effective management and accurate compensation systems, thorough knowledge about the carnivore populations is required, including continuous monitoring of their numbers and distribution. Moreover, comprehensive knowledge about the genetic and ecological needs of each species will certainly be needed from the conservation point of view.

Table 1. The national goals for sizes of carnivore populations in Sweden and Norway, respectively. In both countries the goals are expressed as the number of reproductions per year. The approximate number of individuals corresponding to these numbers is provided in brackets.

| | Lynx | Brown bear | Wolverine | Grey wolf |
|---|---|---|---|---|
| Population size - Sweden | 300 (1500) | 100 (1000) | 90 (575) | 20 (200) |
| Population size - Norway | 65 (325) | 15 (150) | 39 (250) | 3 (30) |

## Non-invasive genetic techniques

In general, studies and precise monitoring of large carnivores are difficult to conduct because the animals are rare and roam over large and remote areas. However, combining field data with genetic analysis is helpful and, fortunately, the opportunity to gain genetic data from natural populations has recently been enhanced by the development of non-invasive genetic techniques.

During the 1990s several researchers demonstrated the possibility to use e.g. faeces, shed hair, feathers and sloughed skin to extract DNA for individual identification (e.g. Ellegren 1991, Gerloff et al. 1995, Taberlet et al. 1996, Kohn et al. 1997, Palsbøll et al. 1997, Reed et al. 1997). Since then the methodology has been applied to increasing numbers of species and populations (e.g. Kohn et al. 1999, Sloane et al. 2000, Ernest et al. 2000, Constable et al. 2001, Parsons 2001, Eggert et al. 2003, Fernando et al. 2003, Frantz et al. 2003, Valière et al. 2003, Maudet et al. 2004, Prugh et al. 2005) and proved to be very useful in several types of research, as well as in wildlife forensics, management and conservation.

Although non-invasive genetic sampling may allow studies of wild animals without having to catch or even observe them, the approach inherently involves with certain limitations. The amount of DNA obtained from non-invasive samples is usually small and/or of poor quality and may contain PCR inhibitors (Gerloff et al. 1995, Gagneux et al. 1997, Taberlet et al. 1999, Taberlet and Luikart 1999, Morin et al. 2001). As a consequence, it may be difficult to perform accurate genotyping.

The most frequent problem in non-invasive genetic analysis is referred to as allelic dropout and results in the identification of false homozygotes be-

cause only one of the two alleles present at a heterozygous locus is amplified during PCR. When the number of template molecules is low, one allele may happen to amplify more efficiently than the other at an early stage, thus resulting in an extremely biased amplification. Another, less frequent, PCR-generated error associated with non-invasive samples is that of false alleles (Taberlet et al. 1999). During amplification of microsatellites, fragments that are one or a few repeat units shorter than the main allele (i.e. stutter bands) are often produced as a result of slippage mutation processes (Ellegren 2004). When a very dilute template is used, such fragments may be amplified in equal amounts as the actual allele. False alleles are typically less common than allelic dropouts and in general they constitute a relatively small problem in non-invasive analysis (Taberlet et al. 1996, Gagneux et al. 1997).

As a means to achieve reliable genotypes from non-invasive DNA samples, a multiple-tube approach can be applied (Taberlet et al. 1996, Pompanon et al. 2005). This approach includes multiple amplifications of each extract in several independent replicates for each locus (Gerloff et al. 1995, Gagneux et al. 1997). A number of studies have been performed to assess genotyping error rates and the number of replicates required to obtain correct genotypes (e.g. Taberlet et al. 1996, Gagneux et al. 1997, Flagstad et al. 1999, Bayes et al. 2000). However, the results vary considerably between studies and several factors have been shown to affect the success rate, e.g. time of year when samples were collected (Lucchini et al. 2002, Maudet et al. 2004), storage of samples (Wasser et al. 1997, Frantzen et al. 1998, Murphy et al. 2002) and diet (Murphy et al. 2003). Conclusions on how to obtain reliable results can thus not be directly transferred and applied to studies involving other species and conditions.

## The wolverine – the main study species

The wolverine is an elusive animal that is very difficult to study due to its solitary lifestyle, large home ranges, low population density and distribution in remote areas. Among the species of large carnivores in northern Europe, the wolverine is the least studied and least known in regard to its biology. Nevertheless, field based research performed in Scandinavia during the last 10-15 years has provided essential data on wolverine ecology, including information on home ranges, choice of prey and predation on livestock, dispersal, juvenile mortality and reproductive rates (Landa et al. 1997, Landa et al. 1999, Vangen et al. 2001, Persson et al. 2003, Persson et al. 2006). For example, this research has shown that females in general do not reproduce before the age of three and that the mean proportion of females (≥ 3 years old) that produce offspring in a given year is 53%, implying low reproductive potential (Persson et al. 2006). It has also been revealed that intraspeci-

fic predation is the most important cause of juvenile mortality and that these killings occur in two distinct time periods: in May-June when juveniles are still dependent and in August-September, soon after independence (Persson et al. 2003). Furthermore, population viability analysis has been conducted on the basis of demographic data obtained through telemetry (Saether et al. 2005). This analysis showed that the current rate of human caused mortality of wolverines in Norway is likely to lead to rapid extinction over large parts of the country, provided that no immigration occurs from neighbouring countries.

In 2001, genetic analysis was used for the first time to study Scandinavian wolverines (Walker et al. 2001). This study provided a necessary tool for analysis on the population and individual level by identifying microsatellite markers that were polymorphic among the wolverines of Scandinavia. Since then, genetic applications have become an increasingly important part in the studies and monitoring of wolverine populations, as described in my studies presented below.

# Research aims

In the thesis I develop and improve genetic methods for studies of natural wolverine populations. Moreover, I perform investigations that contribute to the knowledge about the genetics and ecology of the endangered Scandinavian wolverine population.

The main objectives were the following:

1. Develop and improve genetic methods for individual identification of wolverines from non-invasive samples.

2. Develop genetic markers specific for sex determination of wolverines from non-invasive samples.

3. Investigate the possibility to successfully extract and amplify DNA from claws left on tanned carnivore hides.

4. Apply non-invasive genetic methods to Scandinavian wolverine populations to address aspects such as population size, sex ratio, genetic isolation, number of founders, territoriality and breeding.

5. Perform paternity tests to gain insight in the wolverine mating system.

# Present investigations

## Paper I. DNA-based individual and sex identification from wolverine (*Gulo gulo*) faeces and urine

Accurate monitoring of wolverines is difficult because of their elusive nature in remote mountainous areas. A genetic approach where samples that are relatively easily collected (e.g. faeces and urine) can be used for individual and sex identification would thus be helpful. The objectives of this study were to investigate the possibility of using non-invasive samples for individual identification of wolverines and to develop wolverine-specific markers for sex determination.

### Methods

Thirty-two faecal samples and 139 blood or tissue samples were collected in northern Sweden. Individuals represented by faecal samples were also represented by tissue or blood to enable comparison of results obtained from faeces with those obtained from conventional samples. In addition, 22 urine samples were collected in northern Norway. DNA from frozen excrement was extracted using a commercial kit, with a slight modification of the manufacturers protocol for urine samples.

Extracted samples were analysed across 10 microsatellite loci. A multiple-tubes approach, including a minimum of four independent amplifications for loci appearing homozygous, and a minimum of two amplifications for loci appearing heterozygous, was applied for faecal and urine samples. Multi-locus genotypes obtained from excrement samples were then compared to the genotypes obtained from tissue or blood in a blind test.

For sex determination, two Y-chromosome linked markers were developed. First, conserved exon primers (*DBY*3 and *DBY7*; Hellborg & Ellegren 2003) flanking two different introns on the Y-linked *DBY* gene were used to amplify and sequence these introns in one wolverine. The sequences were subsequently used to construct two internal primer pairs (*DBY3Ggu* and *DBY7Ggu*). These new markers were tested for amplification on tissue and faeces samples from male and female wolverines. In addition, to control for wolverine specificity the primers were tested on male samples of a range of other mammalian species including carnivores, potential prey species, humans and one other mustelid (the otter).

## Results and discussion

Twenty-one of the faecal samples (65%) could be amplified for the 10 microsatellite loci. The average allelic dropout rate among amplifications from heterozygotes was 12%. False alleles were observed in less than 1% of all amplifications from faeces. Three samples accounted for more than half of all allelic dropouts, thereby showing that allelic dropout rates vary considerably among samples. The consensus genotype inferred from conducted PCR replicates for each faecal sample proved to be identical to the genotype obtained from tissue or blood of the same individual. Moreover, all faecal genotypes were correct already after consideration of a maximum of three PCR replicates. This suggests that a multiple-tubes approach including three independent amplifications may be sufficient for reliable genotyping of wolverine faecal samples provided our sampling strategy and analysis approach. Discarding samples that are clearly performing worse than average will further reduce the risk of obtaining incorrect genotypes.

Nine of the 22 urine samples (40%) could be genotyped at all microsatellite loci. Although the success rate for urine samples was lower, the observed error rate among amplifiable extracts was comparable to that of faecal samples. Allelic dropouts were recorded in 14% of heterozygous amplifications whereas no false alleles were detected.

A test of the Y-chromosome markers, using tissue samples from 20 individuals (ten of each sex), consistently gave the anticipated result, i.e. a single fragment of expected length for males and no fragment for females. However, the possibility to test the markers on faecal samples from individuals of known sex was limited as only one of the samples that amplified microsatellites successfully represented a male. Nevertheless, this male sample amplified both markers whereas none of the female samples did. *DBY3Ggu* appeared to be wolverine-specific as no amplification product was obtained for males of other species. For *DBY7Ggu*, a product was obtained for the otter, suggesting that this marker may be mustelid-specific.

The study demonstrates that wolverine excrements collected in the field can be used for individual and sex identification through genetic analysis. Moreover, the comparison of faecal genotypes to those obtained from tissue of the same individuals suggests that reliable genotypes in general are obtained using a limited number of PCR replicates.

# Paper II. Colonization history and noninvasive monitoring of a reestablished wolverine population

The wolverine was considered functionally extinct in southern Norway in the 1960s. However, wolverines reappeared in the area a few years later and a new population has been established since then. In this study, crucial popu-

lation parameters such as population size, sex ratio and immigration rate were assessed through application of the non-invasive genetic technique. To trace the colonization history of the population, the current genetic status was compared to that of wolverines present in the area shortly after reappearance and to that of northern Scandinavian wolverines.

## Methods

Two hundred and eleven presumed wolverine faecal samples were collected during 2000 and 2001 in southern Norway. In addition, tissue samples from wolverines present in the area during 1980-1987 (n = 11), 1988-1995 (n = 10) and after 1995 (n = 20) were available. Forty-seven tissue samples representing wolverines in northern Scandinavia during 1998-2000 was also included. Individuals were analysed using 18 microsatellite markers. Faecal microsatellite analysis was carried out according to the procedures recommended in Paper I. Molecular sexing was performed using the Y-chromosome marker *DBY7Ggu*, also as described in Paper I.

Levels of genetic variation, as assessed by heterozygosity and number of alleles, were assessed for each population and sampling period. To detect possible immigrants, a Bayesian clustering approach as implemented in the program STRUCTURE (Pritchard et al. 2000) was used. The observed immigration rate was further evaluated in terms of whether it would be sufficient to maintain the current level of genetic diversity. Population size was assessed both through a minimum population size estimate based on the number of unique multi-locus genotypes observed and through application of a capture-recapture approach.

Potential parent-offspring relations were inferred from likelihood based parentage analysis. Assuming that reproducing females show strong home fidelity throughout life, inferred mother-offspring relationships were used to assess dispersal distances.

## Results

Among the 211 faecal samples, 147 were successfully genotyped and both the proportion of samples amplifying (70%) and the observed proportion of allelic dropouts (10%) were similar to those obtained in Paper I. The 147 samples were found to represent 68 different individuals. Of these, eight were detected only in 2000 and 55 only in 2001. A minimum of 60 individuals (31 females and 29 males) were thus alive in southern Norway in 2001. About half of these individuals were however sampled only once, which indicates that the population comprised more than these 60 individuals. Accordingly, the capture-recapture estimate based on the observed resampling rates, suggested a population size of 89 wolverines (95% CI = 74-104).

The southern Norway population was significantly differentiated from the northern Scandinavian population during all investigated time periods with the differentiation being most significant during 1980-1987. Two private alleles were present in southern Norway during all time periods. No significant difference in the amount of genetic diversity was detected between southern Norway and northern Scandinavia. Bayesian clustering analysis suggested that animals with a recent northern ancestry, i.e. immigrants or descendants to immigrants, were present in southern Norway during all time periods.

Forty-six potential parent-offspring relationships were inferred from parentage analysis. The inferred parent pairs were generally sampled at moderate distances from each other (mean = 35 km, median = 29 km). In contrast, a large variance in distance was observed between mother and offspring (indirect estimates of dispersal distance: mean = 125 km, median = 83 km). The variance was significantly higher for male (mean = 164 km, median = 64 km) than for female offspring (mean = 78 km, median = 75 km).

## Discussion

The fact that two private alleles were found in the southern Norway population may argue against a sole origin from northern Scandinavia after the eradication during the 1960s. It seems unlikely that these two alleles have arisen through mutation among the few individuals present from 1970 to 1985. Instead, we favour the idea that a few individuals survived during the 1960s and contributed to reproduction when the population started to increase again. A sole northern Scandinavian origin cannot however, be totally rejected as the two alleles potentially were present at low frequencies in the northern population around 1970.

It appears clear that northern migrants have been present in the population from 1980 and onwards: four individuals sampled in southern Norway between 1980 and 1995 were inferred as immigrants, and in the current population an immigration rate of 0.062 was suggested by the Bayesian approach. Furthermore, parentage analysis in the current population suggested that at least three females with a recent northern ancestry contributed to reproduction. Thus, regular immigration from the major Scandinavian population and successful breeding of those in the southern Norway population appear likely.

According to simulations of genetic drift in a small wolverine population with the observed migration rate, about 90% of the current level of genetic diversity will remain after 100 generations. On the other hand, if no immigration is assumed, genetic diversity will erode rapidly with only 10% being left after 100 generations. Therefore, maintenance of gene flow from the main Scandinavian population is crucial in future management of the southern Norway wolverine population.

# Paper III. Microsatellite genotyping of DNA isolated from claws left on tanned carnivore hides

Preparation of hides through tanning is a common form of preservation of mammalian specimens, both in museums and as trophies. The chemicals involved in tanning cause DNA degradation and act to inhibit enzymatic reactions required in DNA analysis. Consequently, attempts to perform DNA analysis from tanned material commonly fail. This is unfortunate as genetic analysis can assist in forensic cases of illegal trade with trophies of endangered species, e.g. carnivores. Moreover, for many mammals the only access to historical specimens may be in the form of tanned hides and the possibility to analyse these genetically would facilitate studies of temporal variation in genetic diversity and even allow analysis of extinct species.

Even though tanning in general results in a skin that is inadequate for DNA analysis, it is possible that blood in the pulp of claws left on tanned hides is a useful source of DNA as it is encapsulated by hard tissue. This study investigates the possibility to extract and amplify nuclear DNA from claws left on tanned hides of wolverines and lynxes.

## Methods

Eight wolverine and eight lynx hides that had been preserved through tanning between 1981 and 1998 were used. A sample was taken from one claw of each hide by first removing the outermost keratinized layer underneath the claw and then drilling with a dentists drill into the pulp. The released material was collected and used for DNA extraction. For all wolverines and four lynxes, reference material in the form of tissue was available. DNA extraction from claw material was carried out according to a procedure described for bone fragments (Yang et al. 1998). DNA concentration was measured on a spectrophotometer and microsatellite amplification was attempted for five species-specific markers in wolverines and four in lynxes. Three independent amplifications were performed for each sample and marker. The genotypes obtained from claws were subsequently compared to those obtained from available tissue samples of the same individuals. The proportion of PCRs yielding a product possible to score on a sequencing instrument and the rate of genotyping error was calculated for claw amplifications.

## Results and discussion

The concentration of DNA in extracts from claws was 5-60 ng/µl, which is far higher than what is usually needed for successful DNA analysis. Accordingly, all samples of both species amplified microsatellites very well. The overall amplification success was high, with 93% and 98% of all amplifica-

tion attempts providing a distinct PCR product for wolverines and lynxes, respectively. The genotyping error rate was low (< 1%); there was only a single sample in which the three replicates did not provide an identical genotype. In this case, the deviating replicate was inferred to be the result of an allelic dropout, i.e. the failure of one allele to amplify in a heterozygous individual. This was confirmed by the analysis of the corresponding tissue sample, in which the individual was recorded as a heterozygote. Otherwise all claw genotypes were identical to those of tissue samples.

As the samples included hides tanned up to 20 years ago, it appeared that long-time storage did not significantly affect the ability to obtain successful results. The body (claw) size of the animals had no effect either, the small claws of juveniles amplifying equally well as larger ones.

The high consistency between replicate amplifications and the agreement between claw and tissue genotypes demonstrate that claws can be a useful source of DNA. It can thus be concluded that while tanning normally precludes DNA analysis from skin, claws left on the same hide offer a means for successful genetic analysis.

## Paper IV. A test of the multiplex pre-amplification approach in microsatellite genotyping of wolverine faecal DNA

The aim of this study was to further improve the non-invasive technique for genetic analysis of wolverine faeces. A two-step PCR approach – referred to as the multiplex pre-amplification approach – was recently proposed by Piggott et al. (2004) to improve amplification success and reduce error rates in genotyping of faecal DNA. This approach was here applied to wolverine samples and the results were evaluated in comparison to the standard PCR method described in Paper I. Moreover, we offer a modification of the new approach to reduce labour and costs. The multiplex pre-amplification approach is based on performing an initial multiplex PCR including primers for all markers to be genotyped, and the subsequent use of these products as templates in separate amplifications of each marker.

### Methods

For comparison of the two PCR approaches we used 48 wolverine faecal DNA extracts that provided PCR products in an initial test of the extraction success. These extracts were genotyped across 18 loci using both standard PCR and the multiplex pre-amplification approach. For the former, three replicates of each marker were amplified in PCR using 2 µl of DNA tem-

plate. For the multiplex pre-amplification approach, the 18 markers were divided into four groups. A multiplex PCR was then performed for each group in 50 µl volumes containing 12 µl of DNA template. The number of PCR cycles for multiplex reactions were restricted to 25. Subsequently, the PCR products from the multiplex amplifications were used as template in a second PCR step. In this step, each marker was amplified separately three times with 2 µl template in each. For 22 extracts the whole multiplex pre-amplification procedure (initial PCR and second step PCR) was replicated.

Amplification success was calculated separately for standard PCR and multiplex pre-amplification as the proportion of PCRs providing a distinct product possible to score on a MegaBACE capillary instrument. Consensus genotypes were constructed according to the following: a homozygous genotype was not accepted unless three or more independent amplifications provided an identical homozygous pattern; a heterozygous genotype was not accepted unless each allele had been observed in two independent amplifications. A false allele was interpreted when a spurious allele occurred in a single replicate but not in any of four other positive amplifications, or when a third allele occurred at heterozygous loci. Amplification replicates through standard PCR are independent from each other and all positive replicates were therefore considered in the construction of consensus genotypes. On the other hand, amplification from the same multiplex PCR is not independent and only one of the three replicates was therefore considered. However, for the 22 extracts where the whole multiplex pre-amplification procedure was repeated, two independent replicates from multiplex pre-amplification could be considered for the construction of consensus genotypes. Only amplifications at loci for which a consensus genotype could be constructed were included in subsequent estimates of genotyping errors (i.e. allelic dropouts and false alleles).

## Results and discussion

The overall amplification success was significantly higher for the multiplex pre-amplification approach (91%) compared to standard PCR (80%; $P < 0.002$). Moreover, the allelic dropout rate was significantly lower for the multiplex pre-amplification approach (2.4%) then for standard PCR (12.5%; $P < 0.0001$). The rate seen with standard PCR is comparable with that found in Paper I and II. However, allelic dropouts obtained in the multiplex pre-amplification approach were to a high extent repeated in all replicates of the second PCR. If consensus genotypes had been inferred only from the three second-step replicates of the multiplex pre-amplification approach, there would have been eight cases where the final genotype of a heterozygous locus had been misinterpreted as a homozygous locus. This should be compared to two such cases for the standard approach. Thus, the proportion of allelic dropouts leading to undetected errors was much higher for the multi-

plex pre-amplification approach (24/29) than for standard PCR (6/123; $P < 0.0001$). The high proportion of instances with repeated allelic dropouts in second-step PCRs of the multiplex pre-amplification approach indicates that these errors were generated already in the first PCR (i.e. the multiplex reaction). This leads to the conclusion that replication of the second step is of limited value in detecting genotyping errors. The number of detected false alleles was equally low for both methods (< 1% of all amplifications) but may not, for the same reason as for allelic dropout, be possible to detect through replication of the second step in the multiplex pre-amplification approach. In one case the same false allele was repeated in all three replicates of this step.

This study shows that the multiplex pre-amplification approach is advantageous over standard PCR for DNA analysis of wolverine faeces. The approach provides higher amplification success and a considerably lower rate of allelic dropout. However, errors generated during the first PCR are likely to remain undetected throughout replications of the second step. Therefore, we suggest performing two separate multiplex pre-amplifications with subsequent analysis of a single second-step PCR from each of them. In this way, two independent multiplex pre-amplification replicates are obtained. This was tested for 22 extracts, and for all loci (n = 383) the correct genotype was obtained at least once, except for two loci where no PCR product was yielded. Together these two replicates provided considerably better results than the three standard PCR replicates carried out for the same extracts (Figure 3).

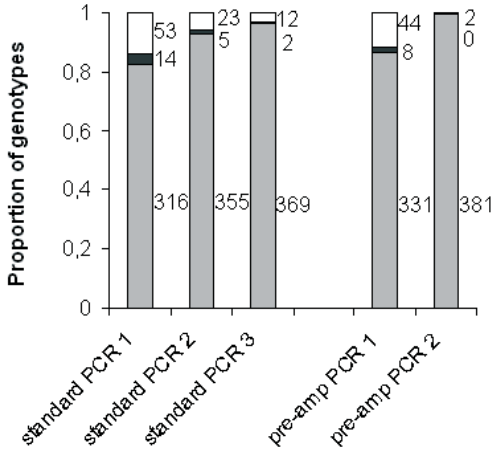


Figure 3. Cumulative proportions of correct (grey), incorrect (black) and absence of result (white) after 1, 2 and 3 independent PCR replicates for standard PCR and multiplex pre-amplification. Numbers to the right of each bar refer to number of genotypes in each category.

## Paper V. Paternity and mating system in wolverines

The knowledge of the wolverine mating system is limited. In this study we used genetic analysis to perform paternity tests for 145 wolverine offspring with known mothers. The results of the tests were used in combination with available telemetry data to address issues of e.g. polygamy, multiple paternity and consistence between breeding pair formation and the observed pattern of territoriality.

### Methods

Samples were obtained from wolverines in northern Sweden and southern Norway. In northern Sweden, tissue biopsies from 111 offspring of radiomarked females were collected during 1993-2004. These offspring were also reared in 64 litters by 37 different females, which were represented by tissue samples. In addition, 29 males of unknown age and relation to other individuals were sampled for tissue. In southern Norway, 34 offspring with known mothers were sampled during 2001-2004. These were reared in 17 litters by 15 different females. All offspring and mothers were represented by tissue samples, except for one mother which was represented by a faecal sample. In addition, faeces or tissue samples from 80 males and 9 individuals of unknown sex were available among samples collected in southern Norway during 2000-2004.

All individuals were analysed at 19-20 microsatellite loci. Paternity tests were performed in CERVUS (Marshall et al. 1998) in which the principles of Mendelian inheritance and population allele frequencies are used to determine which male among a pool of candidates is the most likely father. However, if relatives such as brothers or uncles are present among the candidates – and this is likely to be the case in our populations – there is a risk that these obtain high likelihood scores and are interpreted as fathers. To reduce this risk, we also considered relatedness estimates between pairs of individuals when assigning paternity. For example, we did not assign paternity to a male that was related to the offsprings mother because of the potential risk that he was a brother or uncle.

### Results

In total, paternity was resolved for 74 of the 145 offspring (51%). In northern Sweden, 57 paternities were resolved with 14 different males being assigned as fathers. Eight of these males reproduced with more than one female (six males with two females and two with three). There were six occasions where one male reproduced with two females in the same year. Telemetry data from northern Sweden revealed that male and female home ranges overlapped in 20 of 23 cases where such data were available. In the

remaining three cases home range borders were located 2-5 km from each other.

Females often reproduced with the same male in subsequent breeding years. Only two of 11 females that produced more than one sampled litter for which paternity was resolved, bred with two different males. However, if data also from females with at least one litter with resolved paternity and at least one with unresolved paternity was included – and assuming that the father in the latter case was different from the former – eight out of 13 females bred with different males.

In southern Norway, 17 paternities were resolved for offspring belonging to seven different females. All these females reproduced with different males. The only female in southern Norway for which two litters were sampled reproduced with the same male.

In all litters, a single male genotype could explain the paternal alleles observed among the siblings. The possibility of detecting multiple paternity in our study was, however, limited as there were only eight litters of three offspring and limited numbers of alleles at each locus. Nevertheless, we found one potential case of multiple paternity from the paternity assignment. In this case, one offspring in a litter was assigned a father while none of the male candidates matched its sibling (all candidates had at least three mismatching alleles and obtained very low likelihood scores in the paternity analysis).

## Discussion

This study provides evidence for a polygamous mating system among wolverines. Several occasions where one male reproduced with more than one female during the same season were recorded. A polygamous system is also in agreement with previous suggestions based on field observations (Rausch & Pearson 1972). Moreover, females often reproduced with the same male in subsequent breeding years and partner shift potentially occurred as a consequence of a change of territory-holding male as no female was observed to shift back to a previous male. In general, where both genetic and telemetry data was available, breeding pairs were found to have overlapping territories. Breeding pair formations thus appear to be consistent with the territories held by males and females.

Despite there being a polygamous mating system, the difference between the number of breeding male and female wolverines appeared to be relatively small. No male was found to reproduce with more than two females in a single year or with more than three across all years. The observed male-to-female ratio of reproducing adults was 0.84.

In the vast majority of litters, siblings were unambiguously assigned the same father, thus indicating that multiple paternity rarely takes place among Scandinavian wolverines. Multiple paternity seemed likely only in one of 32 litters, which is a lower proportion than that reported for other mustelids, e.g.

mink, stoat and badger. The low frequency of multiple paternity among wolverines should probably be seen in relation to their solitary lifestyle, low population densities and strong male territoriality.

## Paper VI. DNA-based monitoring of two newly founded Scandinavian wolverine populations

During the 1990s, wolverines started to appear regularly in two new areas in central Sweden, approximately 200 km east of the Swedish/Norwegian border and quite close to the coastland of the Gulf of Bothnia (Figure 4). By current Scandinavian standards these wolverines are unique in that they mainly reside in forested habitat outside the distribution of wild and semi-domestic reindeer. In this study, non-invasive genetic techniques were used to monitor these new populations. We compare them genetically to the main Scandinavian wolverine population and address aspects such as genetic isolation, number of founders, population size, territoriality and breeding.

Figure 4. The two areas recently colonized by wolverines are indicated by squares. The shadowed areas illustrate the main wolverine distribution in Fennoscandia.

## Methods

Faeces and glandular secretions were collected in the two areas by local trackers during the five winter seasons 2000/2001-2004/2005. In the southernmost area (SF), a total of 274 samples were collected, whereas 142 samples were collected in the second area (NF) 70 km further north. Individual identification through microsatellite genotyping and sex determination by the use of two wolverine Y-chromosome markers were carried out according to the procedures described in Paper I. Individuals were genotyped across 20 microsatellite loci.

For genetic comparison to the main wolverine population, 63 individuals sampled in the central parts of the Swedish/Norwegian mountain range were used, representing the closest area with a regular wolverine population. Potential differentiation from the main population was assessed through estimates of $F_{ST}$. Mean relatedness and relatedness between pairs of individuals were calculated for SF, NF and the main population. Furthermore, we assessed the numbers of founding individuals of the new populations using a simulation approach where groups of two to seven synthetic individuals (generated from the allele frequencies of the main population) were drawn at random 1000 times. This generated a distribution of the numbers of alleles that would be expected in a population founded by a defined number of individuals. Finally, attempts were made to resolve parent-offspring relations through parentage tests.

## Results and discussion

In total, 22 individuals were detected in the newly colonized areas. Seventeen (11 males and six females) were found in SF and five (one male and four females) were found in NF. Thirteen individuals were sampled in more than one season, and among those, four were sampled in all five seasons. Overall, the re-sampling rate was high with a mean of 4.4 samples per individual and season. Across all seasons, only two individuals were represented by a single sample. The high re-sampling rate suggests that large proportions of the populations were sampled.

With one exception, all individuals in SF showed relatedness values corresponding to first-order relationship against one to six other individuals in the population. In NF, four of the five individuals showed very high relatedness values, i.e. values exceeding what would be expected in the absence of inbreeding, towards one or two other individuals. These observations suggest that the newly founded populations are reproducing and that the majority of the identified individuals represent local recruits.

One individual in each population appeared to be completely unrelated to the others, and these also carried alleles that were not observed in the respective population. One of these individuals was sampled only once, whereas

the other was sampled twice within a period of two weeks. The high rate of re-sampling therefore suggests that they were only present during a short period of time in the respective area. Moreover, both were sampled in the periphery of the respective region. Potentially these two animals represent migratory individuals originating from the main population. This suggests that the newly colonized areas are not completely isolated.

The average relatedness between pairs of individuals was significantly higher in both SF and NF compared to in the main population. The mean relatedness between individuals in SF and NF was low, suggesting separate colonization events. According to simulations, the numbers of alleles observed in each population was consistent with two to four founders of the SF population and two founders of the NF population.

For nine individuals in SF, potential parents were tentatively inferred from parentage tests. These nine individuals were likely offspring to two different breeding pairs sampled throughout the study period. As judged from the location of 10-40 samples representing each parent, the male and female of each breeding pair had overlapping territories, whereas neither the territories of the two males nor the two females overlapped. The male and female of one pair appeared to be related to each other, and the two breeding females also appeared to be related. This indicates that the inferred offspring-parent combinations should be treated with some caution as the ability to make correct parent assignments is lowered in the presence of inbreeding and/or close relatives. In NF it was not possible to fully resolve internal relationships, as several parent-offspring combinations were possible.

$F_{ST}$ estimates indicated that the new populations were slightly genetically differentiated from the main population, and from each other. This is likely to be an effect of few founding individuals and genetic drift.

The SF population shows an increasing yearly number of individuals and in the most recent season there were at least ten individuals present. The NF population does not seem to be expanding and the detection of only two individuals in the last winter season shows that it is highly vulnerable. Given the restricted number of individuals, the occurrence of inbreeding and the limited number of founders, both populations are likely to be dependent on gene flow from the main population even for short-term survival. The finding of stray individuals shows that gene flow is indeed feasible.

# Concluding remarks

The papers presented in this thesis demonstrate that the non-invasive genetic approach provides a useful tool in studies of wolverine populations. The technique allowed reliable individual and sex identification of approximately 65% of wolverine faecal samples. This made an assessment of a range of parameters that are important in the management of threatened populations possible, e.g. population size, sex ratio, genetic isolation, number of founders, territoriality and breeding. As non-invasive sampling is particularly suitable for elusive animals and threatened populations, the approach is likely to become important in future studies of wolverine biology and genetics. Perhaps most importantly, it has already been implemented in national plans for management in Sweden and Norway.

Immigration from other populations appears to be crucial for Scandinavian wolverines both on a smaller and on a larger scale. In the small southern Norway population, it was found that erosion of genetic diversity has been counteracted by reproductive contribution from northern immigrants, illustrating the value of immigrants in maintaining levels of genetic variation in small populations. There may be an immediate need of immigrants or gene flow from the mountain range population into the two newly established populations in east-central Sweden. On the larger scale, the entire Scandinavian population is likely to depend on gene flow from populations in Finland/Russia for survival in the long term.

In the future, continuous genetic monitoring of Scandinavian wolverines will be important to allow observations of population trends, such as if genetic diversity is decreasing in the population. Non-invasive sampling of populations in Finland and western Russia could potentially be used to assess immigration rates. Furthermore, analysis of Finnish and Russian samples would provide valuable information on to what extent the genetic diversity of Scandinavian wolverines (and potentially also wolverines in the entire Fennoscandia) is deprived. It is not known if there is a gradient with increasing genetic diversity towards the east, or if there at some point is a pronounced differentiation between Scandinavian and eastern animals.

In general, for a population to be viewed as long-term viable, management strategies must be based on profound knowledge. Long-term population surveys combining genetic and field methods probably represent the most effective way to gain deeper insight in the biology of wolverines.

# Svensk sammanfattning

## Bakgrund

På den skandinaviska halvön finns fyra stora rovdjursarter: lodjur *Lynx lynx*, brunbjörn *Ursus arctos*, järv *Gulo gulo* och varg *Canis lupus*. Under det senaste seklet, har populationerna av dessa arter upplevt en dramatisk nedgång eller total utrotning, men fredning och fridlysning under 1900-talet har också inneburit återhämtning och populationsökning.

Före de kraftiga nedgångarna förekom alla arterna i stora sammanhängande populationer över nästan hela Skandinavien. Historiskt sett var utbredningen också betydligt mer omfattande på andra håll i världen. Den huvudsakliga orsaken till nedgången anses vara mänsklig förföljelse och förlust av lämpligt habitat på grund av en ökande befolkning. Rovdjuren har sedan länge varit illa ansedda, i huvudsak på grund av predation på tamboskap. Under 1800-talet när jakten effektiviserades genom en ökad tillgång på skjutvapen började samtliga rovdjursstammar att minska kraftigt. Ungefär 100 år senare hade lodjur, björn och järv jagats till gränsen för utrotning och fanns endast kvar i mycket små bestånd. För vargen blev situationen ännu allvarligare och vid slutet av 1960-talet ansågs den vara utrotad i Skandinavien.

Rovdjuren fridlystes vid olika tidpunkter under 1900-talet och till följd av detta har populationerna ökat. Även vargen, som vid tidpunkten för fredande (1966 i Sverige och 1972 i Norge) redan var försvunnen, har återvänt till Skandinavien. Dagens lilla vargpopulation är resultatet av att ett fåtal individer från öster har anlänt och förökat sig.

De skandinaviska populationerna av lodjur, järv och varg visar idag begränsad genetisk variation jämfört med de större populationerna längre österut. Den begränsade variationen kan förklaras av genetisk drift och få individer från vilka populationerna grundats. Björnstammen visar ett annorlunda mönster med en nivå av genetisk variation motsvarande stora björnpopulationer i Nordamerika. Att björnen uppvisar en så hög grad av variation kan ha flera förklaringar. Björnstammen var när den var som minst uppdelad i fyra olika områden. Detta medförde separata förlopp av genetisk drift och en möjlighet för fler genetiska varianter att bevaras. Inom björnstammen finns dessutom två distinkta genetiska grenar eftersom björnar koloniserat Skandinavien både från norr (öster) och från söder. De två grenarna kan ha bidragit

till att öka heterogeniteten bland björnarna på ett sätt som inte skett hos lodjur, järvar och vargar.

Genetisk variation är väsentligt för livskraftigheten hos arter och populationer. På lång sikt är genetisk variation nödvändigt för möjligheten till anpassningar, t.ex. till miljöförändringar. Låg genetisk variation kan också ha en omedelbar negativ effekt eftersom skadliga gener kan komma att uttryckas i högre grad. I små och perifera populationer är risken att förlora genetisk variation stor på grund av slumpmässiga processer och inavel. För de små Skandinaviska rovdjurspopulationerna är därför genetiskt utbyte med östliga populationer viktigt då det kan medföra tillförsel av nytt genetisk material och därmed motverka de negativa effekterna av inavel och genetisk drift.

Under 1900-talet har den allmänna attityden gentemot de stora rovdjuren delvis förändrats. Från att man tidigare önskat utrota dem helt är nu myndigheternas målsättning att bevara livskraftiga populationer. Detta är dock kontroversiellt och förenat med konflikter, framför allt på grund av rovdjurens predation på tamren och lösgående får. Samhällets tillvägagångssätt för att minska konflikter består främst av att ersätta ekonomiska förluster orsakade av rovdjur och av att reglera rovdjurspopulationernas storlek och utbredning. Att skapa livskraftiga rovdjurstammar samtidigt som de begränsas till storlek och utbredning är emellertid svårt och kommer att kräva effektiva metoder för kontinuerlig bevakning av populationerna samt gedigen kunskap om respektive arts genetiska och biologiska behov.

Stora rovdjur är ofta svåra att studera. En kombination av fältdata och genetiska analyser har visat sig brukbart och tack vare teknisk utveckling är det nu möjligt att samla in omfattande genetiska data från vilda populationer. Under 1990-talet demonstrerades möjligheten att extrahera DNA från material som samlats in i fält utan att man vare sig sett eller varit i kontakt med djuret, t.ex. spillning och hårstrån. Även om detta lovar gott för studier av vilda djur i framtiden så finns en hel del svårigheter. DNA utvunnet ur t.ex. spillning har betydligt lägre koncentration och sämre kvalitet än DNA utvunnet ur blod och vävnad. Detta gör att analyserna blir krävande och att resultaten ibland blir felaktiga. Sedan 1990-talet har tekniken testats på ett flertal arter och metoder för alltmer pålitlig analys har utarbetats. Studierna har visat att framgång och tillförlitlighet beror på en rad olika faktorer, t.ex. art, hur och när proverna samlats in, hur proverna förvarats och vad djuret ätit. En analysteknik som ger tillförlitliga resultat i en studie behöver alltså inte göra det i en annan. Därför behöver tekniken utformats speciellt för just de omständigheter som råder i den tänkta studien.

Studierna i min avhandling fokuserar på järven som av norra Europas stora rovdjur är den där kunskapen är mest begräsad. Detta beror förmodligen på att järven är ovanligt besvärlig att studera; den är sällsynt, har låg populationstäthet, lever solitärt och rör sig över stora områden i otillgängliga trakter. Skandinaviska studier baserade på fältdata har emellertid bidragit med en del kunskaper och det pågående projektet med märkta järvar i norra Sverige

är det mest omfattande av sitt slag i världen. Den första studien där genetik användes för att studera järvar i Sverige och Norge publicerades år 2001. Detta var en viktig studie eftersom det i den utvecklades genetiska basredskap nödvändiga för att kunna utföra genetiska studier av skandinaviska järvar på populations- och individnivå. Att utveckla och använda genetiska metoder för studier av jäven och dess populationer i Skandinavien utgör målet för min forskning. Nedan följer en sammanfattning av mina studier.

## Artikel I. DNA-baserad individ- och könsbestämning av järv (*Gulo gulo*) genom analys av spillning och urin

Målet för den här studien var att utveckla en tillförlitlig genetisk metod där individ- och könsbestämning av järvar kan utföras med DNA-analys från insamlade järvexkrementer. I studien användes 32 spillningsprover och 139 vävnadsprover från järvar i norra Sverige. Alla individer som fanns representerade bland spillningsproverna fanns också representerade bland vävnadsproverna, för att genom en blindtest möjliggöra en kontroll av resultaten från spillning. Utöver dessa prover användes även 22 urinprover insamlade i norra Norge. DNA från frysta exkrementer extraherades och analyserades med tio genetiska markörer (mikrosatelliter). Alla analyser av DNA från exkrementer replikerades ett flertal gånger enligt ett i förväg definierat kriterium. Tjugoen spillningsprover (65%) var möjliga att analysera för samtliga mikrosatellitmarkörer. Det vanligast förekommande felet vid genotypning med mikrosatelliter är att en av de två alleler som finns hos en heterozygot individ faller bort. Detta inträffade i 12% av amplifieringar från heterozygota loci. De slutliga genotyperna (dessa baserades på flera replikat enligt kriteriet) från de 21 spillningsproverna var identiska med resultaten från vävnadsproverna från samma individer. I samtliga fall erhölls korrekt genotyp efter maximalt tre upprepade analyser per prov och markör. Tillförlitliga genotyper från järvspillning tycks alltså kunna uppnås efter ett relativt begränsat antal replikat. Genotypning kunde utföras för 40% av urinproverna, bortfall av en allel för heterozygoter inträffade i 14% av amplifieringarna. För könsbestämning utvecklades två Y-kromosommarkörer specifika för järv, *DBY3Ggu* och *DBY7Ggu*. Markörerna testades på både vävnadsprover och spillning och gav i samtliga fall det förväntade mönstret för hanar respektive honor. Markörerna testades även på ett flertal andra arter, *DBY3Ggu* visade sig vara järv-specifik medan *DBY7Ggu* förmodligen är specifik för mårddjur då den även gav resultat för utter.

## Artikel II. Kolonisationshistoria och uppföljning av en återetablerad järvpopulation med hjälp av DNA analys baserat på spillningsprover

Järvpopulationen i södra Norge ansågs vara utrotad under 1960-talet, men en återetablering skedde under 1970-talet. För att kunna förvalta populationen är man beroende av att känna till viktiga parametrar så som populationsstorlek, könsfördelning och omfattning av immigration av individer från Skandinaviens huvudsakliga järvpopulation i fjällkedjan längre norrut. Studien innefattar genetisk analys av att stort antal spillningsprover insamlade under 2000 och 2001. Sextioåtta olika individer detekterades bland 147 analyserade prover. Av dessa konstaterades 60 individer (29 hannar och 31 honor) under 2001 och detta utgör därför ett minimimått av populationsstorleken. Eftersom endast hälften av individerna var representerade med mer än ett prov kan man dock anta att populationen i själva verket är större. Genom en fångst-återfångst beräkning baserad på antalet prover insamlade för respektive individ uppskattades populationsstorleken till 89 ± 15 individer. Resultaten visar bland annat också att djur från järvpopulationen i fjällkedjan mellan Sverige och Norge fortlöpande vandrat in och lyckats reproducera sig i södra Norge. Detta har bidragit till att motverka förlust av genetisk variation i denna lilla population.

## Artikel III. Genotypning med mikrosatelliter baserat på DNA extraherat från klor på garvade rovdjurshudar

Garvning är en vanlig form för beredning av hudar. Garvningsprocessen medför oftast att materialet blir oanvändbart för DNA-analys, men i den här studien visar jag att DNA extraherat från blod inne i pulpan av klor kan användas för genotypning med mikrosatelliter. För studien användes åtta järvhudar och åtta lodjurshudar som garvades för 5-20 år sedan. Varje analys (PCR amplifiering) upprepades tre gånger och distinkta resultat åstadkoms i 93-98% av fallen. Genotyperna från klorna jämförelse med genotyper från vävnadsprover av samma djur och visade att resultaten överensstämde väl. Studien demonstrerar därmed att DNA i pulpan av klor på garvade rovdjurshudar utgör en möjlighet för pålitlig genetisk analys. Detta kan man t.ex. få användning för vid utredningar rörande illegal handel med hotade arter, studier av temporära skillnader i genetisk variation och kanske till och med för genetiska studier av utrotade arter.

## Artikel IV. Ett test av en metod baserad på PCR i två steg för mikrosatellit-genotypning av DNA från järvspillning

Nyligen visade några forskare att en metod baserad på PCR i två steg (kallad multiplex pre-amplifiering) kan förbättra möjligheten att utföra genotypning med mikrosatelliter från spillning. I den här studien testades metoden på järvspillning och resultaten bedömdes i förhållande till resultat från den mer traditionella metoden utvecklad i Artikel I. Totalt analyseras 48 prover med 18 mikrosatelliter för båda metoderna. Två-stegs metoden baseras på att först utföra en PCR inkluderande alla markörer som ska analyseras och att därefter utföra ytterligare PCR-reaktioner, där produkten från den första reaktionen används som underlag för separata reaktioner för varje markör. Resultaten visade att två stegs metoden var fördelaktig både med avseende på andelen analyserbara PCR produkter (91% vs. 80%) och på felfrekvens (2.4% vs. 12.5%). Även om felfrekvensen var låg i två-stegs metoden så gjordes en viktig observation angående dessa fel (huvudsakligen bortfall av en allel hos heterozygoter), nämligen att samma fel upprepades i replikerade amplifieringar i det andra steget. Detta betyder att fel huvudsakligen uppstår redan i den inledande multiplex-reaktionen och att replikering av andra steget därför har en begränsad betydelse för att upptäcka fel som kan uppstå under genotypning. I studien rekommenderar jag därför att man genomför två oberoende multiplex-reaktioner och sedan utför en PCR per prov och markör utifrån var och en av dem. Detta testades för 22 prover och det visade sig vara ett effektivt sätt för att åstadkomma korrekta genotyper.

## Artikel V. Faderskap och parningssystem hos järv

I den här studien kombinerades genetiska data med data från radiomärkta järvar i syftet att studera järvens parningssystem. Faderskapsanalyser baserade på data från 20 mikrosatelliter utfördes för 145 järvungar med kända mödrar. Totalt kunde en sannolik fader hittas till 51% av ungarna. De flesta fall där ingen fader hittades kan sannolikt förklaras av att fadern inte fanns representerad bland insamlade prover. Flera fall där en hane reproducerade sig med mer än en hona kunde påvisas och studien bekräftar därmed att järven har ett polygamt parningssystem. Antalet honor per hane tycks dock vara begränsat, ingen hanne kunde påvisas reproducera sig med mer än två honor under samma år eller med mer än tre honor under flera år. Honor reproducerade sig ofta med samma hane under flera år och skifte av partner skedde potentiellt som en konsekvens av att den revirhållande hannen ersatts av en annan. I 20 av 23 tillfällen där samtida pejlingsdata fanns tillgängliga hade reproducerande par överlappande hemområden. I de tre fall där områ-

dena inte överlappade var ytterkanterna 2-5 km från varandra. Detta antyder att vilka järvar som bildar reproducerande par generellt överensstämmer med deras hävdande av revir. Fenomenet ”multiple paternity” (dvs. att en eller flera ungar i samma kull har olika fäder) tycks vara ovanligt bland järvar eftersom endast ett fall där syskonen i en kull eventuellt hade olika fäder kunde påvisas.

## Artikel VI. DNA-baserad bevakning av två nyligen etablerade Skandinaviska järvpopulationer

I Skandinavien är järvens utbredning begränsad till fjällkedjan och alpina delar av södra Norge. Under 1990-talet etablerades dock två förekomster av järv i skogsområden ungefär 100 km öster om fjällkedjan i höjd med Sundsvall respektive Örnsköldsvik. I denna studie användes DNA-analys för att utifrån ett stort antal spillningsprover insamlade under fem års tid följa och studera järvarna i de nybildade populationerna. Genetiska data tyder på att antalet individer som grundat populationerna är så få som två till fyra i det sydligare området och endast två i det mer nordliga. Analysen visar också att ingen eller endast liten genetisk kontakt med populationen i fjällkedjan skett sedan populationerna etablerades under 1990-talet. En hög grad av genetisk likhet mellan individerna i respektive område vittnar om att inavel ägt rum, eventuellt till och med genom syskonparning. Under perioden 2001-2005 påträffades totalt 17 individer i södra området och fem i det norra. Populationen i södra området ser ut att växa och för närvarande finns där cirka tio djur. I motsats till detta, så minskar antalet djur i det nordligare området och under vintern 2004/2005 fåträffades endast två djur. Eftersom båda populationerna karaktäriseras av få individer och inavel, är genetiskt utbyte med populationen i fjällkedjan förmodligen viktigt för deras överlevnad även under ett ganska kort tidsperspektiv. Under studiens gång påvisades ett par förbipasserande individer med förmodad härkomst i fjällpopulationen. Detta visar att genetiskt utbyte med fjällpopulationen inte är osannolikt.

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