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Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline

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Abstract

The grey wolf (*Canis lupus*) was numerous on the Scandinavian peninsula in the early 19th century. However, as a result of intense persecution, the population declined dramatically and was virtually extinct from the peninsula by the 1960s. We examined historical patterns of genetic variability throughout the period of decline, from 1829 to 1979. Contemporary Finnish wolves, considered to be representative of a large eastern wolf population, were used for comparison. Mitochondrial DNA (mtDNA) variability among historical Scandinavian wolves was significantly lower than in Finland while Y chromosome variability was comparable between the two populations. This may suggest that long-distance migration from the east has been male-biased. Importantly though, as the historical population was significantly differentiated from contemporary Finnish wolves, the overall immigration rate to the Scandinavian peninsula appears to have been low. Levels of variability at autosomal microsatellite loci were high by the early 1800s but declined considerably towards the mid-20th century. At this time, approximately 40% of the allelic diversity and 30% of the heterozygosity had been lost. After 1940, however, there is evidence of several immigration events, coinciding with episodes of marked population increase in Russian Karelia and subsequent westwards migration.

Keywords: *Canis lupus*, genetic drift, immigration, loss of genetic variability, microsatellites, mitochondrial DNA, population decline

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Introduction

Interactions between extrinsic factors, such as environmental stochasticity, and intrinsic factors, like social behaviour, reproductive strategy and migration patterns, are important in determining the demography of populations. However, human interference has increasingly skewed the balance between populations and their environment in many areas. Over the last two centuries, human persecution has led to markedly reduced population sizes of many species and also to a considerable number of extinctions (Leakey & Lewin 1995). Large carnivores, among them the grey wolf (*Canis lupus*), have been particularly affected (Ewer 1973). As a result of habitat fragmentation and hunting over the last two centuries, the wolf has vanished from much of its former geographical range in Western Europe (Mech 1970). Also in Eastern Europe, predator removal programmes had a strong impact on the density of wolves from the 1800s until the mid-20th century (Pulliainen 1980; Danilov *et al.* 1985). A similar pattern is evident in North America (Mech 1970).

Low population density, together with maintained hunting pressure, may affect wolves in various ways. For example, the ability to form and maintain stable social

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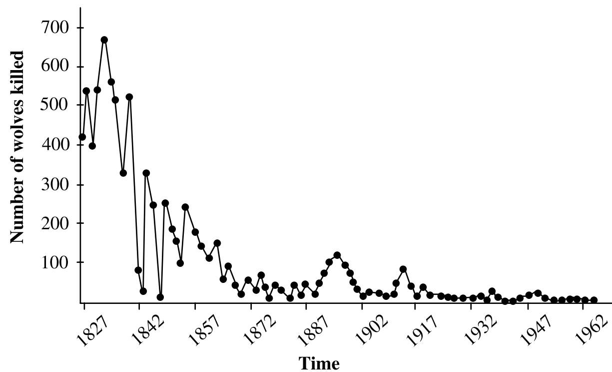

Fig. 1 Wolf hunting statistics for Sweden from the early 1800s until the species was legally protected in 1966 (redrawn from Persson & Sand 1998).

groups is strongly reduced. This may influence juvenile survival, which in turn affects reproductive potential (Mech 1970). Moreover, low population density and disruption of packs may increase the probability of hybridization with dogs (Boitani 1983; Vilà *et al*. 2003a). Small populations are also prone to loss of genetic variability, which in turn may lead to reduced individual fitness in cases of close breeding (Hedrick & Kalinowski 2000; Keller & Waller 2002). However, immigration may strongly counteract these negative effects of small population size (Ingvarsson 2001). Importantly, empirical data suggest that the immigration rate may increase in cases where the density of wolves in the recipient population is low (Pulliainen 1980; Ballard *et al*. 1987). The existence of large neighbouring populations may thus be of considerable importance to restore small wolf populations on the verge of extinction.

As in the rest of Western Europe, the Scandinavian wolf population declined dramatically during the 19th century. An abrupt reduction around 1840 was followed by continued decline for almost 150 years (Fig. 1). When the population became legally protected in 1966 (Sweden) and 1973 (Norway), the species was considered functionally extinct from the Scandinavian peninsula (Wabakken *et al*. 2001). However, in the early 1980s, a few wolves were unexpectedly discovered in southern Sweden, more than 900 km from the nearest known occurrence in Finland and Russia. The first breeding event was recorded in 1983 and today the population is estimated to number approximately 100 individuals (Aronson *et al*. 2002).

The contemporary Scandinavian wolf population is likely to originate from a very limited number of eastern immigrants (Vilà *et al*. 2003b; Ingvarsson 2002). This indicates that the extant population may be severely inbred and that further immigration may be important to ensure its continued persistence (Ellegren 1999). An assessment of the degree of isolation experienced by the historical population may contribute to an understanding of the underlying mechanisms associated with gene flow from eastern populations.

In this study, we use mitochondrial (mt) DNA sequencing, Y chromosome haplotyping and autosomal microsatellite genotyping to examine historical patterns of genetic variability and migration during the long-term population decline of the Scandinavian wolf population. Wolf teeth were obtained from museum specimens and used as the source of DNA. Possible immigration from the east is discussed in relation to the observed variability patterns. Specifically, we test whether there is a correlation between population decline and levels of genetic variability.

Materials and methods

Sampling

Samples from the historical Scandinavian population as well as from the contemporary Finnish population were used. The historical material, comprising 55 teeth and two tissue samples (Appendix I), represented a time span of almost 150 years. They were divided into three temporal groups 1829–1889, 1890–1939 and 1940–1980, each group comprising 18, 19 and 18 samples, respectively. The contemporary Finnish population was represented by 22 tissue samples.

DNA extraction and authenticity of DNA templates from old specimens

Each tooth sample was sanded off with a separate piece of autoclaved sandpaper to eliminate possible surface contaminants and subsequently drilled with a Dremel tool (Dremel®) to obtain small fragments from inside the tooth. To avoid heating and potential DNA degradation, the tip of the drill was never in contact with the tooth for more than one second at any time. Fragments of teeth stained with blood from the pulp were considered as particularly promising sources of DNA. The drilling process continued until about 20–50 mg of blood-stained tooth fragments had been obtained.

DNA was extracted employing the Isoquick DNA extraction kit (Orca Research Inc.), following a modification of the manufacturer's protocol. A 100-μL aliquot of a buffer containing thiocyanate (Isoquick sample buffer A) was added to the tooth fragments and the samples were incubated for 15 min at room temperature. The tubes were inverted several times to increase the contact surface between the liquid and the tooth fragments. A 100-μL aliquot of lysis buffer (Isoquick sample buffer 1) was subsequently added and incubation at room temperature for 45 min was again accompanied by frequent tube inversion. The next steps of the extraction procedure were performed according to the manufacturer's protocol. DNA was precipitated with isopropanol at −20 °C overnight and recovered by centrifugation at 16 060 *g* for 45 min.

Finally, pellets were resuspended in 75 µL of RNAse-free water.

The authenticity of DNA from old specimens is sometimes questioned because contaminating DNA from recent samples and polymerase chain reaction (PCR) products readily out-compete low copy number and potentially degraded templates recovered from old sources of DNA. A critical concern in our study was the possibility of accidental carryover of mtDNA sequences previously amplified in our laboratory for recent wolf samples. To minimize the risk of contamination, DNA extraction and PCR set-up were undertaken in a spatially separated room dedicated to low-copy DNA research, which is located as far as possible away from any source of PCR products. A maximum of eight samples were handled simultaneously during DNA extraction. Two extraction blanks were included in each round of extraction and separate PCR blanks were used in all amplifications. All genotyping experiments were independently replicated in at least two replicates and extraction and mtDNA sequencing were replicated by different researchers for some of the samples.

DNA from tissue samples was extracted by a standard phenol : chloroform protocol (Sambrook *et al*. 1989).

mtDNA sequencing

All tooth extracts were amplified for 229 base pairs (bp) of the mtDNA control region, using the canid-specific primers LS1 5′ TGTGCTATGTCAGTATCTCCAGG-3′ and HS1-NC 5′ GCAAGGGTTGATGGTTTCTC-3′. Primers are located 101 bp (LS1) and 330 bp (HS1-NC) downstream of the start of the control region, respectively. Amplifications were performed in 50-µL reactions containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, 2.5 µg of bovine serum albumin, 1.25 units HotStar DNA polymerase (QIAGEN GmbH) and 5 µL of template. Forty-two cycles of amplification with 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C were preceded by a 15-min pre-denaturation step at 95 °C and were followed by an additional 10-min extension step at 72 °C. Tissue extracts were amplified and sequenced according to Vilà *et al*. (1999).

PCR products were purified using a PCR purification kit (Qiagen). Sequencing was performed using BigDye terminator cycle sequencing chemistry on an ABI 377 instrument (Applied Biosystems) following the protocol provided by the manufacturer.

Microsatellite genotyping

We used 15 microsatellite loci comprising six dinucleotide [*UCB109*, *UCB173*, *UCB213*, *UCB225*, *UCB250* and *UCB253*; Ostrander *et al*. (1993)], one tri-nucleotide (*PEZ03*; Perkin Elmer, Zoogen; see dog genome map at www.fhcrc.org/science/dog_genome/dog.html), seven tetranucleotide [*FHCR2001*, *FHCR2054*, *FHCR2088*, *FHCR2096*, *FHCR2159*; Francisco *et al*. (1996) and *PEZ01* and *PEZ05*; Perkin Elmer, Zoogen], and one hexanucleotide [*vWF*; Shibuya *et al*. (1994)] repeat loci. Amplification of the tooth extracts was performed in 10-µL reactions containing 1.5 $MgCl_2$, 0.2 mM of each dNTP, 3.2 pmol of each primer, 0.45 units of HotStar DNA polymerase (Qiagen) and 2 µL of template. PCR profiles were the same as for the mtDNA amplification except for the number of cycles, which in this case was 37, and the annealing temperature that was 52 °C for the tri-, tetra- and hexanucleotide and 55 °C for the dinucleotide repeat loci. For samples that proved difficult to amplify, the $MgCl_2$ concentration was increased to 3.0 or 4.0 mM. All amplifications were performed at least twice. An additional amplification was performed when a sample appeared homozygous after two replicates. Tissue extracts were amplified under identical conditions as the tooth extracts except for the number of cycles, which in this case was 32. All PCR products were separated in 5% Long Ranger polyacrylamide gels on an ABI 377 instrument and gel analysis was performed using the software packages GENESCAN 3.1 and GENOTYPER 2.1 (Applied Biosystems).

Y chromosome haplotyping

Four Y chromosome-specific microsatellite markers [MS34A, MS34B, MS41A, and MS41B; Sundqvist *et al*. (2001)] were applied. Using the original primers, these four markers give rather long PCR products [172–228 bp, cf. Sundqvist *et al*. (2001)] and are not likely to give reliable amplification products for DNA from old sources that may be highly degraded. Therefore, we redesigned the reverse primers to obtain shorter products. The new primer sequences were 5′-TCATTCGTGAATGTGAACACATGGATGC-3′ for MS34 and 5′-GAAGTGAGACCCTTTACCC-3′ for MS41, which gave amplification products of 108–118 and 88–104, respectively. Amplification conditions for the tooth extracts were the same as for autosomal microsatellites except for an increased amount of $MgCl_2$ to 4.0 mM and of primers to 5 pmol for MS34, and an increased $MgCl_2$ concentration to 3.0 mM for MS41. The PCR profile was also identical to autosomal profiles for MS34 except for the number of cycles, which in this case was 40, and the annealing temperature, which was 68 °C. The profile for MS41 included an initial denaturation step of 95 °C for 15 min, 11 touch-down cycles with 94 °C for 30 s, 58 °C for 30 s decreasing 0.5 °C each cycle, and 72 °C for 1 min followed by 33 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min. An extension step of 72 °C for 10 min was added at the end. Tissue extracts were amplified as described in Sundqvist *et al*. (2001) using the original primers. As for autosomal microsatellites, PCR products were run on an ABI 377 instrument and the subsequent determination of allele lengths was performed with GENESCAN and GENOTYPER (Applied Biosystems).

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Data analysis

The mtDNA and Y chromosome haplotypes were determined and compared to previously published data for northern European populations (Sundqvist *et al*. 2001). Haplotype diversity (Nei 1987) was calculated using DNASP 3.0 (Rozas & Rozas 1999).

Since DNA extracted from old teeth may be dilute and of poor quality, a measure of the genotyping reliability was required. The most common scoring error associated with amplifications from low concentration DNA is allelic dropout, where one allele at a heterozygous locus fails to amplify (Taberlet *et al*. 1996). A heterozygous individual may thus be misinterpreted as being homozygous at that particular locus. Allelic dropout was detected in cases where at least one replicate showed a heterozygous pattern while the others were homozygous. The detected incidence of allelic dropout was used to obtain an estimate of the probability of false homozygotes after n replicates, i.e. cases where all independent replicates would show allelic dropout for the same allele. We used the estimate suggested by Gagneux *et al*. (1997); $P_{(\text{false homozygote})} = (K) \times (K/2)^{n-1}$, where K is the observed frequency of detectable allelic dropouts averaged over all heterozygous individuals and loci. This estimate assumes that allelic dropouts are randomly distributed across individuals and loci (cf. Gagneux *et al*. 1997), which is not always the case. Rather, samples and markers may vary considerably in quality. We dealt with this potential problem by running a fourth replicate for samples that had shown more than five detectable allelic dropouts across the 15 loci. Moreover, markers that were more sensitive to dropouts than an average marker were carefully checked for deviating genetic patterns.

Levels of genetic variability were described by the number of alleles and heterozygosity, the latter measure corrected for small sample size according to Levene (1949) and Nei (1978). In cases where the sample size differed significantly between entities to be compared, a randomization approach was used to correct for the difference. From the largest entity, 1000 subsamples of the same size as the smallest entity were drawn at random, and the average value was used in the comparison. To detect possible immigrants, a Bayesian clustering approach was used, as implemented in the program STRUCTURE (Pritchard *et al*. 2001). This approach uses allele frequencies to infer the probability that a given multilocus genotype is found in either of the populations under study. A genotype that is sampled in one population but has a higher probability of occurring in another may be interpreted as a migrant from the latter.

A test to detect genetic effects as a result of a population bottleneck (Luikart *et al*. 1998a) was performed for a subsample of the historical population as well as for the Finnish one. For this test 10 allele frequency classes are defined. All alleles that have a frequency of ≤ 0.1 belong to allele frequency class I, alleles that have a frequency of > 0.1 but ≤ 0.2 belong to class II, and so forth. Populations that show a lower occurrence of class I alleles as compared to alleles of any other class have a genetic signature compatible with a recent and severe bottleneck. This approach has a low statistical power and only very strong bottlenecks have a reasonable probability ($P > 0.80$) of being detected. Another potential problem may be that it is designed to test bottlenecks over only a few generations while our historical samples span a long time period. Also, it has been recommended that at least 30 samples should be included (Luikart *et al*. 1998), which was not possible in our case when partitioning samples in separate time intervals. As the probability of missing rare alleles increases with a reduced number of samples, a small sample size may increase the chances of falsely detecting a bottleneck that has never occurred. Obviously, the results from such an approach must be interpreted with caution.

To illustrate the distribution of genetic variability across individuals and visualize the effects of random genetic drift in the Scandinavian wolf population, a factorial correspondence analysis (Benzecri 1973) as implemented in GENETIX 4.0 (Belkhir *et al*. 1999) was carried out. This approach clusters individual microsatellite profiles in a multidimensional space. Separate comparisons were made between Finnish wolves and individuals representing each of the three historical time intervals.

Results

Uniparental markers

mtDNA sequence data. Fifty-one out of 55 (91%) tooth extracts and all tissue samples were successfully amplified and sequenced for 229 bp of the mtDNA control region. Four different mtDNA haplotypes were found among the historical Scandinavian wolves. One animal, B428 (Appendix I), displayed a haplotype commonly found among dogs and not yet reported in wolves. Microsatellite profiles strongly suggested that this sample actually originated from a pure dog (data not shown). It was therefore excluded from all subsequent analyses, resulting in three different wolf haplotypes. All wolves sampled prior to 1950 ($n = 33$) displayed the mtDNA haplotype that is fixed in the contemporary Scandinavian population (Ellegren *et al*. 1996). After 1950 however, four individuals [B2 (1950), 1974–590 (1974), A77 5097 (1977), and A79 5007 (1979)] showed two deviant haplotypes (Appendix I) common in eastern populations (unpublished data). The haplotype diversity in the historical Scandinavian population apparently increased from zero prior to 1950 to 0.368 [95% confidence interval (CI) = 0.312–0.424] between 1950 and 1979. Nevertheless, the diversity remains significantly lower ($P < 0.001$, t-test) than the corresponding figure

Table 1 Y chromosome haplotypes and allele sizes found among historical Scandinavian wolves

Accession number	Sampling year	MS34A	MS34B	MS41A	MS41B	Haplotype designation
A58 0058, A58 3537, A58 35321974–590	1829, 1847, 1852, 1974	108 (172)	118 (182)	88 (208)	96 (216)	O
M3965, A58 2958, A58 2864	1863, 1899, 1965	108 (172)	116 (180)	88 (208)	94 (214)	J
M5852, A60 3043, BM2268	1875, 1900, 1900	116 (180)	114 (178)	88 (208)	104 (224)	S
BM2631, B2, B432, 176–64	1915, 1950, 1955, 1963	114 (178)	112 (176)	88 (208)	102 (222)	T
A77 5097	1977	110 (174)	114 (178)	88 (208)	106 (226)	C

Numbers in parenthesis represent the corresponding allele sizes that would have been obtained from amplification with the original primers described by Sundqvist *et al*. (2001).

for the contemporary Finnish population (0.640, 95% CI 0.616–0.663).

Y chromosome data. Five different Y chromosome haplotypes were found among the 15 individuals successfully genotyped across all four microsatellite loci (Table 1). This gives a haplotype diversity of 0.829 (95% CI = 0.804–0.853), which is comparable to that of the Finnish population (0.758, 95% CI = 0.721–0.795). None of the five paternal lines have been detected among contemporary Scandinavian wolves. Two of the haplotypes (haplotypes J and O, Table 1) are present in eastern wolf populations (Sundqvist *et al*. 2001), while three have not been previously detected outside Scandinavia (haplotypes C, S and T, Table 1).

Autosomal microsatellite markers

Amplification success and consistency across independent replicates. Thirty-three (57%) of the tooth samples were successfully genotyped across at least 12 out of 15 microsatellite loci. There was a tendency for amplification success to be negatively correlated to the age of the teeth. For the oldest samples (collected between 1829 and 1889), nine out of 18 teeth (50%) were successfully genotyped. The corresponding figures for more recent time intervals were 57% (1890–1939) and 65% (1940–74), respectively. Genotypes generally appeared consistent across parallel replicates although 49 allelic dropouts, 9.0% of the total number of amplifications of heterozygous loci, were detected. The number of dropouts did not differ between the shorter and the longer allele of each heterozygote pair ($P = 0.14$, Wilcoxon signed-rank test), as might be expected given that shorter alleles are, in general, more readily amplified. However, the size difference between most alleles was limited. A dropout ratio of 9.0% gave a low probability of false homozygotes after two independent replicates and a negligible probability for most loci after three replicates (Table 2). None of the parallel amplifications showed detectable inconsistencies other than allelic dropouts; for example, three or more alleles were never found in a single individual.

Detection of immigrants. The Bayesian clustering approach demonstrated a strong differentiation between the historical Scandinavian population and contemporary Finnish wolves. All Finnish wolves and a large proportion of the historical animals clustered with a probability of > 95% to their respective populations. This is illustrated in a factorial correspondence analysis (Fig. 2), where the Scandinavian population appears as a distinct entity outside the distribution of eastern wolves. However, six historical individuals had multilocus genotypes that could indicate an eastern ancestry (Table 3, Fig. 2). Two of these animals showed genotypes that could be found in Scandinavia with moderate to high probability, when using all available samples as the basis for comparison. However, the presence of private alleles in both animals, an unusually high level of individual heterozygosity for A58 2864 (1965), and a deviant mtDNA haplotype for A79 5007 (1979) could point towards an eastern ancestry also for these animals. To examine this possibility more closely, we carried out separate analyses for each time interval and found that these two animals carried multilocus genotypes that were different from the genotypes of other Scandinavian wolves sampled during the same time interval. The probabilities of having a pure Scandinavian origin were in this comparison reduced to 20% and 5%, respectively.

The remaining four individuals showed very low probabilities of having pure Scandinavian origin. Three animals carrying a deviant mtDNA haplotype [B2 (1950), 1974–590 (1974), and A77 5097 (1977)] were, by the Bayesian approach, suggested to be pure immigrants (Table 3). The fourth animal, M7385 from 1862, showed a high probability of being a first-generation offspring from a mating between an immigrant and a Scandinavian wolf (Table 3). Five of the six animals with presumed eastern ancestry were sampled after 1940, suggesting a significant increase ($P < 0.001$, χ^2-test) in the proportion of immigrants in the population.

Levels of genetic variability through time. Table 4 compares indices of genetic diversity from three historical time intervals. High levels of diversity were found in time interval

Table 2 Number of correct genotypes and allelic dropouts, and the estimated probabilities (*P*) of false homozygotes for individual loci

	C109	*C173*	*C225*	*U213*	*U253*	*C2001*	*C2088*	*C2096*	*C2159*	*VWF*	*C2054*	*U250*	*PEZ01*	*PEZ03*	*PEZ05*	Total
Correct genotype	28	38	19	35	30	24	41	37	22	30	29	42	27	28	40	502
Allelic dropout:																
shorter allele missing	4	0	1	1	2	1	4	1	1	3	2	0	0	0	1	21
longer allele missing	8	2	0	2	2	1	1	0	1	5	2	2	3	0	0	28
Percentage allelic dropout	30.0	5.0	5.0	7.9	11.8	7.7	10.9	2.6	8.3	21.1	12.1	4.8	5.0	0.0	2.4	9.0 ± 7.8
P (false homozygotes after 2 replicates)	0.045	0.001	0.001	0.003	0.007	0.003	0.006	< 0.001	0.003	0.022	0.007	0.001	0.001	0.000	< 0.001	0.005
P (false homozygotes after 3 replicates)	0.007	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001

Only samples that appeared heterozygous in at least one replicate are considered.

Table 3 Posterior probabilities (*P*) for having a recent eastern ancestry for six individuals in the historical Scandinavian wolf population

Accession number	Sampling area, time	*P* (pure Scand. origin)	*P* (pure eastern origin)	*P* (one parent is an immigrant)
M-7385	South 1862	0.087	0.016	0.897
B-2	South 1950	0.016	0.662	0.323
A58–2864	South 1965	0.921	0.000	0.079
1974–590	North 1974	0.036	0.595	0.369
Ma77/5097	North 1977	0.000	0.999	0.001
Ma79/5007	North 1979	0.587	0.080	0.333

1829–1889, with an average expected heterozygosity of 0.75 and an average of 4.9 alleles per locus. These figures are comparable to those found in the contemporary Finnish population [H_E = 0.72, average number of alleles (A) = 4.75; corrected for difference in sample size]. Expected heterozygosity (0.75–0.68; P = 0.09, pairwise *t*-test) and allelic diversity (4.9–4.4; P < 0.05, pairwise *t*-test) decreased to the period of 1890–1939, but there was no further decrease in the most recent time interval 1940–80 (H_E = 0.69, A = 5.5). Given that the number of wolves on the Scandinavian peninsula continued to decrease during the 20th century, the high levels of genetic variability after 1940 may appear as an unexpected observation. However, as five of the 14 samples successfully genotyped from this period presumably had an eastern ancestry, immigration seems to have counteracted further loss of genetic variability. In fact, these five individuals (Table 3) account for a large proportion of the total genetic diversity detected between 1940 and 1980. Excluding them (Table 4), the expected heterozygosity and average number of alleles decrease to 0.52 and 3.1, respectively, which is significantly lower (P < 0.01 for both measures, pairwise *t*-tests) than in 1890–1939. The magnitude of the decrease is 25% or more for all diversity indices.

Also, at the individual level, there is an overall decrease in genetic variability through time in the historical population. A linear regression model suggests a reduction of approximately 2% per decade (r^2 = 0.27, P < 0.01) (Fig. 3). Notably, the three individuals with lowest heterozygosity (< 30%) were sampled after 1950.

To illustrate the relationship between loss of genetic diversity and differentiation towards eastern wolves, we carried out separate factorial correspondence analyses for the three historical time intervals, and related the observed patterns to average individual heterozygosity within each of the intervals (Fig. 4). The loss of diversity is accompanied by increased population differentiation, expressed through increased values of θ. As for the estimates of genetic variability through time, immigration after 1940 apparently counteracted the differentiation process.

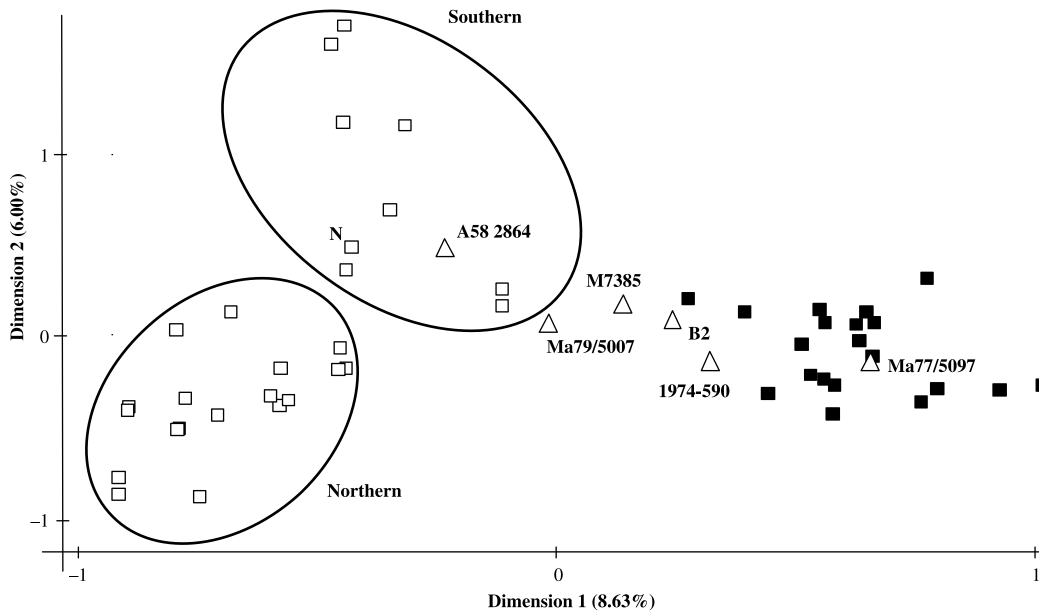

Fig. 2 Factorial correspondence analysis (FCA) of individuals from the historical Scandinavian (□) and contemporary Finnish (■) wolf populations. Individuals having a presumed eastern ancestry (see text) are represented by △. The distribution of individual microsatellite profiles suggests a differentiation between northern and southern Scandinavian wolves. The single northern wolf that falls within the southern distribution is indicated with the capital letter N. The first two factorial dimensions are represented with the proportion of the total variation explained indicated at the axes.

Table 4 Levels of genetic variability in the Scandinavian wolf population during different time intervals. A is the number of alleles and H_{obs} and H_{exp} are observed and expected heterozygosity, respectively. 1940–80* refers to the most recent time interval excluding five individuals with a presumed eastern ancestry (see Table 3). Numbers in parentheses for Finnish wolves are the average number of alleles in 1000 random draws of only 9 individuals (to make sample size comparable to that of the oldest historical time interval)

	1829–1889				1890–1939				1940–80				1940–80*				Contemporary Finnish wolves			
Locus	*N*	*A*	H_O	H_E	*N*	*A*	H_O	H_E	*N*	*A*	H_O	H_E	*N*	*A*	H_O	H_E	*N*	*A*	H_O	H_E
C2001	9	3	0.22	0.62	10	3	0.30	0.43	14	5	0.57	0.59	9	2	0.56	0.42	22	5 (3.7)	0.82	0.67
C2088	9	6	0.78	0.78	10	8	0.80	0.84	14	6	0.71	0.80	9	4	0.89	0.67	22	6 (4.1)	0.55	0.66
U213	9	7	1.00	0.86	10	4	0.50	0.62	14	8	0.50	0.77	9	4	0.44	0.58	22	11 (6.9)	0.50	0.81
U253	9	6	0.78	0.86	10	4	0.30	0.54	14	5	0.79	0.69	9	3	0.67	0.50	20	6 (5.4)	0.85	0.78
VWF	9	5	0.44	0.77	9	6	0.78	0.87	14	7	0.71	0.84	9	4	0.67	0.69	22	7 (6.0)	0.77	0.78
C2096	9	3	0.78	0.70	10	2	0.60	0.44	14	4	0.71	0.62	9	2	0.56	0.53	21	5 (3.2)	0.62	0.61
PEZ01	9	6	0.78	0.76	8	5	0.50	0.71	13	6	0.62	0.73	8	4	0.50	0.68	22	4 (3.8)	0.73	0.64
PEZ03	9	5	0.67	0.69	7	4	0.57	0.79	12	9	0.50	0.78	7	3	0.29	0.48	17	9 (6.3)	0.82	0.85
PEZ05	9	5	0.67	0.78	8	4	0.75	0.76	13	4	0.08	0.50	8	2	0.00	0.23	19	8 (5.8)	0.53	0.81
C2054	6	5	0.83	0.85	7	5	0.43	0.79	12	5	0.33	0.59	7	4	0.14	0.49	18	4 (3.7)	0.61	0.67
C2159	7	5	0.57	0.73	9	5	0.56	0.71	11	5	0.36	0.77	7	5	0.43	0.79	21	11 (7.8)	0.81	0.87
U225	8	3	0.13	0.58	9	3	0.67	0.66	14	3	0.29	0.54	9	2	0.22	0.21	17	3 (3.0)	0.71	0.64
U109	9	7	1.00	0.84	10	4	0.80	0.73	14	5	0.50	0.57	9	2	0.33	0.29	21	6 (4.3)	0.52	0.62
U173	8	4	0.5	0.51	8	4	1.00	0.69	14	5	0.64	0.74	9	3	0.44	0.63	22	6 (4.6)	0.77	0.65
U250	8	6	0.75	0.83	9	5	0.56	0.69	13	6	0.69	0.77	9	3	0.56	0.54	21	5 (4.7)	0.71	0.72
Mean	8.5	5.0	0.66	0.74	9.0	4.4	0.61	0.68	13.3	5.5	0.53	0.69	8.5	3.1	0.45	0.52	20.5	6.4 (4.9)	0.69	0.72
SD		1.3	0.25	0.11		1.4	0.19	0.13		1.1	0.22	0.12		1.0	0.23	0.17		2.4 (1.4)	0.12	0.09

Test for an historical bottleneck. The low mtDNA variability observed among the historical samples may indicate that previous demographic fluctuations may have had an impact on genetic diversity in the population. Therefore, to test for the existence of a bottleneck prior to the 19th century, the distribution of microsatellite allele frequencies among wolves sampled prior to 1900 was examined (Fig. 5a). This historical subset showed no sign of having

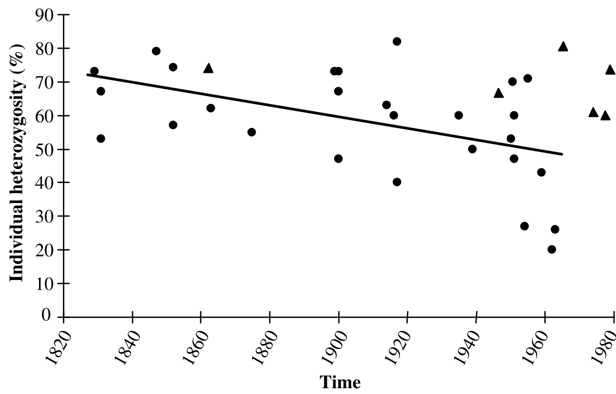

Fig. 3 Linear regression model ($r^2 = 0.27$; $P < 0.01$) of individual heterozygosity through time in the historical Scandinavian wolf population. Individuals with presumed eastern ancestry (▲) were excluded from the regression analysis.

gone through a bottleneck as allele frequency class I (see Materials and methods) had a higher occurrence than any other class. The observed pattern was comparable to that found among contemporary Finnish wolves (Fig. 5b).

Discussion

Consistency and reliability of methodology

The DNA extraction method employed in this study on museum teeth gave a high success ratio in obtaining DNA of adequate quality for mtDNA amplification, with successful amplification of a 229-bp fragment for more than 90% of the 25–170-year-old samples. As expected, a lower success ratio was obtained for microsatellite amplifications. However, more than 50% of the samples were successfully genotyped across at least 12 loci.

Detectable allelic dropouts were observed for one-half of the amplifiable extracts, giving an average dropout ratio of 9.0% across loci and independent amplifications. Given this ratio, the probability of false homozygotes after three replicates was estimated to be 0.005% (Table 2). However, this estimate assumes that allelic dropouts are randomly distributed across individuals and markers (cf. Gagneux *et al.* 1997), an assumption that is partly violated in our case. While allelic dropout appears randomly distributed across markers ($P = 0.13$, *G*-test), the distribution across individuals is significantly different from a Poisson distribution ($P < 0.01$, *G*-test).

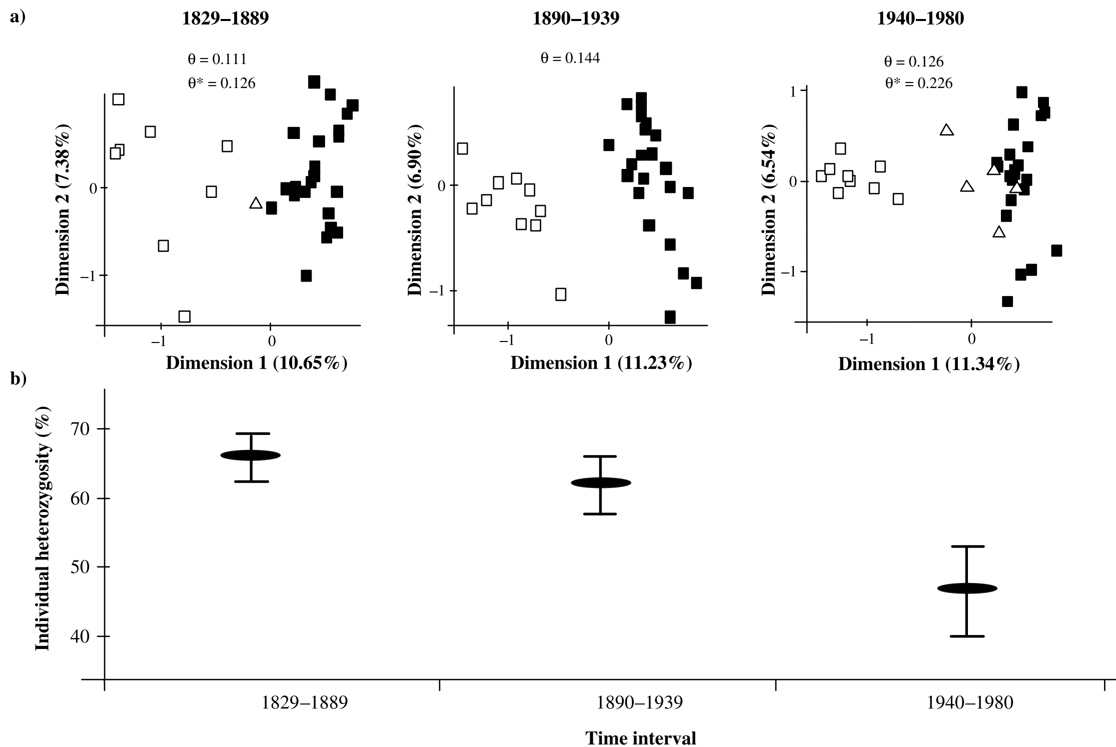

Fig. 4 (a) Factorial correspondence analyses for the contemporary Finnish wolf population (■) together with historical Scandinavian wolves (□) from three different time intervals. Individuals with a presumed recent eastern ancestry are represented by △. Estimates of population differentiation in terms of θ are indicated for each of the time intervals. θ* refers to differentiation with individuals of presumed eastern ancestry excluded. (b) Average individual heterozygosity (excluding presumed immigrants) in the same time intervals with standard errors indicated.

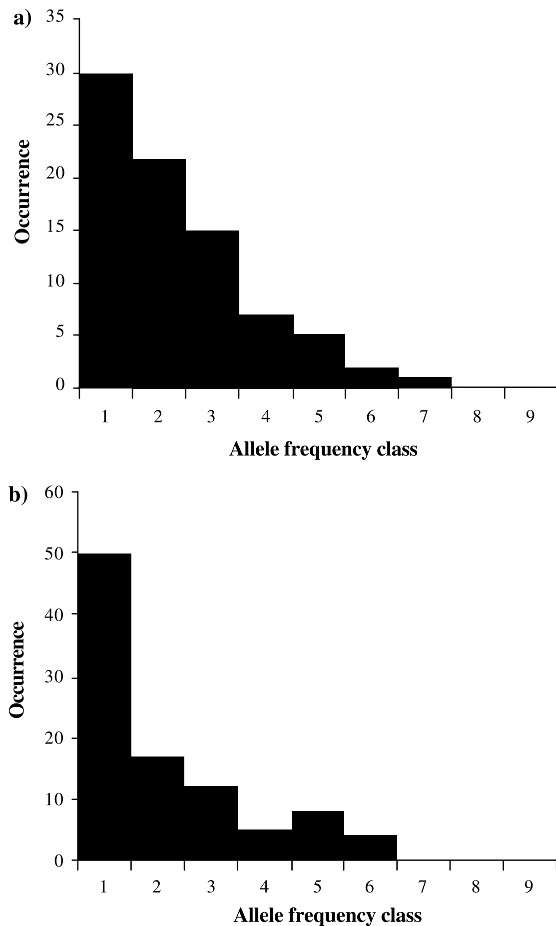

Fig. 5 Distribution of microsatellite allele frequency classes among (a) historical Scandinavian wolves sampled prior to 1900 (n = 13), and (b) contemporary Finnish wolves (n = 22). The classes reflect allele frequency distributions where class 1 comprise frequencies ≤ 0.1, class 2 comprise frequencies > 0.1 but ≤ 0.2 and so on.

This may suggest that the probability of false homozygotes is underestimated to some degree and could thus explain the lower observed than expected heterozygosity for some loci (Table 4). However, this discrepancy may alternatively be because of clustering in space and time for the historical population and/or the presence of null alleles. In fact, our data indicate a partitioning of genetic variability between southern and northern individuals (Fig. 2). Moreover, a clustering effect in time is likely as samples within each group represent a period of several decades. Strong effects of genetic drift in the declining population may have changed allele frequencies considerably through time. A Wahlund effect (Wahlund 1928) may thus have been acting in time as well as in space. Therefore, we conclude that although rare cases of undetected dropouts may still exist after three or four replicates, it seems reasonable to assume that our data set is robust.

Contrasting levels of genetic variability in mitochondrial and nuclear DNA

Given the presumably large size of the historical population in the early 1800s (Fig. 1) and the close distance to the large and genetically diverse populations in Finland (Table 4) and Russia (unpublished data), historical mtDNA variability may seem surprisingly low. In fact, Scandinavian wolves appear to have been fixed for a single mtDNA haplotype already in 1830. Even though levels of mtDNA variability in wolf populations are often limited (Vilà *et al.* 1999), most large populations previously screened show higher haplotype diversity than that found in the historical Scandinavian population (Wayne *et al.* 1992; Randi *et al.* 2000). One possible explanation for the low level of mtDNA diversity in Scandinavia could be that the population went through a bottleneck prior to the 19th century. However, although we cannot formally exclude this possibility, the bottleneck test applied to autosomal microsatellite data of the oldest historical animals did not reveal any signs of recent bottlenecks. Since relatively few samples were included in the test (n = 13), it is conservative with respect to false rejection of a bottleneck.

Alternatively, some general characteristics of the species may explain the limited mtDNA diversity seen in many wolf populations and which, in combination with attributes specific to the Scandinavian population, has led to very low mtDNA variability. For instance, the low overall effective population size associated with the social structure of the wolf (Mech 1970) may lead to rapid fixation of mtDNA haplotypes in small and medium-sized populations. In addition, populations on peninsulae may be partially isolated from potential source populations, as indicated by the low mtDNA variability found among wolves on the Iberian peninsula (Vilà *et al.* 1999) and in Italy (Randi *et al.* 2000). An additional barrier to genetic exchange specific to the Scandinavian peninsula is the extensive reindeer herding of the northern parts of Sweden, Norway and Finland, where the existence of large predators is controversial.

In contrast to the low mtDNA haplotype diversity, genetic variability at autosomal microsatellite loci was high among historical Scandinavian wolves, in particular among the oldest samples (Table 4). Wolves sampled before 1890 showed levels of heterozygosity and average number of alleles comparable to those seen in the contemporary Finnish population. Some of the aspects discussed above that potentially could have involved a reduction in mtDNA variability are likely to have a weaker impact on nuclear DNA. First and obviously, the effective population size for nuclear DNA is higher than that for mtDNA (Birky *et al.* 1983). Moreover, as suggested in some previous reports (e.g. Ballard *et al.* 1983; Peterson *et al.* 1984; Wabakken *et al.* 2001), female wolves may be more philopatric

than males. This would imply particularly low introgression of mtDNA haplotypes from neighbouring populations. In this sense, male-biased migration from the east may potentially explain the apparent incongruence between low historical mtDNA variability and high levels of autosomal variability. This hypothesis is consistent with the Y chromosome data, which revealed significant historical variability compared to mtDNA. While mtDNA haplotype diversity is significantly lower for historical Scandinavian than Finnish wolves, diversity levels for Y chromosome markers are comparable between the two populations. Male-biased gene flow from the east is supported by the observations of Pulliainen (1965, 1980), who found a significant excess of males among wolves migrating westwards in connection with population expansion events in Russian Karelia.

Levels of genetic variability and patterns of gene flow through time — to what extent have Scandinavian wolves been isolated from eastern populations historically?

Factorial correspondence analysis shows the historical Scandinavian population clearly differentiated from the contemporary Finnish population (Fig. 2). The history of the differentiation process is illustrated in Fig. 4, suggesting that the Scandinavian population was already significantly differentiated from eastern wolves by the mid-19th century (Fig. 4a). Barriers to gene flow may thus have existed for a long time, although historical levels of genetic variability were high until the mid-19th century (Table 4, Fig. 4a). The next two time intervals show increasing levels of differentiation towards eastern populations (Fig. 4a) accompanied by an overall reduction in levels of genetic variability (Table 4, Fig. 3, Fig. 4b). The increased differentiation and loss of variability are compatible with the effects of genetic drift in the declining population, which is also reflected by an increasingly clumped distribution of individual microsatellite profiles through time (Fig. 4).

As in Western Europe, wolves were intensively hunted in Eastern Europe from the 1800s until the mid-20th century (Pulliainen 1980). Consequently, the species was rare in westernmost Russia during this period (Danilov *et al.* 1985). Our data suggest that the immigration rate to the Scandinavian peninsula was low during the same period, with only one detectable immigration event, increasing differentiation between Scandinavian and eastern wolves, and finally an overall reduction in genetic variability through time. After 1940, however, the wolf population in Russian Karelia (Pulliainen 1980) expanded northwards. Three distinct population expansion events — in the late 1940s, the late 1950s and in the mid-1970s — were recorded, leading to extensive movements of animals across the Russian-Finnish border (Pulliainen 1965, 1980). The increased migration is reflected in our data set as five out of six individuals with presumed eastern ancestry are sampled after 1940, pointing towards a connection between the density of wolves in westernmost Russia and immigration to the Scandinavian peninsula. These immigration events apparently slowed down the differentiation process (Fig. 4a) and counteracted the loss of genetic diversity (Table 4).

Conclusion

To summarize the relationship between population decline, genetic erosion and immigration to the Scandinavian peninsula, we note that there was a significant decrease in levels of genetic variability from the early 1800s to the mid-1900s. The observed strong differentiation from eastern populations, already evident in the early 19th century, suggests that the immigration rate has been low for long periods of time. However, immigration events after 1940 contributed to increased levels of genetic variability, and population expansion from the east was apparently of importance in connection with the founding of the contemporary Scandinavian wolf population as shown by Vilà *et al.* (2003b). That study demonstrated that the original historical gene pool did not survive the bottleneck and that the population was founded by two eastern immigrants, only. Immigration is also likely to be important in the future to retain or even increase levels of genetic variability and counteract the potential harmful effects of inbreeding (Spielman & Frankham 1992).

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Appendix I

Historical wolf samples represented by teeth or tissue from museum specimens and their corresponding mtDNA haplotypes [designations as in Ellegren *et al*. (1996) for wolf haplotypes and Vilà *et al*. (1997) for the dog haplotype]. Samples amplifiable for nuclear DNA are indicated (+)

Accession number	Sampling locality	Geographical region*	Year of collection	mtDNA haplotype	Microsatellite amplification
A58 0058	Stockholm	Southern Sweden	1829	W1	+
A58 0057	Västerhaninge	Southern Sweden	1830	W1	–
A58 3534	Västerhaninge	Southern Sweden	1831	W1	+
A58 3536	Västerhaninge	Southern Sweden	1831	W1	+
BM280	Finnmark county	Northern Norway	1840–1860	W1	–
A58 3537	Jämtland	Southern Sweden	1847	W1	+
A58 3547	Örebro	Southern Sweden	1852	W1	+
A58 3532	Jokkmokk	Northern Sweden	1852	W1	+
BM 3029	Finnmark county	Northern Norway	1860–1910	W1	–
BM 3028	Finnmark county	Northern Norway	1860–1910	W1	–
M7385	Aurskog-Höland	Southern Norway	1862	W1	+
M7224	Öyestad	Southern Norway	1862	W1	–
3965	—	Norway	1863	W1	+
BM 3219	—	Norway	1864	W1	–
M 5172	Ringebu	Southern Norway	1873	W1	–
5851	Karasjok	Northern Norway	1875	W1	–
5852	Karasjok	Northern Norway	1875	W1	+
A58 3531	Jokkmokk	Northern Sweden	1878	W1	–
A58 2958	Jämtland	Southern Sweden	1899	W1	+
A59 3044	Jämtland	Southern Sweden	1900	W1	+
A60 3043	Jämtland	Southern Sweden	1900	W1	+
BM 2268	Karasjok	Northern Norway	~1900	W1	+
A581198	Kiruna	Northern Sweden	1908	—	–
A60 0125	Sorsele	Northern Sweden	1914	W1	–
BM2631	Bæivargiedd	Northern Norway	1915	W1	+
BM2933	Karasjok	Northern Norway	1915	W1	–
BM2851	Karasjok	Northern Norway	1916	—	–
BM2850	Karasjok	Northern Norway	1916	W1	+
BM2954	Karasjok	Northern Norway	1917	W1	+
BM2925	Kautokeino	Northern Norway	1917	W1	–
BM2924	Kautokeino	Northern Norway	1917	W1	–
BM2926	Kautokeino	Northern Norway	1917	W1	+
A59 0010	Vilhelmina	Northern Sweden	1935	—	–
A59 0011	Västerbotten	Northern Sweden	1935	W1	+
A59 0009	Vilhelmina	Northern Sweden	1937	W1	–
BM 4347	Snåsa	Northern Norway	1939	W1	+
M7830	Snåsa	Northern Norway	1939	—	–
B2	Naustdal	Southern Norway	1950	W3	+
B10	Finnmark county	Northern Norway	1950–51	W1	+
B9	Finnmark county	Northern Norway	1950–51	W1	+
B20	Finnmark county	Northern Norway	1951	W1	+
B21	Finnmark county	Northern Norway	1951	W1	+
B308	Röyrvik	Northern Norway	1952	W1	–
B428	Femunden	Southern Norway	1953	D4	+
B413	Karasjok	Northern Norway	1954	W1	+
B432	Dividalen	Northern Norway	1955	W1	+
A58 0112	Kiruna	Northern Sweden	1959	W1	+
A77 5021	Isfjorden	Southern Norway	1960	W1	–
73–78	Nesseby	Northern Norway	1962	W1	–
175/64	Nordreisa	Northern Norway	1962	W1	+
176/64	Dyröy	Northern Norway	1963	W1	+
177/64	Målselv	Northern Norway	1963	W1	–
A58 2864	Dalarna	Southern Sweden	1965	W1	+
A58 3538	Lappland	Northern Sweden	1965	W1	–
1974–590	Finnmark county	Northern Norway	1974	W3	+
A77 5097†	Lappland	Northern Sweden	1977	W3	+
A79 5007†	Lappland	Northern Sweden	1979	W4	+

*63°30′ N defines an arbitrary border between southern and northern parts of Norway and Sweden.
†Tissue sample.