Genetic variability and population differentiation in Scandinavian wolverines

by

Andrew J. Duffy

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Dedication

For my parents, Elizabeth Duffy (1939-1990) and James Laurence Duffy (1936-1995).

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Abstract

Five polymorphic microsatellite markers (four developed in wolverine, the fifth in American mink) were used to examine genetic variability and population differentiation in wolverine (*Gulo gulo*) populations in northern Norway, south-central Norway and Sweden. Levels of genetic variability at microsatellite loci were found to be slightly lower than in other mammals. There were between three and six alleles at each microsatellite locus, and expected heterozygosity values across populations were 0.533 to 0.566. Population differentiation and genetic distance were examined by infinite alleles model measures (F_{ST} and Nei's genetic distance, respectively) and stepwise mutation model measures (R_{ST} and $(\delta\mu)^2$, respectively). There was significant differentiation among the populations examined (R_{ST}=0.113). These results provide baseline data for future genetic management of the wolverine in Scandinavia.

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Introduction

Wolverine biology

The wolverine (*Gulo gulo*) is the largest terrestrial mustelid and one of the least studied of all large carnivores (Banci 1994). Wolverines are generally solitary scavengers (Nowak and Paradiso 1983) which live in large home ranges that overlap ranges of members of the opposite sex (Banci 1994). The wolverine's circumpolar distribution has been greatly reduced, mainly due to human activity (Nowak and Paradiso 1983). Conservation programs for the wolverine would be assisted by greater knowledge of population structure and genetic diversity (Banci 1994).

Like most mustelids, wolverines appear to have a system of intrasexual home ranges (Powell 1979). Home ranges are relatively large, with those of adult males (237-1281 km^2) generally being larger than those of subadult males (435-526 km^2), solitary adult females (56-963 km^2), and adult females with young (55-139 km^2) (Banci 1994, Landa *et al.* 1997). The range of one male generally overlaps with that of 2-6 females (Magoun 1985, Banci 1987). This pattern is consistent with a carnivore spatial strategy where home ranges of females reflect the minimum size required to obtain sufficient food, while male home ranges reflect the spacing of females, at least during the breeding season (Sandell 1989, Banci 1994). Some overlap of home ranges in members of the same sex is seen; individual females may tolerate neighbours if they are related (Banci 1994). One

study (Hornocker and Hash 1981) has also reported overlap in male home ranges. Defence of home ranges is not seen, probably because home ranges are relatively large and because a system that allows movement to areas of food abundance is more advantageous to a scavenger like the wolverine (Hornocker and Hash 1981). Adults may also make temporary long-distance movements outside their usual home ranges; the reason for this behaviour is not known (Banci 1994).

With some exceptions, the wolverine leads a solitary existence (Nowak and Paradiso 1983). Scent marking (using anal gland secretions and urination) is used to maintain spacing in time, but not in space (Koehler *et al.* 1980). Strict mutual avoidance of wolverines is the rule (Hornocker *et al.* 1983), though tolerance increases at large food sources, where adults of the same sex may feed concurrently (Banci 1987). The only groups of wolverines are natal groups, composed of mother and kits; males are not involved in the rearing of young (Banci 1994). Dispersal of young usually occurs after the first summer, though it may occur during the second year (Banci 1994). Young females usually establish residency in or adjacent to the natal home range (Magoun 1985). Young males tend to disperse much further, often as a consequence of aggressive encounters with older males (Banci 1987, Gardner 1985).

Wolverine in Scandinavia

The wolverine population in Europe is vulnerable to extinction due to low numbers and a history of persecution. In Europe, the wolverine's range once extended through Scandinavia, west to Germany and east throughout Finland and northern Russia (Corbet 1978). Its range today has been reduced to mountainous and taiga areas of northern and south-central Norway, northwestern Sweden, northeastern Finland, and northern Russia, though there have been reports of sightings in more southerly parts of Scandinavia and in Estonia (Sandell 1995). In Norway, there are 200-280 animals; in Sweden, 100-150; in Finland, 98-109; and in the European part of Russia, 1500 (Sandell 1995). The International Union for the Conservation of Nature and Natural Resources has listed the wolverine as "rare" in Norway and "vulnerable" in Sweden, Finland, Russia, and Estonia.

Wolverines have been hunted in Scandinavia due to conflicts with animal husbandry. Wolverines kill large numbers of domestic sheep and reindeer in areas where they coexist (Sandell 1995). While wolverines have been protected in Sweden since 1969, in southern Norway since 1973 and in Finland and northern Norway since 1982 (Sandell 1995, Landa and Skogland 1995), they are hunted illegally in all three countries (Sandell 1995). Efforts to reduce this

conflict have included issuing of a small number (<20) of hunting licences in areas of high livestock predation in northern Norway, compensation payments to farmers for loss of livestock, and trials of volatile wolverine repellents (Sandell 1995, Landa and Skogland 1995).

The extent of population differentiation of Scandinavian wolverines is unclear. For the purposes of this study, wild wolverine populations from three areas (northern Norway, south-central Norway, and northern Sweden) were examined. The small (approximately 20 individuals) south-central Norwegian population is clearly separate, and was founded between 1976 and 1979 (Landa and Skogland 1995). The northern Norwegian and Swedish populations form one apparently continuous distribution, with a reduction of population density along the Norwegian-Swedish border (Sandell 1995). Wolverines are exchanged between Finland and Russia, and in the north among Norway, Sweden and Finland (Sandell 1995). The proximity of these populations, and movement among populations, might be expected to reduce population differentiation. However, populations may not become completely genetically mixed, even where individuals make long-distance movements. In polar bears, gene flow between populations is restricted despite long-distance seasonal movements (Paetkau *et al.* 1995), while in grey seals there is clear genetic differentiation between two breeding colonies even though movement between the two sites is within the seals' capabilities (Allen *et al.* 1995).

The extent of genetic variability of Scandinavian wolverines has not previously been investigated. There may be as few as 250 wolverines in Fennoscandia (that is, Finland, Norway and Sweden), a small number for the purposes of long-term population preservation (Sandell 1995). Such small numbers may lead to a reduction of genetic variability, which could hamper recovery efforts. Captive breeding efforts have begun in Sweden (Blomqvist 1995), but their success will depend to a great extent on the amount of genetic variability in the stock animals.

In this study, molecular genetic markers were developed and used to investigate the population differentiation and genetic diversity of three wild wolverine populations. Such genetic markers may also be useful for investigations of other wild or captive wolverine populations, and help in the management of conservation and captive breeding programs for this species. Since the markers used needed to be polymorphic, even in a situation where genetic variability was potentially reduced, the type of markers chosen for this study were microsatellites.

Microsatellites

Microsatellites are short stretches (10-100bp) of DNA composed of tandemly arranged di-, tri- or tetranucleotide repeats, flanked by (usually) unique sequence (Wright and Bentzen 1994). Repeat arrays are highly susceptible to length polymorphism by the addition or subtraction of repeat units, apparently caused by slipped-strand mispairing during DNA synthesis (Levinson and Gutman 1987).

Microsatellite loci are suitable for amplification by the polymerase chain reaction (PCR) because they are relatively short and they are usually flanked by unique sequence. Once the sequence of a microsatellite locus has been determined, primers complementary to the flanking sequence can be constructed, permitting amplification of the repeat array. PCR allows for amplification even from small, degraded or archival samples (Bruford and Wayne 1993). PCR products can be size-fractionated and scored for polymorphism on polyacrylamide gels, without the need for Southern blotting (Wright and Bentzen 1994, Bruford and Wayne 1993).

Several properties of microsatellites make them suitable as genetic markers. Levels of polymorphism at microsatellite loci are generally greater than those of unique-sequence DNA and equivalent to those of minisatellite loci (Estoup *et al.* 1993); for example, over 50 alleles have been found at a single

microsatellite locus (Amos *et al.* 1993). Heterozygosities exceeding 0.90 have been reported in some microsatellite loci (Brooker *et al.* 1994), although many microsatellites show much lower levels of heterozygosity (Wright and Bentzen 1994). Unlike RAPDs and mitochondrial DNA markers, microsatellites are codominant alleles inherited in a Mendelian fashion, increasing their usefulness in population-level studies (Wright and Bentzen 1994). Finally, microsatellites are very common in the genome, with frequencies of 10^3-10^5 per genome (Wright 1993), allowing the development of suites of markers for various purposes.

Microsatellites are useful as genetic markers at a variety of levels. At the individual level, microsatellites have been used for individual identification (Hagelberg *et al.* 1991) and parentage analysis (Morin and Woodruff 1992, Evans 1993). Population-level studies have mostly examined variation in humans (Edwards *et al.* 1992, Fornage *et al.* 1992, Roewer *et al.* 1993), but such studies have also been performed in other mammals (Amos *et al.* 1993, Gotelli *et al.* 1994, Roy *et al.* 1994, Paetkau *et al.* 1995), amphibians (Scribner *et al.* 1994), and fish (Brooker *et al.* 1994, Nielsen *et al.* 1994, Morris *et al.* 1996).

Population studies of large mammals

Studies of the genetic population structure of natural populations of large mammals can be hampered by low genetic variability and small sample size. In large part the first difficulty, low genetic variability, can be mitigated by the use

of microsatellite loci; typical expected heterozygosity values at microsatellite loci in such populations are 0.60-0.65. The second problem is the low number of samples available. Such low numbers are due to the difficulty in obtaining samples from large wild mammals, and the small numbers of many large mammals, which are often top carnivores and frequently threatened by human activity. Sample sizes in microsatellite-based studies of large mammals include: 22-30 samples from each of four populations in a study of Canadian polar bears (Paetkau *et al.* 1995); 16.6 to 22.1 in 14 populations of wolflike canids (Roy *et al.* 1994); 23-32 from three populations of Canadian black bears (Paetkau and Strobeck 1994).

In this study, sample sizes are relatively small, and they vary widely for each population, reflecting the relative sizes of the populations in question. In addition, effective sample sizes are reduced due to non-amplification of some loci. Statistical approaches to the microsatellite data can reduce the effects of small, unequal sample sizes.

Measures of population differentiation and genetic distance

In any comparison of natural populations, it is valuable to know the extent of population differentiation and the distance between any pair of populations. Such measures are dependent on models of mutation for the genetic markers

used to undertake comparisons. Two main models are generally used for microsatellite data: the infinite allele model and the stepwise mutation model.

Under the infinite allele model, each mutation at a locus is assumed to result in a new allele not previously found in the population (Wright 1949, Kimura and Crow 1964). This assumption underlies the most common measures of population differentiation, Wright's (1951) F statistics, which are estimated for multi-locus problems by Weir and Cockerham's (1984) statistics F, θ and f. The most common measure of genetic distance under this model is Nei's (1972) standard genetic distance.

Under the stepwise mutation model, it is assumed that mutations do not always result in a new allelic state, but can often result in a reversion to another allelic state already present in the population (Ohta and Kimura 1973, Shriver *et al.* 1993). A slipped-strand mispairing mechanism of mutation would seem to be consistent with this model of mutation. As a result, new measures of population differentiation and genetic distance, which assume stepwise mutation, have been derived. RST (Slatkin 1995) is a measure of population differentiation analogous to Wright's FST. Of the several genetic distance measures derived for use with microsatellite data (Goldstein and Pollock 1997), the most generally applicable is $(\delta\mu)^2$ (Goldstein *et al.* 1995).

Computer simulations have shown that microsatellite data do not conform to either the infinite alleles model or a strict one-step stepwise mutation model. Though generally in agreement with the stepwise mutation model, microsatellite data show deviations in the direction of the infinite alleles model (Shriver *et al.* 1993). Deviations from a one-step stepwise mutation model may be the result of rare multi-step mutations (Di Rienzo *et al.* 1994).

Measurements of genetic diversity and population differentiation assuming the infinite alleles model and the stepwise mutation model are both used in microsatellite-based population studies. Both types of measurements are used and compared in this study.

Aims of project

The aims of this study, one of the first to examine the population genetics of the wolverine, are as follows:

1. To clone single locus microsatellites from the wolverine (*Gulo gulo*) genome.
2. To determine the nucleotide sequence of cloned microsatellite loci (flanking regions and repeats).
3. To determine the extent of polymorphism at wolverine microsatellite loci using the polymerase chain reaction (PCR).

4. To assess the amount of genetic variability in wild Norwegian and Swedish wolverine populations.
5. To determine the extent of genetic differentiation among wolverine populations in northern Norway, south-central Norway, and Sweden, using both traditional and stepwise mutation model measures.

Materials and Methods

Study area and collection of samples

Norwegian wolverine samples, collected between 1983 and 1996, were provided by Arild Landa of the Norwegian Institute for Nature Research. Samples from Nord-Trøndelag, Nordland, Troms and Finnmark counties were designated as belonging to the northern Norwegian population, while those from Hedmark, Oppland, Møre og Romsdal and Sør-Trøndelag counties were designated as belonging to the south-central Norwegian population (Figure 1). Sample tissues included skin, heart, brain, muscle and liver.

Swedish wolverine samples were provided by Arild Landa. Swedish samples were collected between 1989 and 1996 from Jamtlands, Vasterbottens and Norrbottens counties (Figure 1). All Swedish samples consisted of skin or muscle tissue.

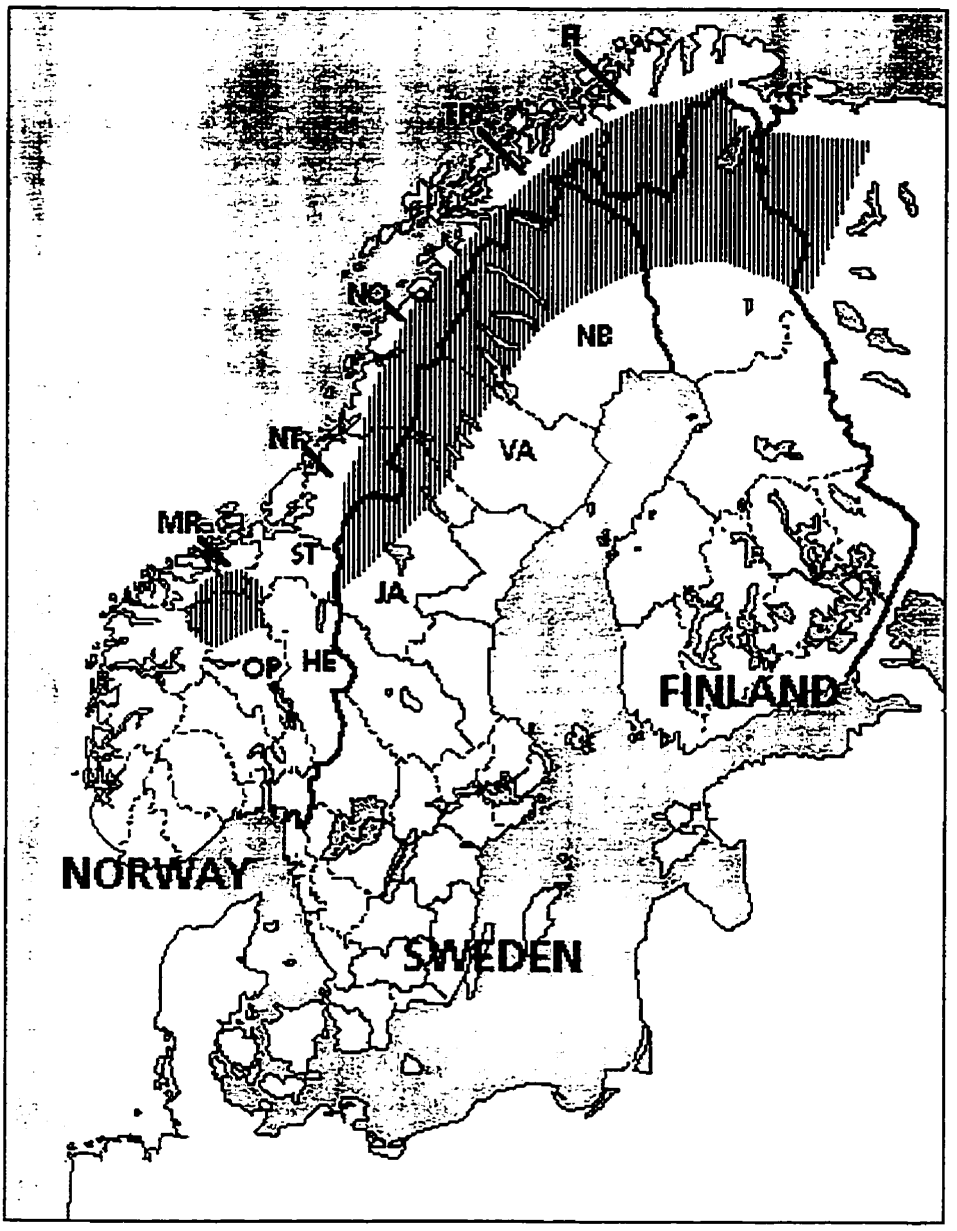


Figure 1: Current range of wolverines in Norway, Sweden and Finland. Swedish and Norwegian counties are designated by two-letter abbreviations as follows: HE - Hedmark; OP – Oppland; MR – Møre og Romsdal; ST – Sør-Trøndelag; NT – Nord-Trøndelag; NO – Nordland; TR – Troms; FI – Finnmark; JA – Jamtlands; VA – Vasterbottens; NB – Norrbottens.

Extraction of wolverine DNA

Wolverine tissue samples were either stored dry or suspended in ethanol. Prior to extraction, ethanol-stored samples were allowed to air-dry. Approximately 50 mg of each tissue sample was desiccated using liquid nitrogen, homogenized, and suspended in 500 µL STE (sodium chloride/Tris/EDTA buffer). 15 µL Proteinase K (20 mg/mL) and 30 µL 10% SDS were added to this mixture, which was incubated at 50°C for 12-18 hours. The samples were then extracted three times: once using an equal volume of phenol, once using an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol, and once using chloroform. DNA was precipitated by addition of three volumes of absolute ethanol; the samples were subjected to centrifugation at 14 000 g for 15 minutes, and the supernatants were aspirated. The resulting DNA pellets were washed by addition of 1 mL of 70% ethanol and subjected to centrifugation for 10 minutes. The samples were air-dried for 10 minutes and then resuspended in 50-150 µL TE (Tris/EDTA buffer). The concentration of DNA in each sample was estimated by spectrophotometry. Samples were stored at 4°C.

Size-selection of DNA

Genomic DNA from four to five individuals was digested overnight using restriction endonucleases *Rsa*I, *Pal*I and *Hinc*II in a volume of 250 µL according to

the manufacturer's instructions (Pharmacia and Upjohn, Stockholm, Sweden). This procedure was performed twice, the first time using 80 μg DNA, and the second time using 55 μg DNA. 50 μL of 6X loading dye was added, and the DNA was size-fractionated by electrophoresis through a 1% low melting point agarose gel. Fragment size was determined by comparison to an ethidium bromide-stained 100 base pair ladder (Pharmacia and Upjohn, Stockholm, Sweden). Those parts of the gel corresponding to 300-700 bp were excised using a razor blade. Agarose was removed from the gel mixture by centrifugation through a paper slurry (Chuang and Blattner 1994).

For the first completion of this size-selection procedure, DNA was precipitated from the resulting liquid (approximate volume 500 μL) by addition of 50 μL 3M sodium acetate, 1 μL yeast tRNA and 1000 μL absolute ethanol, with centrifugation for 15-25 minutes. The supernatant was aspirated and the resulting DNA pellet was washed with 750 μL 70% ethanol and subjected to centrifugation for 10 minutes. The supernatant was aspirated, the pellet was allowed to air dry, and the DNA was resuspended in 25 μL TE. The samples were then stored at 4°C.

For the second completion of this size-selection procedure, DNA was extracted twice from the resulting liquid, using an equal volume of phenol followed by an equal volume of chloroform. Precipitation occurred by addition

of sodium acetate, absolute ethanol and glycerol, followed by cooling to -70°C for 1 hour, -20°C overnight, and -70 °C for 45 minutes. The samples were subjected to centrifugation for five minutes, washed with 70% ethanol for five minutes, air dried for five minutes, and dissolved in a total of 20 μL TE. The concentration of DNA was examined by size-fractionation of 1 μL of the DNA by electrophoresis through a 1% agarose gel.

Ligation

Two sets of ligation reactions were carried out. The first set was used in four libraries, while the other set was used for a fifth library.

For the first set of ligation reactions, *SmaI/BAP*-digested pUC18 vector (Pharmacia and Upjohn, Stockholm, Sweden) was diluted to 25 ng/μL. Three ligation reactions were set up, each in a total volume of 25 μL, including 1.5 μL T4 DNA ligase (Pharmacia and Upjohn, Stockholm, Sweden), genomic DNA, vector, and 5 μL T4 ligation buffer (Life Technologies, Rockville, MD). Each reaction used a different ratio of genomic DNA:vector; the reactions, labelled A, B and C, had ratios of 1:0.5, 1:1 and 1:2. The reactions were incubated at 15°C for 24 hours, and stopped by heating at 60°C for 20 minutes.

The success of ligation reactions was examined by electrophoresis. 1 μL of 6X loading dye was added to 5 μL of each ligation reaction. The samples were then fractionated by electrophoresis through a 1% agarose gel. The gel was

stained with ethidium bromide and examined by transillumination with ultraviolet light. All reactions appeared to have been successful, as shown by a smear above the sharp pUC18 band.

This process was repeated for ligations used in the fifth library, except that the genomic DNA:vector ratios used were 0.25:1, 0.5:1 and 1:1, a different ligation buffer was used, and the length of ligation reactions was 16 hours. Electrophoresis, performed as above, showed that the reactions were successful.

Library construction

The efficiency of the ligation reactions was tested by using them to transform MAX-Efficiency DH5-α (Life Technologies, Rockville, MD) cells according to the manufacturer's instructions. The transformed cells were spread on LB (Luria-Burani) Agar plates containing 0.1 mg/mL ampicillin (LB Amp^+ plates). The most efficient ligation reaction from the first set of reactions was reaction A, with a genomic DNA:vector ratio of 0.5:1. For the second set of reactions, the most successful reaction had a genomic DNA:vector ratio of 1:1 (here called reaction B). These ligation reactions were used to construct a total of 5 wolverine genomic libraries.

The first library was constructed by the addition of 1 μL ligation reaction A to 40μL MAX-Efficiency cells, and proceeding according to the manufacturer's instructions (Life Technologies, Rockville, MD). The transformed cells were

spread on LB Amp$^+$ plates (200 μL of cell solution/plate) and grown overnight. From a total of 27 plates, 22 showed distinct bacterial colonies; the rest showed streaks or lawns of bacteria and were discarded. The library was then screened, as described below.

The second and third libraries were constructed and plated in the same way as the first, except that 300 μL of bacterial culture was used per plate. 16 of the resulting 18 plates from the second library and all 20 plates from the third library were screened as described below.

After an unsuccessful attempt with apparently expired cells, a fourth library was constructed as described above, except that 120 μL or 200 μL of bacterial culture was spread on each LB Amp$^+$ plate. The resulting 20 plates were screened as described below.

The fifth library was constructed using MAX-Efficiency DH5-α cells and ligation reaction B according to manufacturer's instructions (Life Technologies, Rockville, MD), except that only 0.5 μL ligation reaction (rather than the standard 1 μL) was added to the cells prior to the transformation procedure. 300 μL of the resulting transformed cell mixture was added to each LB Amp$^+$ plate. On average, there were 300 colonies/plate, with 20 plates screened as described below.

Library screening

Colonies were lifted onto Hybond-N and Hybond-N+ membranes according to the manufacturer's instructions (Amersham, Little Chalfont, UK). Briefly, membranes were placed onto the surface of LB Amp^+ plates for one minute, then onto denaturing solution-saturated Whatman paper for seven minutes and neutralizing solution-saturated Whatman paper twice for three minutes each. Membranes were placed into 2X SSC (sodium chloride/sodium citrate solution) for five minutes, allowed to air dry, then baked at 80°C for 2 hours. Membranes were pre-hybridized at 62°C for two hours in a solution of 5X SSPE, 5X Denhardt's, 0.5% SDS and 10 mg/mL RNA; each hybridization bottle contained 40 mL prehybridization solution and four to five membranes. (Denhardt's solution contains Ficoll, polyvinylpyrrolidone and bovine serum albumin.) After pre-hybridization, the bottles were emptied. A $(GT)_{15}$ oligonucleotide probe was labelled with $[\gamma^{32}P]$ATP using T4 polynucleotide kinase (Pharmacia and Upjohn, Stockholm, Sweden) and then added to the hybridization bottles, along with 10 mL 5X SSPE/5X Denhardt's/0.5% SDS/(10 mg/mL) RNA. (In the case of the fourth library, a labelled $(GA)_{15}$ oligonucleotide probe was also used.) Hybridization with this probe occurred overnight at 62 or 65°C. Membranes were then washed twice with 2X SSC/0.2% SDS for 15 minutes each at room temperature, and once with 0.5X SSC/0.2% SDS

for 15 minutes at 42 or 45°C. Films were wrapped in Saran Wrap and exposed to X-ray film overnight at -80°C. The screening process was repeated as a secondary screen for the positive clones of the second and third libraries.

After films were developed, they were compared to the original plates to determine which clones were positive, and therefore likely to contain microsatellites. Positive clones were picked with a sterile toothpick and spread on a master LB Amp^+ plate. Colonies were then picked from the master plate and incubated overnight at 37°C, 225 rpm in LB broth containing ampicillin. Glycerol stocks were prepared by the addition of 0.15 mL sterile glycerol to 0.85 mL of bacterial culture. Plasmid DNA was recovered from bacterial cultures by a miniprep procedure.

Extraction of plasmid DNA

Plasmid DNA was isolated from bacterial cultures by the miniprep procedure of Sambrook *et al.* (1989), modified by doubling of all amounts. Cultures were subjected to centrifugation to yield a bacterial pellet, and pellets were suspended in 200 µL ice-cold 50 mM glucose/25mM Tris-Cl/10mM EDTA (ethylenediaminetetraacetate). 400 µL 0.2N NaOH/1% SDS (sodium dodecyl sulphate) was added, followed by 300 µL 5M potassium acetate/11.5% glacial acetic acid. After mixing, the samples were placed on ice for five minutes, and subjected to centrifugation for 15 minutes; the supernatant was removed and

extracted with phenol/chloroform. DNA was then precipitated, concentrated into a pellet, and resuspended in TE.

DNA Sequencing

Positive clones were sequenced using the Sequenase T7 DNA polymerase kit (Pharmacia and Upjohn, Stockholm, Sweden). Sequencing reactions were carried out according to the manufacturer's instructions, with the following exceptions: the 37°C and 65°C incubations were replaced by one 20 minute 37°C incubation, and in some reactions all amounts were reduced by 1/3. Clones were initially sequenced in the forward direction, and were sequenced in the reverse direction as warranted. Sequences were determined by running sequencing mixes on 8% polyacrylamide gels, and results were visualized by autoradiography. Most gel electrophoreses lasted 2.5-3 hours, but longer electrophoreses were performed as appropriate.

PCR primer design and testing

Complete insert sequences were assembled using the Easy Sequence Eyeball Editor (Cabot and Beckenbach 1989). Sequences were used for primer design if they had an unbroken run of at least 12 dinucleotide repeats and did not have two or more long repeat arrays. Primers were designed using the computer program GeneRunner to minimize interactions within or between

primers, to ensure that the predicted annealing temperatures were between 50 and 60°C, and to ensure that the predicted annealing temperatures of the two primers in a set did not differ by more than 2°C. Suitable primers were obtained in a powdered form (Oligos Etc, Wilsonville, OR) and diluted in water as appropriate.

A total of 27 primer pairs were tested on wolverine DNA. Of these, 17 primers were derived from the wolverine genomic libraries described above, one was a human-derived primer set which had been shown to be polymorphic in other carnivores (D. Coltman, pers. comm.), and nine were developed in American mink (*Mustela vison*) (O'Connell *et al.* 1996). Primer sets were initially tested in a panel of eight unrelated wolverine from the northern and south-central Norwegian populations. PCR reactions were performed in a total volume of 5 μL containing approximately 20 ng genomic DNA, 200 μM of each dNTP, 1 mM $MgCl_2$, 10mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 μM of each primer, 0.01% gelatin, 0.1% Tween-20, and 0.5 units *Taq* polymerase. To determine which primer should be radiolabelled, two sets of reactions were run; in each, one of the two primers was labelled using T4 polynucleotide kinase (Pharmacia and Upjohn, Stockholm, Sweden) and [γ^{32}P]-ATP. Reactions were conducted in an MJ thermal cycler (MJ Research, Watertown, MA) as follows: five cycles of 20s at 94°C, 20s at the annealing temperature, 20s at 72°C, followed by 35 cycles of 20s

at 90°C, 20s at the annealing temperature and 20s at 72°C. Initially, an annealing temperature of 5°C below the expected annealing temperature of the primers was used. The temperature was increased or decreased as appropriate: higher temperatures were used when too many bands appeared on the autoradiograph, and lower temperatures were used when product bands on the autoradiograph were faint or absent. As required, different PCR reaction conditions were tried, including an increase in the proportion of labelled to unlabelled primer, a change in the concentration of $MgCl_2$ in the buffer, and a decrease in the concentration of dNTPs.

PCR primer application

After the PCR conditions had been optimized, the primers were used in all the Norwegian and Swedish samples of wolverine. Amplification was attempted for 68 samples from the northern Norwegian population, 23 samples from the south-central Norwegian population, and 46 samples from the Swedish population. All PCR reactions used the conditions described previously (see Table 1 for the specific annealing temperatures associated with each primer set). The size of alleles was determined by comparison to the M13 sequence. A minimum of two rounds of amplification was carried out for every sample collected. Often, several rounds of amplification were performed to ensure that multi-locus genotypes were available for a minimum number of samples.

Other species

The four polymorphic, single-locus wolverine-derived primer sets were used to attempt amplification in samples of other species. The samples used were obtained by workers in Dr J.M. Wright's laboratory at Dalhousie University. All primer sets except Ggu 216 were tested with two stoat (*Mustela erminea*) samples, two European otter (*Lutra lutra*) samples, two pine marten (*Martes martes*) samples, two American mink (*Mustela vison*) samples, one dog (*Canis familiaris*) sample and one harbour seal (*Phoca vitulina*) sample. Ggu 216 was tested in two samples each of stoat, otter and pine marten and one mink sample. PCR conditions were as described above.

Genetic diversity determination

The genetic profile of each individual was determined by scoring of all successful amplifications. This information was used to determine genetic diversity and population differentiation measures.

Allelic numbers and frequencies were determined by use of the computer program Microsoft Access 97. Expected heterozygosities for individual loci in each population, and for individual loci in the total population, were calculated according to the formula

$$h = 1 - \frac{n\sum_i p_i^2 - 1}{n-1}$$

(Nei and Roychoudhury 1974) using a module written in Microsoft Access 97. Overall expected heterozygosities at each population were determined as arithmetic means.

Probabilities of identity for individual loci at each population were calculated according to the formula

$$I = \sum_i p_i^4 + \sum_i \sum_{j>i} (2p_i p_j)^2$$

using a module written in Microsoft Access 97. Overall probabilities of identity for each population were calculated as products of probabilities of identity at the five loci.

Conformity of the populations to Hardy-Weinberg proportions was determined by use of the computer program Genepop 2.0 (Raymond and Rousset 1995). Data were examined by locus within populations, with test results combined for each locus and for each population. Tests were calculated by a Markov chain method, using a dememorization number of 1000 and 100-175 batches of 1000 iterations.

The microsatellite data were tested to determine the presence or absence of genetic disequilibrium using the computer program Genepop 2.0. An exact

test was calculated using a Markov chain method, using a dememorization number of 1000 and 600 batches of 1000 iterations. Bonferroni corrections (Rice 1989) were performed on the results where appropriate.

Population differentiation determination

The infinite alleles model measures of population differentiation, Wright's (1951) *F*-statistics, were estimated by Weir and Cockerham's (1984) estimators using the computer program Fstat 1.2 (Goudet 1995). Mean values and standard deviations for *F* (estimating FIT), θ (estimating FST) and *f* (estimating FIS) were calculated by jackknifing over loci. Confidence limits and probability values for *F* were calculated by permutting alleles within the total population 1000 times. Confidence limits and probability values for θ were calculated by permutting genotypes within the total population 1000 times. Confidence limits and probability values for *f* were calculated by permutting alleles within individual samples 1000 times. Bonferroni corrections were performed on the results as appropriate (Rice 1989).

A stepwise mutation model measure of population differentiation, RST, was determined by use of the computer program RST-Calc (Goodman 1997). Allele sizes were standardized before RST was calculated. This procedure allows use of data from populations with unequal sample sizes. RST was estimated by

1000 bootstraps over loci; confidence limits and probability values were calculated by 1000 permutations.

Genetic distances between pairs of populations were calculated by using the computer program Microsat (Goldstein *et al.* 1995). The infinite alleles model measure, Nei's (1972) standard genetic distance, was estimated by bootstrapping 1000 times over loci. The stepwise mutation model measure $(\delta\mu)^2$ (Goldstein *et al.* 1995) was estimated (adjusting for small sample size) by bootstrapping 1000 times over loci.

The number of genetic migrants (N_m) was estimated by three methods. The computer program Genepop was used to estimate N_m by the private alleles method of Slatkin (1985). The computer program RST-Calc was used to estimate N_m by calculation from RST (Slatkin 1995). Finally, N_m was estimated from FST by use of the equation

$$F_{ST} = \frac{1}{4N_m + 1}$$

(Wright 1951).

The assignment test of Paetkau *et al.* (1994) was calculated by use of a module written in Microsoft Access 97. For each individual, the probability of finding the individual's genotype in that population was determined by multiplying the expected genotype frequency at each of five loci. For the purposes of this calculation, the individual's genotype was added to the

populations in which it is not found (*ie* two of three populations), so that expected genotype frequencies of zero were not obtained. The individual was then assigned to the population where the probability of finding its genotype was highest.

Results

Library creation and screening

Five libraries were constructed, and a total of 274 clones appeared positive. For the first library, the two first attempts at the hybridization procedure were unsuccessful because the first two washes were erroneously performed at 62°C. Under these stringent conditions, probing was unsuccessful and only one clone appeared positive. The third attempt at the hybridization procedure was more successful, but positive colonies were rare and a new library was constructed. For the second library, 16 plates were screened and 58 clones appeared positive and were streaked onto a master plate. These clones were designated as 1-58. For the third library, 20 plates were screened and 67 clones (designated 59-125) appeared positive and were streaked onto a master plate. The master plate itself was screened using the hybridization procedure described earlier; after this secondary screening 22 clones appeared positive and were sequenced. For the fourth library, 20 plates were screened and 69 clones (designated as 301-369) appeared positive and were streaked onto a master plate. All 69 apparently positive clones from this library were sequenced. For the fifth library, 20 plates were screened and 80 colonies appeared positive. In this library, positive colonies were not streaked onto a master plate and no glycerol stocks were made; clones were picked directly off the plate and used to establish

bacterial cultures from which plasmid DNA was isolated. All 80 clones from this library were sequenced.

Sequencing and primer design

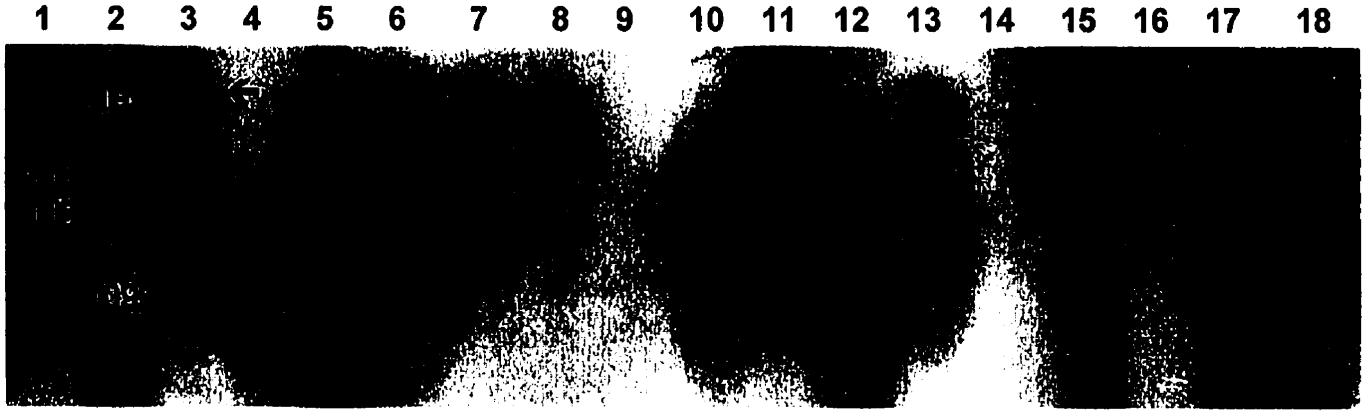
In total, 171 plasmid inserts were sequenced. Of these, a total of 29 (nine from the second and third libraries, eight from the fourth library and 12 from the fifth library) contained suitable microsatellite repeat arrays. Primers were designed for 17 of these putative microsatellite loci using GeneRunner; in other cases primers could not be designed due to a lack of flanking sequence or failure of potential primer sets to meet temperature and interaction requirements.

PCR testing

Amplification of wolverine DNA was attempted using 27 primer sets. Of these, five amplified microsatellite loci appeared variable and readily interpretable (Figure 2); their names, derivations, sequences, amplified repeat arrays, annealing temperatures and labelling conditions are given in Table 1. A list of primers that did not amplify readily interpretable, variable microsatellite loci, and the reasons for their rejection, is given in Table 2.

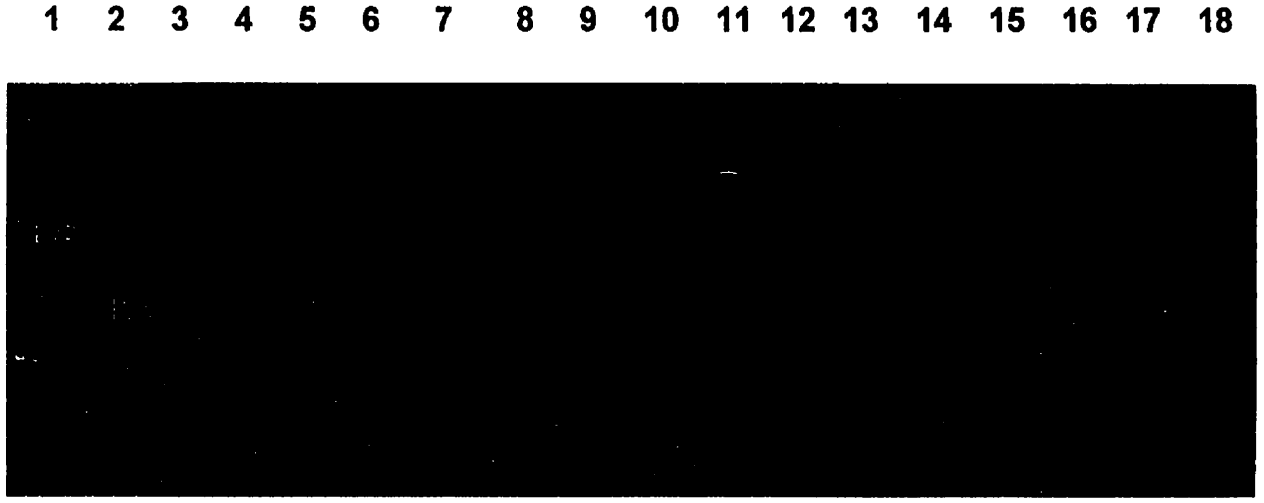
Figure 2. Examples of autoradiographs of microsatellite loci amplified in wolverines. M13 size standards are present for comparison, as indicated. Sizes of some alleles are also indicated.

a. Mvi 57 amplified in wolverines. M13 size standards are present in lanes 1, 2 and 11. Arrows indicate alleles 119 and 113 in lane 3 and alleles 117 and 109 in lane 5.

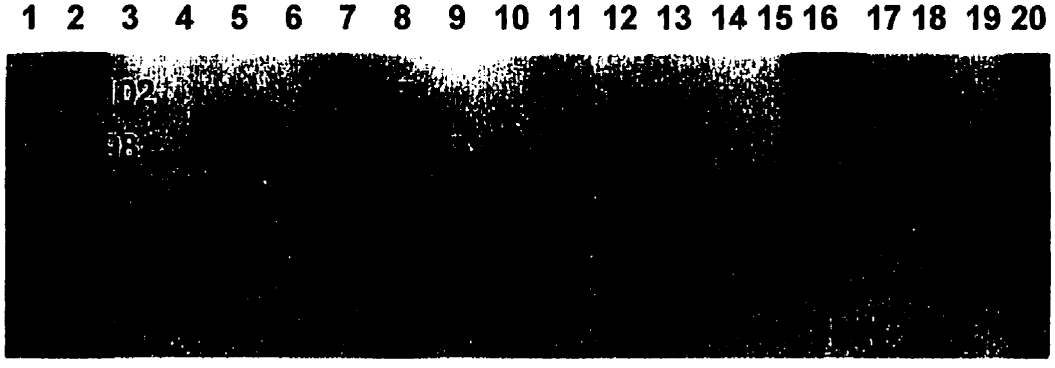


b. Ggu 101B amplified in wolverines. M13 size standards are present in lanes 1, 2 11, 17 and 18. Arrows indicate alleles 152 and 145 in lane 6 and alleles 152 and 143 in lane 8.

c. Ggu 216 amplified in wolverines. M13 size standards are present in lanes 1, 2, 3, 16 and 17. Arrows indicate alleles 180 and 172 in lane 4 and alleles 180 and 174 in lane 7.



d. Ggu 234 amplified in wolverines. M13 size standards are present in lanes 1, 2, 11 and 20. Arrows indicate allele 102 in lane 7 and alleles 98 and 92 in lane 8.



e. Ggu 238 amplified in wolverines. M13 size standards are present in lanes 1, 2, 3, 16 and 17. Arrows indicate alleles 148 and 142 in lane 4 and allele 148 in lane 13.

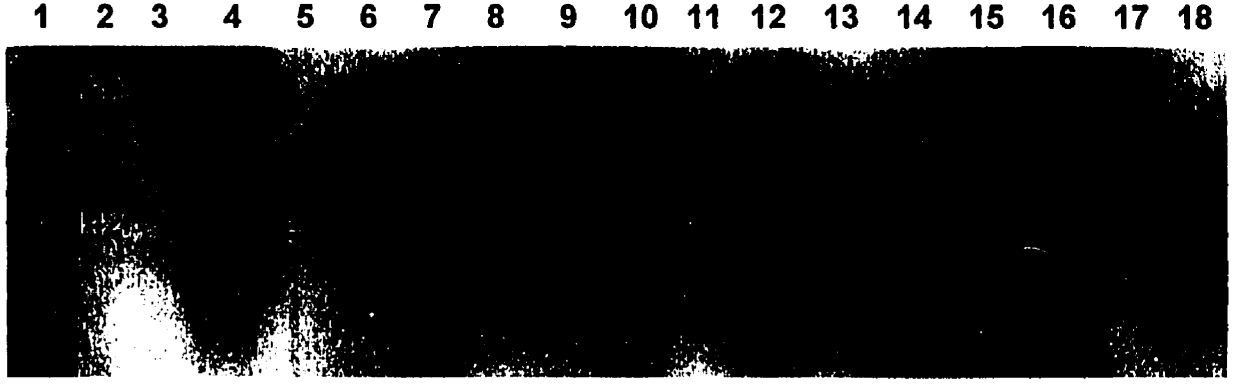


Table 1: Names, derivations, PCR primers, repeat sequences and annealing temperatures for wolverine microsatellite loci. * represents the radiolabelled primer.

Name	Derivation	PCR primers	Repeat sequence	Annealing temperature (°C)
Ggu 101B	Wolverine	GCATTTATTACCTATTTggAg* ggTgTAgAATTgTATTTAAgTg	$(CA)_{22}$	57
Ggu 216	Wolverine	CAAAgACACATCTAATTCAAg TTCCCTTCCATTCTgCTC*	$(GT)_{22}$	49
Ggu 234	Wolverine	TTACTTAgAggATgATAACTTg* gAACTCATAggACTgATAgC	$(CA)_{15}$	53
Ggu 238	Wolverine	TTTgAgAACTgCTgATTTgg ACATATATAggATgAATTTACTC*	$(CA)_{19}$	51
Mvi 57	American mink	gAACAggACCAgCCCTgC gTTggAAATgAggATCTCAC*	$(GT)_{16}$	58

Table 2: Names, derivations and reasons for rejection for rejected primers

Name	Derivation	Reason for rejection
Ggu 3	Wolverine	Unspecific priming
Ggu 78	Wolverine	Amplified two loci; bands not readily interpretable
Ggu 96	Wolverine	Unspecific priming
Ggu 101A	Wolverine	Too close to Ggu 101B on chromosome
Ggu 103	Wolverine	Amplified multiple loci
Ggu 116	Wolverine	Primers were designed on the same strand
Ggu 118	Wolverine	Unspecific priming
Ggu 232	Wolverine	Unspecific priming; bands not readily interpretable
Ggu 278	Wolverine	Unspecific priming
Ggu 316	Wolverine	Amplified two loci; band at expected product size was monomorphic
Ggu 322	Wolverine	Did not amplify
Ggu 327	Wolverine	Did not amplify
Ggu 355	Wolverine	Did not amplify
Mvi 24	American mink	Unspecific priming
Mvi 39	American mink	Did not amplify
Mvi 54	American mink	Unspecific priming
Mvi 87	American mink	Monomorphic
Mvi 111	American mink	Did not amplify
Mvi 114	American mink	Amplified multiple loci
Mvi 219	American mink	Amplified multiple loci
Mvi 232	American mink	Unspecific priming
Quinta	Human	Monomorphic

Determination of genetic variability

Genetic profiles for each sample at the five microsatellite loci used were determined as given in Appendix 1.

The number of alleles at each locus ranged from three (at Ggu 101B) to six (at Ggu 216) (Table 3). Expected heterozygosities within populations at individual loci ranged from 0.330 to 0.703, while overall probabilities of identity ranged from 1.84×10^{-3} to 8.64×10^{-4} (Table 4).

The microsatellite data were tested to determine the presence or absence of genetic disequilibrium. Thirty pairs of loci (ten pairs at each of three populations) were tested for statistical independence using a Markov chain method. After a Bonferroni correction, all locus pairs were found to be statistically independent. When the results from populations were combined using Fisher's method, all locus pairs were found to be independent after a Bonferroni correction.

Table 3: Allele sizes and frequencies

Name	Allele size (bp)	Allele frequencies (n)			
		Northern Norway	South-Central Norway	Sweden	Overall
Mvi 57	109	0.294 (30)	0.100 (4)	0.040 (2)	0.188 (36)
	111	0.059 (6)	0	0.020 (1)	0.036 (7)
	113	0.245 (25)	0.250 (10)	0.240 (12)	0.396 (76)
	117	0.392 (40)	0.500 (20)	0.700 (35)	0.495 (95)
	119	0.010 (1)	0.150 (6)	0	0.036 (7)
Ggu 101B	143	0.146 (19)	0.048 (2)	0.162 (12)	0.134 (33)
	145	0.177 (23)	0.143 (6)	0.081 (6)	0.142 (35)
	152	0.677 (88)	0.810 (34)	0.757 (56)	0.724 (178)
Ggu 216	170	0.016 (2)	0.158 (6)	0	0.033 (8)
	172	0.161 (20)	0.658 (25)	0.200 (16)	0.364 (88)
	174	0.161 (20)	0.053 (2)	0.388 (31)	0.219 (53)
	176	0.073 (9)	0	0.025 (2)	0.045 (11)
	180	0.540 (67)	0.132 (5)	0.388 (31)	0.426 (103)
	182	0.048 (6)	0	0	0.025 (6)
Ggu 234	92	0.637 (79)	0.500 (20)	0.556 (50)	0.587 (149)
	96	0.016 (2)	0	0.044 (4)	0.024 (6)
	98	0.290 (36)	0.275 (11)	0.378 (34)	0.318 (81)
	102	0.056 (7)	0.225 (9)	0.022 (2)	0.071 (18)
Ggu 238	142	0.288 (34)	0.400 (16)	0.542 (39)	0.387 (89)
	146	0	0	0.069 (5)	0.022 (5)
	148	0.669 (79)	0.600 (24)	0.347 (25)	0.557 (128)
	150	0.042 (5)	0	0.042 (3)	0.035 (8)

Table 4: Heterozygosities and probabilities of identity

Locus	Heterozygosity				Probability of identity		
	Northern Norway	South-Central Norway	Sweden	Overall	Northern Norway	South-Central Norway	Sweden
Mvi 57	0.703	0.672	0.460	0.661	0.171	0.150	0.361
Ggu 101B	0.493	0.330	0.400	0.440	0.490	0.311	0.405
Ggu 216	0.652	0.537	0.667	0.705	0.268	0.163	0.186
Ggu 234	0.510	0.640	0.552	0.551	0.212	0.315	0.296
Ggu 238	0.471	0.492	0.588	0.541	0.386	0.360	0.253
Overall	0.566	0.534	0.533	0.580	1.84×10^{-3}	8.64×10^{-4}	2.03×10^{-3}

The microsatellite data were examined for their adherence to Hardy-Weinberg expectations (Table 5). Two loci within the northern Norwegian population did not conform to Hardy-Weinberg expectations (Ggu 101B, P=0.015; Ggu 216, P=0.019). In addition, Ggu 216 did not conform with Hardy-Weinberg expectations across the three populations (P=0.030). The northern Norwegian population showed a significant departure from expectations over all loci (χ^2=20.3, df=10, P=0.027). When a global test was applied to the data from this population, the alternative hypothesis of heterozygote deficit was accepted (P=0.0439, SE=0.0069). All other loci within populations, loci and populations were consistent with Hardy-Weinberg expectations. In particular, the south-central Norwegian population (χ^2=12.8, df=10, P=0.236) and the Swedish population (χ^2=5.5, df=10, P=0.854) were consistent with Hardy-Weinberg expectations. Finally, when the tests on the three populations were combined using Fisher's method, the three populations taken together were found to be consistent with Hardy-Weinberg expectations (χ^2=38.6, df=28, P=0.082).

Table 5: Probabilities that loci within populations conform to Hardy-Weinberg proportions.

Population	Locus					
	Mvi 57	Ggu 101B	Ggu 216	Ggu 234	Ggu 238	All loci
Northern Norway	0.619	0.015	0.019	0.837	0.265	0.027
South-Central Norway	0.239	0.559	0.078	0.416	0.385	0.236
Sweden	1	0.414	0.618	0.471	0.525	0.854
All populations	0.701	0.080	0.030	0.728	0.440	0.088

Examination of population differentiation

The results of F-statistic estimation showed that there is evidence of inbreeding and population differentiation for these three populations. There was evidence that there was inbreeding in the population as a whole (F=0.062, σ=0.035, $P_{(F_{IT} \leq 0)}$=0.015). Such inbreeding can be the result of inbreeding within individual populations (as estimated by F_{IS}) or of the differentiation of the individual populations (as estimated by F_{ST}). The estimation of F_{IS} showed that there was no evidence of inbreeding within the three populations (f=-0.014, σ=0.032, $P_{(F_{IS} \leq 0)}$=0.647). However, there was evidence that there was significant population differentiation (Table 6). After a Bonferroni correction, the null hypothesis that there was no population differentiation (ie $F_{ST} \leq 0$) was rejected at the adjusted significance level (0.01) at three loci (Mvi 57, Ggu 238 and Ggu216). Over all loci, there was also evidence for significant population differentiation (θ=0.075, σ=0.027, $P_{(F_{ST} \leq 0)}$<0.001).

Table 6: F_{ST} (estimated by θ) by loci within populations. * indicates significant deviation from $F_{ST}=0$

Locus	θ	Standard deviation
Mvi 57	0.083*	0.044
Ggu 101B	0.008	0.005
Ggu 216	0.079*	0.132
Ggu 234	0.006	0.016
Ggu 238	0.131*	0.076

R_{ST} was determined for individual loci (Table 7), between populations and over the total population. The unbiased R_{ST} values for the three populations, measured by averaging over variance components, were: 0.183 between the northern Norwegian and south-central Norwegian populations; 0.088 between the northern Norwegian and Swedish populations; 0.082 between the south-central Norwegian and Swedish populations; and 0.113 (SE=0.002, P=0.000) for the total population. The probability value associated with the final test indicates significant population differentiation.

Both Nei's genetic distance and the $(\delta\mu)^2$ genetic distance were calculated for all three populations. Nei's genetic distance for the three populations was as follows: 0.130 (SE=0.106) between the northern Norwegian and south-central Norwegian populations; 0.089 (SE=0.037) between the northern Norwegian and Swedish populations; and 0.127 (SE=0.090) between the south-central Norwegian and Swedish populations. The $(\delta\mu)^2$ genetic distance for the three populations was 1.373 (SE=0.837) between the northern Norwegian and south-central Norwegian populations; 0.511 (SE=0.417) between the northern Norwegian and Swedish populations; and 0.461 (SE=0.210) between the south-central Norwegian and Swedish populations.

Table 7: RST by loci within populations.

Locus	RST
Mvi 57	0.179
Ggu 101B	0.010
Ggu 216	0.271
Ggu 234	0.019
Ggu 238	0.079

The number of genetic migrants per generation (N_m) was calculated by three different methods. The private alleles method (Slatkin 1985) estimated that N_m, adjusted for population size over the three populations, was 1.39. The unbiased RST method estimated that N_m was 1.31. Finally, calculation from FST estimated that N_m was 3.08.

A final method of population differentiation measurement, the assignment test, was able to assign individuals to their correct populations approximately 65% of the time (Table 5). Considering only those individuals for which a full genotype was available, the assignment test correctly assigned 13/14 individuals in the south-central Norwegian population, 27/43 individuals in the northern Norwegian population, and 9/18 individuals in the Swedish population.

Amplification in other species

The four wolverine-derived primer sets were tested in several other carnivore species. Only Ggu 234 amplified in other species; it amplified two alleles in pine marten and one in each of otter, mink and dog.

Table 8: Assignment test results.

Source population	Assigned population Northern Norway	South-Central Norway	Sweden
Northern Norway	27	6	10
South-Central Norway	1	13	0
Sweden	9	0	9

Discussion

Importance of research

This work is one of the first genetic studies of the wolverine, one of the least known of all carnivores. The study characterized genetic markers that can be used in future studies of wolverines and provides baseline genetic data for future examinations of Scandinavian wolverine populations.

Genetic variability

Five variable single-locus microsatellite loci were found in wolverine, despite the construction of five genomic libraries and testing of 27 primer sets. The overall low number of microsatellite loci available reduced the amount of genetic data on which the estimates of genetic variability and population differentiation were calculated. Since data on the number of primer sets tested and primer test success rates are not generally published, the low success rate here cannot be quantitatively compared with the results of other investigators. However, it is now known that it is technically difficult to obtain variable microsatellites that amplify consistently in mustelids (J.F. Dallas, University of Aberdeen; A. Davidson, University of Leeds, pers. comm.). This is partially due to the frequent association of poly-A tracts with microsatellites and to the frequently short length of mustelid microsatellite repeat arrays (O'Connell *et al.* 1996).

Full genotypes could not be obtained for all samples despite at least two PCR attempts at every sample for every locus. This could be due to factors in the PCR process, or it may be due to the conditions of the samples themselves. All data collected from the samples were included for the purposes of data analysis, except for the assignment test, when only those individuals for whom full genotypes were obtained were included.

The number of alleles at the five loci used to examine the wolverine populations was low compared to other similar studies. The number of alleles at each locus ranged from three (at Ggu 101B) to six (at Ggu 216). Other large carnivores showed more overall allelic variety. At eight microsatellite loci, polar bears had from four to nine alleles (Paetkau *et al.* 1995). Grizzly bears examined at eight microsatellite loci showed six to ten alleles at each locus (Craighead *et al.* 1995), while black bears had six to 14 alleles at each of four microsatellite loci (Paetkau and Strobeck 1994). Grey seals had six to 11 alleles at each of eight microsatellite loci (Allen *et al.* 1995).

The levels of expected heterozygosity found at wolverine microsatellite loci were slightly lower than those seen at microsatellite loci in other mammals. The population heterozygosity levels ranged from 0.533 to 0.566, and the overall expected heterozygosity was 0.580. By comparison, polar bear populations showed average expected heterozygosities of 0.610 to 0.643 (Paetkau *et al.* 1995), black bear average expected heterozygosities ranged from 0.360 to 0.801 (Paetkau

and Strobeck 1994), and grey seal average expected heterozygosities for two populations were 0.740 and 0.751 (Allen *et al.* 1995). Lower expected heterozygosities were seen at microsatellite loci in the harbour seal, with values of 0.00-0.47 at 7 loci (Coltman *et al.* 1996).

Examination of the microsatellite data showed significant deviations from Hardy-Weinberg expectations at several levels. These results may indicate inbreeding or there may be technical or molecular explanations.

When data from all loci were combined, the northern Norwegian population was shown to deviate significantly from Hardy-Weinberg expectations. This result may actually indicate that the conditions for the Hardy-Weinberg situation (*i.e.* no drift, no migration, no selection, no mutation and random breeding) are not satisfied. A second possibility is that this result is due to a Wahlund effect – that is, subdivision of the population increased the homozygosity of the sample "population", changing proportions of genotypes such that Hardy-Weinberg expectations were not met. However, given that only two of the five loci deviated from Hardy-Weinberg expectations for this population, and that changes from Hardy-Weinberg conditions or Wahlund effects would likely be expected to affect all loci, these possibilities probably do not explain the deviations. Instead, single-locus factors must be examined. It is possible that selection on the loci in question resulted in deviations from Hardy-Weinberg expectations, but unlikely given that microsatellite loci are thought to

be selectively neutral. The most likely explanation for these results is the presence of null alleles. Null alleles are alleles which fail to amplify under normal PCR conditions for a locus, usually due to point mutations in primer sites. When scored, an individual with a single null allele will appear homozygous. When null alleles occur frequently, an apparent excess of homozygous genotypes will arise. However, the presence of null alleles cannot be conclusively proved without investigating allele inheritance in pedigrees. Null alleles have been proposed as being responsible for deviations from Hardy-Weinberg expectations in microsatellite loci in other species e.g. grey seals (Allen *et al.* 1995).

The significant deviation of Ggu 216 from Hardy-Weinberg expectations is most likely due to technical, rather than biological reasons. First, it is possible that Ggu 216 showed large allele dropout—that is, a large number of null alleles due to the difficulty in amplifying such a large locus (O'Connell and Wright 1997). Second, Ggu 216 had many alleles and was difficult to score in many cases; such mis-scoring may have resulted in an excess of homozygotes.

The probability of identity results show that identification of individual wolverines would be difficult using these five loci alone. The chance that two individuals drawn at random from a population would have identical genotypes at all loci ranged from approximately 1/490 in the south-central Norwegian population to 1/1150 in the Swedish population.

In short, while the ability to test these populations with additional loci would have been valuable, the results presented here show that levels of genetic variability in these three populations of wolverine are relatively low. This result is not surprising, given that wolverine numbers are only now recovering after a long period of decline. A comparison of genetic diversity levels in present-day Scandinavian wolverines to those in archival Scandinavian samples and to those in North American wolverines would place the results presented here in greater context. In particular, it would be valuable to know whether levels of genetic diversity in Scandinavian wolverines have declined over time, since declines in levels of genetic diversity are thought to be associated with inbreeding depression and reduced population fitness (Avise 1994). In addition, monitoring of these populations should continue, to ensure that levels of genetic diversity do not decline. The south-central Norwegian population should be a particular target for study, given its recent provenance and the potential for founder effects.

Population differentiation

Two main models of mutation, the infinite alleles model and the stepwise mutation model, have been used to develop measures of population differentiation. Infinite alleles model measures tend to give more conservative results (*i.e.* indicate less population differentiation) than stepwise mutation models (Allen *et al.* 1995, O'Connell and Wright 1997). However, microsatellite

data do not conform exactly to either model's predictions. While the use of infinite allele model statistics has been advised for fisheries data (O'Connell and Wright 1997), stepwise mutation model measures were found to be preferable in a large carnivore, the grey seal (Allen *et al.* 1995). In this study, both types of measure were calculated.

Both the infinite alleles model and stepwise mutation model measures of population differentiation indicate that there is significant differentiation among the three populations examined. In biological terms, this suggests that the populations are separate for breeding purposes, with a limited amount of gene flow among them. The two measures show somewhat different extents of differentiation, notably at the level of individual loci.

The infinite alleles model measure of differentiation, F_{ST}, shows population differentiation over all loci (θ=0.075) and at three of the five individual loci (Mvi 57, θ=0.083; Ggu 216, θ=0.079; Ggu 238, θ=0.131). The stepwise mutation model measure, R_{ST}, also shows significant differentiation over all loci, but shows a higher level of differentiation than F_{ST} (R_{ST} =0.113). While R_{ST} values for individual loci were not tested for significance, once again Mvi 57 (R_{ST} =0.179), Ggu 216 (R_{ST} =0.271) and Ggu 238 (R_{ST} =0.079) show much higher levels of differentiation than the other two loci. Thus, R_{ST} shows higher levels of differentiation than F_{ST}. The differences between the two measures can be seen more clearly when expressed in terms of numbers of genetic migrants.

Calculation of the number of genetic migrants from FST indicates that N_m=3.08 while RST indicates that N_m=1.31.

The finding that there is significant population differentiation is somewhat surprising, given that wolverines are capable of long-distance movements and that the populations along the Norway-Sweden border seem to form a continuous distribution. However, both polar bears (Paetkau *et al.* 1995) and grey seals (Allen *et al.* 1995) showed significant population differentiation, and these animals are also capable of long-distance movements. These data confirm field observations that wolverines are highly philopatric for mating purposes. A mitochondrial DNA (mtDNA) study of these same populations would give additional information on the mating strategies of wolverines, especially given that males disperse much further from the natal den than do females.

The two measures of genetic distance used provide strikingly different results. According to Nei's standard genetic distance which assumes the infinite allele model, the northern Norwegian and Swedish populations were the least distant (D=0.089), while the northern and south-central Norwegian populations (D=0.130) and the south-central Norwegian and Swedish populations (D=0.127) were approximately equally distant from each other. According to the $(\delta\mu)^2$ genetic distance, the most closely related populations were the south-central Norwegian and Swedish populations ($(\delta\mu)^2$=0.461), while the northern and

south-central Norwegian populations are much more distantly related ($(\delta\mu)^2$=1.373).

The assignment test was developed as an alternative to other measures whose biological meaning is difficult to grasp (Paetkau et al. 1995) and indicates whether individual genotypes are characteristic of populations. This test indicated that the Swedish and south-central Norwegian populations are relatively similar. The only individual from the south-central Norwegian population assigned to another population was assigned to the Swedish population, while individuals from the Swedish population were assigned equally to the south-central Norwegian and Swedish populations.

Management implications

The long-term survival of the wolverine in Scandinavia depends on the provision of suitable habitat, the reduction of human interference and the maintenance of genetic diversity. While the situation has clearly improved since the 1960s and 1970s, there is still cause for concern.

Given the large habitat needs of large carnivores like the wolverine and the continuing encroachment of human activity on wilderness areas, provision of adequate habitat for wolverines is extremely difficult. In Norway, core conservation areas for wolverine have been established in northern and south-central Norway (Landa *et al.* 1997). However, these areas are not large enough to

support large numbers of wolverines (Landa *et al.* 1997). The setting aside of larger areas as wolverine refugia, on both public and private lands, would increase the wolverine's chances for long-term survival. However, even such actions may be inadequate if human interference with wolverines is not reduced.

The conflict between wolverines and animal husbandry lies at the root of the wolverine's difficulties in Scandinavia. While measures such as legal protection of the wolverine and the introduction of compensation payments to farmers have improved the situation to some extent, both legal and illegal hunting reduce the wolverine's numbers (Landa and Tømmerås 1995) and long-term viability. In addition, as core conservation areas are introduced, it is possible that tolerance of wolverines outside these areas will diminish, increasing the likelihood of illegal killing (Landa *et al.* 1997). While repellents (Landa and Tømmerås 1995) and improved methods of animal husbandry (Linnell *et al.* 1996) can reduce wolverine depredation, they are expensive and unlikely to be deployed in large areas (Landa *et al.* 1997).

This work provides a baseline for our knowledge of the population genetics of Scandinavian wolverines, but further study will be required before appropriate genetic management plans can be drawn up. The results of this study do, however, indicate directions for further research.

Wolverine genetic diversity is low compared to that of other large carnivores, but the consequences of this are difficult to determine. While it is

generally thought that low genetic variability reduces fitness (Frankham 1995), it has been argued that there is no evidence for this in wild populations (Caro and Laurenson 1994). In order to determine whether current levels of genetic diversity in the wolverine are likely to prove problematic, a time-based study of archival wolverine samples and the samples examined here would be useful. Such a study would show whether the sample size used in this study was adequate, and whether wolverine genetic diversity has increased, decreased or remained stable over the recent past. An examination of genetic diversity in North American wolverines would also be useful in this respect. Continued monitoring of genetic diversity in Scandinavian populations would be useful, especially if transfers among populations or from captive-breeding stock are being considered. Such transfers can improve reproductive fitness, but can occasionally result in outbreeding depression (Frankham 1995).

While lack of habitat and human interference are the most immediate problems facing the wolverine, the possible implications of low genetic diversity should be considered in any long-term management plan. Mitochondrial DNA based studies will provide further information on diversity trends and mating and dispersal strategies. Application of microsatellite typing to captive-breeding programs will help to ensure the genetic diversity of breeding stock, while microsatellite typing of other populations will put the results of this study in context. Further monitoring of the populations examined here will indicate

whether intervention is necessary. In conclusion, the continued application of genetic testing should allow not only enhance our knowledge of the wolverine, but also a greater ability to ensure its long-term survival.

Appendix 1

Genotypes of all individuals tested at the five microsatellite loci used in this study

Sample	Mvi 57		Ggu101B		Ggu 216	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
south-central Norway						
171	117	109	152	145	172	172
178	117	113	152	152	180	172
180	117	113	152	152		
191	119	113	152	152	172	170
714	117	117	152	152	172	172
715	117	113	152	143		
716	119	117	152	152	172	172
717	117	113	152	152	172	170
718	119	117	152	152	172	172
719	117	117	152	152	172	172
720	117	117	145	145		
722	117	117	152	152	170	170
725			152	152	180	172
744					180	170
745	117	109			172	172
746			152	143	180	172
754	117	113	152	152	172	172
756	119	117	152	145	174	174
758	119	113	152	145	172	172
759	117	113	152	145	172	172
803	117	113	152	152		
805	109	109	152	152	180	172
900	119	113	152	152	172	170
northern Norway						
124	111	109	152	145	180	174
146	113	109	152	143	180	180
170	119	113	152	152	180	174
177	113	109	152	152		
182	117	109	152	145	180	172
184	117	113	152	145		
187	117	117	152	152	180	172
189	117	117	152	145	172	172

190			152	152		
197	113	113	152	143	180	172
203	113	111	145	145	180	172
204	117	111	152	152	180	172
205			152	152	180	172
206	117	117	152	145	172	172
250	113	113	152	152	180	172
306	117	113	152	143	180	172
322	117	109	152	145	180	172
353	117	113	152	145	176	176
356	117	113	152	143	180	170
357	109	109	152	152	180	180
360	117	117	152	145	180	180
361	113	109	152	145	180	172
394	117	111	152	143	180	170
467	113	109	145	145	180	172
486			152	145		
524	113	109	152	143	180	180
547	117	111	152	145	180	174
560	113	109	152	152	180	180
594	111	109	152	145	180	176
603	117	113	143	143	180	174
670	109	109	152	145	180	176
700	109	109	152	152	180	172
701	117	117	152	152	180	174
702	117	109	152	152	180	180
703	117	117	152	152	180	180
704	117	117	152	145	180	174
705	113	109	143	143	182	180
706			152	152	180	174
707	117	109	145	145	180	172
708	113	109	152	152	180	174
709	117	109	152	152	180	172
710	117	109	152	145	182	180
711	117	109	152	152	180	180
712			152	152	180	174
713			152	152	180	180
723	117	117	152	152	176	174
724			152	152	182	180
726			152	152	180	174
727			152	143	180	174
728			152	152	180	180
729	117	113	152	152	180	174

731	109	109	152	152	182	180
732	117	117	152	145	174	174
743	113	109	152	143	176	176
743n			143	143	180	172
747			152	152	180	174
748	117	117	152	152	182	180
749	109	109	152	152	180	174
750	117	113	152	143	180	174
751	117	109	152	152	180	180
752	113	109	152	143	174	174
753			152	145	182	180
793	117	113			180	172
796	117	113	152	143		
800			152	152	180	174
801						
804			143	143	180	176
806					180	180
Sweden						
8907	117	117	152	145	180	172
9309	117	117	145	143	180	174
9310	117	117	152	152	180	174
9312			152	143	174	174
9314			152	152	174	172
9315	117	113	152	152	180	172
9316	117	113	152	152	174	172
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9424			152	152	180	174
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9536	117	117	152	145	174	174

9537			152	152		
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9539	117	117	152	152		
9541			152	152	180	180
9542			152	152	174	174
9643	117	111	152	152	180	174
9644			152	152	180	174
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9647	117	109			180	174
9648			152	152		
9649					174	172
9650						
9651					174	174
9652	117	113	152	152	180	174
9653			152	145	180	174
9654						
9655						
9656			152	152	174	172
9657					174	172
9658			152	143	172	172
359					180	174

Sample	Ggu 234		Ggu 238	
	Allele 1	Allele 2	Allele 1	Allele 2
south-central Norway				
171	102	92	148	142
178	98	92	148	148
180	102	98	142	142
191	102	92	148	142
714	98	92	148	148
715				
716	102	92	148	142
717	98	98	148	142
718	98	92	148	142
719	98	92	142	142
720	92	92	148	142
722	92	92	148	142
725	98	92	148	148
744				
745			148	142
746	92	92	148	148
754	102	92	148	142

756	102	102		
758	102	92	148	142
759	102	92	148	148
803	98	92	148	142
805	98	92	148	142
900	98	92	148	148
northern Norway				
124	98	98	150	148
146	92	92	142	142
170	92	92	148	148
177	92	92	148	148
182	98	92	148	148
184	92	92	148	148
187	102	92	148	148
189	98	92	148	148
190			148	142
197	92	92	148	142
203	98	92	148	142
204	98	92	148	142
205	92	92	148	148
206	92	92	148	148
250	102	92	148	142
306	98	98	150	148
322	98	92	150	148
353	92	92	148	148
356	98	92	142	142
357	92	92	150	148
360	98	92	148	148
361			148	148
394	98	92	148	142
467	98	92	148	142
486				
524	92	92	148	142
547	98	92	148	148
560	102	92	142	142
594	102	98	148	148
603	98	96	142	142
670	102	98	148	148
700	98	92	142	142
701	96	92	148	142
702			148	142
703	98	92	148	142
704	98	92	148	142

705	92	92	148	142
706			148	148
707	102	98	148	142
708	92	92	148	142
709	98	92	148	148
710	98	92	148	142
711	92	92	148	148
712	92	92		
713	92	92	148	148
723	92	92		
724	98	92		
726	98	92	148	148
727	92	92	148	148
728	98	92	148	148
729	92	92	148	142
731	92	92	148	148
732	92	92	148	148
743	92	92	148	142
743n	98	92	148	142
747			142	142
748	98	98		
749	98	92	148	148
750	102	92	148	148
751	98	98	148	148
752	92	92	150	148
753	98	92	148	142
793	98	98		
796	98	92	142	142
800	92	92	148	148
801	92	92		
804	92	92		
806	92	92		
Sweden				
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9309	98	92	142	142
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9312	98	92	148	142
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9526	92	92	148	142
9527	98	96	148	142
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9530	98	92	148	142
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9644	98	92	146	146
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9647	92	92	148	142
9648	96	92		
9649	92	92	148	142
9650	92	92		
9651	102	92		
9652	98	98	142	142
9653	102	98		
9654				
9655	92	92	150	148
9656	98	92		
9657	92	92	148	148
9658	98	92		
359	92	92		

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IMAGE EVALUATION
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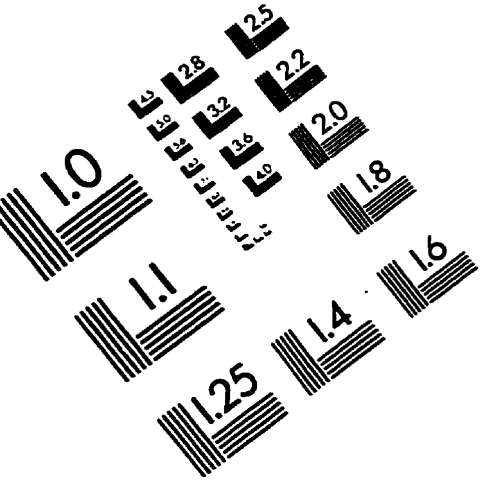

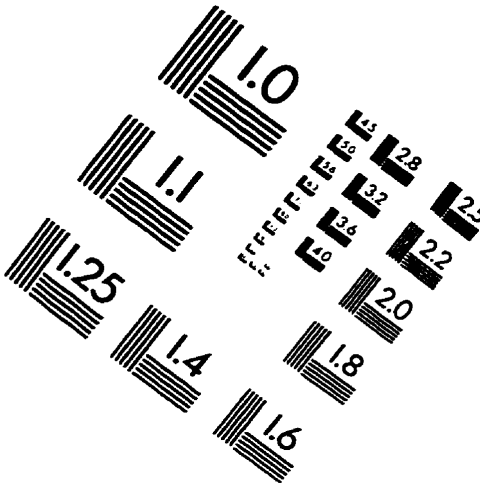

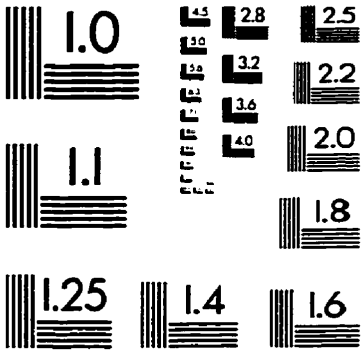

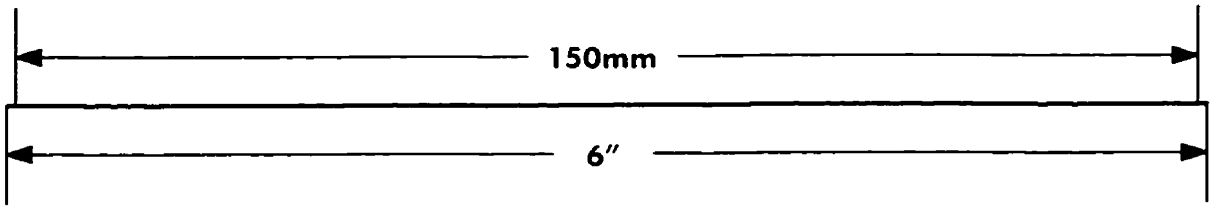

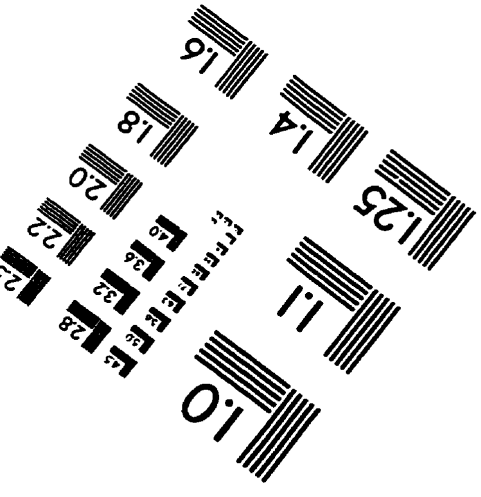

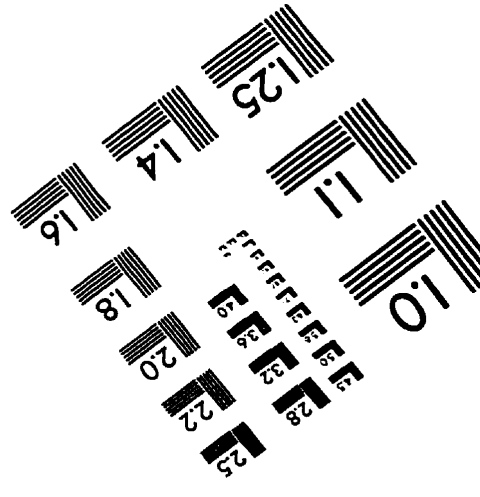

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989