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The genetical history of an isolated population of the endangered grey wolf *Canis lupus*: a study of nuclear and mitochondrial polymorphisms

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SUMMARY

The grey wolf was thought to have been exterminated in the Scandinavian peninsula when the sudden appearance of a few animals in southern Sweden was reported in 1980. These wolves founded a new Swedish population which currently numbers at least 25 individuals, one of the world's smallest populations of the species. The sudden occurrence of the founder animals caused speculation that these had not appeared by 'natural' means but rather were Swedish zoo animals deliberately released by man. To analyse if this was the case and to elucidate the genetic status of this small and isolated population, we assessed nuclear and mitochondrial (mt) genetic variability in wild and captive grey wolves, using microsatellite typing and sequence analysis of the mtDNA D-loop. The new population was found to be monomorphic for a mtDNA haplotype which also was present in the Swedish zoo population. A total of four different mtDNA haplotypes were found among all captive and wild wolves (including two animals from an occasional establishment of a few wolves in northern Sweden in the late 1970s), with a maximum sequence divergence of 3.1%. Despite the mtDNA congruence, animals from the zoo population could most likely be excluded as founders for the wild population since the latter group of animals displayed several unique microsatellite alleles (i.e. alleles not found in the zoo population). Moreover, a phylogenetic analysis of individual wolves, using microsatellite allele sharing as distance measure, placed all wild animals on a branch separated from that of the captive animals. The average degree of nuclear variability as well as allelic diversity was similar in the wild and the captive populations, respectively, but was lower than that reported for North-American populations of grey wolves. Polymorphism has declined in wild wolves born in recent years suggesting that this small population is currently suffering from a loss of genetic variability due to inbreeding. Inbreeding depression is documented in captive wolves and the long-term survival of the wild Swedish population may therefore depend on immigration of animals from Russia. This study illustrates the usefulness of microsatellites for dissecting close genetic relationships and for addressing the genetic status of individuals.

1. INTRODUCTION

The grey wolf *Canis lupus* is usually considered to have been the most widely distributed terrestrial mammal (Goldman 1944), originally inhabiting major parts of the Northern Hemisphere. Although once abundant, severe reductions in population size and also total eliminations have been recorded in many areas of its previous range during the last few hundred years (Young 1944). In North America, the grey wolf now occurs mainly in Canada and Alaska with some small and isolated populations remaining in the rest of the United States and Mexico, areas where the species was previously common (Mech 1970). Many parts of southern, western and northern Europe have also lost their grey wolf populations and the pattern of the present distribution resembles that in the United States, being highly fragmented. Less than 1000 individuals live in mountainous areas in Spain, Portugal and Italy, north of which there are no resident populations in Western Europe. A more continuous distribution is found in eastern Europe and eastwards throughout large parts of Asia (in Japan, the grey wolf became extinct almost 100 years ago). The largest numbers are found in the Asian part of Russia, especially in Kazakhstan. Larsson (1988) assumed the size of the grey wolf population in the former Soviet Union to be 50000 animals. This would imply a pronounced decline in population size since the number of wolves shot annually in the Soviet Union was as high as 40–60000 as late as 1940 (Larsson 1988). It is evident that the widespread decline of the grey wolf in North America and in the Palearctic is caused by habitat destruction and human persecution.

The grey wolf is regarded as an endangered species in several areas where it formerly was abundant.

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Protection and conservation programmes have therefore been planned or initiated in Europe as well as in North America (Mech 1995). A major concern for the preservation of the grey wolf is the genetical consequences of its decline. First, the fact that many of the remaining populations exist as small, isolated groups of animals implies the risk of a loss of genetic variation through inbreeding and genetic drift. Similarly, if animals from these populations disperse, genetic variability may be reduced in recolonized areas due to founder effects. Second, hybridization leading to fertile offspring is known in zones where the grey wolf and other canids overlap, for instance with feral dogs *Canis familiaris* (Mech 1970; Bibikov 1982; Boitani 1982) and the coyote *Canis latrans* (Lehman *et al.* 1991).

As in other parts of Europe, the Scandinavian grey wolf population has experienced a significant decrease in numbers during recent centuries. With annual shooting figures as high as 500 animals at the beginning of the 19th century, a rapid decline led to a total Swedish population size of less than 40 individuals by 1940. Legal protection was declared in 1966 when, at most, 10 grey wolves were thought to remain in the northernmost part of the country (Bjärvall 1988). However, no breeding was recorded after 1964 and in the early 1970s the grey wolf was practically extinct in Sweden. A similar trend was evident in Norway and since no wolves were thought to remain there at that time either, the species would hence have been exterminated from the Scandinavian peninsula. An influx of a few wolves occurred in northernmost Sweden in 1977 and breeding took place in 1978. The appearance of these wolves coincided with an increase in the number of wolves seen passing the Russian border in northern Finland, migrating westwards (Bjärvall 1988). Three of the wolves in northern Sweden were subsequently shot and no animal has been seen in the area since 1981.

In 1980, the sudden appearance of a few grey wolves in Värmland in southern Sweden, and in adjacent parts of Norway, some 1000 km from where the last wolves had been seen in northern Sweden, was reported. In the following years the number of wolves seen in Värmland and surrounding provinces increased and it was evident that breeding took place almost every year from 1983 (Bjärvall 1988). Today, the grey wolf population in southern Sweden has spread and numbers at least 25 individuals (Isaksson 1995), but it still probably comprises one of the world's smallest populations of the species. The distance to the neighbouring Russian population, which extends into Finland, is about 1500 km. No reports of hybridization with dogs have yet been reported.

The sudden occurrence of wolves in southern Sweden in 1980 was indeed surprising. If the founders had migrated into Arctic Sweden or Norway from Finland, and then continued southwards, it is likely that they would have been detected by farmers, hunters or reindeer breeders, for instance via tracks or prey remains (the occurrence of large mammalian predators in Sweden is also carefully monitored by the Environmental Protection Agency). This situation caused speculation that the grey wolves in southern Sweden had not appeared by 'natural' means but rather had been released by man (Larsson 1988). An idea, often cited in the national media, that the wolves originated from Swedish zoos became widespread and almost reached the status of folklore (Klintberg 1994). Since the wolves caused great harm to sheep breeders in Värmland, the release idea was taken by some as an argument in favour of the new wolves being shot (Larsson 1988).

We have conducted a survey of the genetic status of a majority of the wild grey wolves found in Sweden during the last two decades. In order to address the question of whether the recent wolf population descended from captive animals, we analysed possible genetic relationships between the wild animals and animals from the Swedish zoo population. Besides addressing this issue, this study provides some insights into the population genetics of the endangered grey wolf at the border of its range. Moreover, we demonstrate the usefulness of microsatellite polymorphisms for resolving close genetic relationships as well as for addressing the overall genomic variability of individuals.

2. MATERIALS AND METHODS

(*a*) *Animals*

Two main sets of wolves were analysed. The first comprised 21 captive individuals from Swedish zoos sampled in 1980–1994. The Swedish zoo population was founded by two apparently unrelated, wild-caught sib pairs from northernmost Sweden in the 1950s and from northern Finland in the 1960s, and has since been intensively inbred for about 10 generations (the pedigree is extremely complicated). A Russian sib pair (two sisters) from Moscow zoo was introduced into the Swedish breeding programme in 1983 and three siblings from an Estonian zoo have also been used in the programme in recent years. The matriline originating from the Finnish sibling pair quickly became extinct in the pedigree and we did not have access to any individuals carrying the corresponding mitochondrial DNA (mtDNA) haplotype. Neither did our sample include any complete families and hence, we could not firmly check for Mendelian inheritance or mutations at microsatellite loci. The structure of the pedigree did not allow the microsatellite genotypes of the founders to be deduced.

The second set of animals constituted 15 wild grey wolves sampled in Sweden in 1977–1994 (figure 1; one was actually from Norway, but quite close to the Swedish border). These individuals had either been (legally or illegally) shot or had been killed in traffic accidents. Ten of them were aged based on dental morphology (analyses of longitudinal tooth sections) at the Swedish Museum of Natural History. In an analysis of the genetic status of wolves born in different years we arbitrarily assigned an age of 2 years (which was the mean age of the 10 aged animals) to the five unaged animals.

Five DNA samples (generously provided by Deborah

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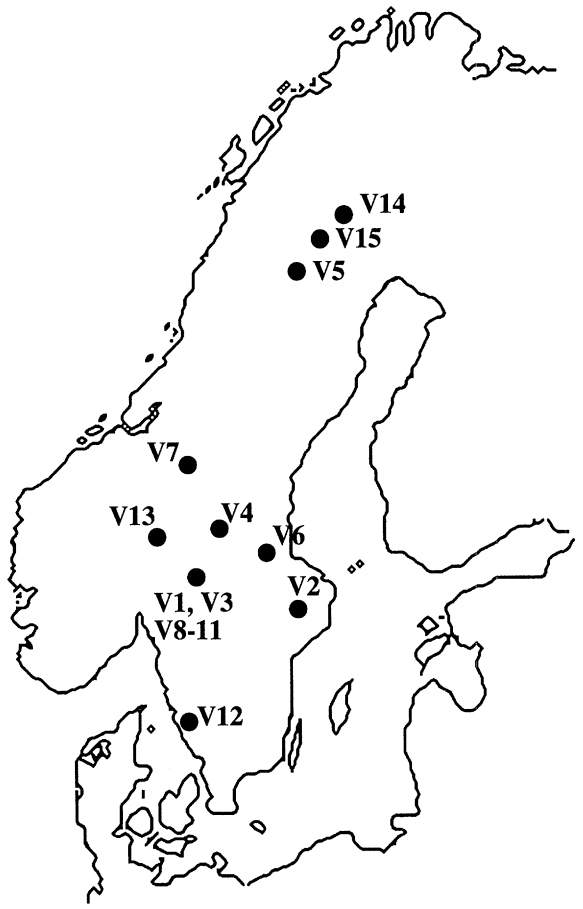

Figure 1. Map showing sampling localities for wild grey wolves (V1–V15) included in this study.

Smith and Robert Wayne) from North American grey wolves were included in an analysis of genetic relatedness between individuals. These wolves came from the Vancouver Island (3 animals; VI1–3), Alberta (ALB1) and Northwest Territories (NW1)

(*b*) *DNA preparation*

Tissue samples from captive or wild wolves had been stored at −20 °C or −70 °C from the time of killing to 1994–95 when they were analysed. Pieces of tissue (1 cm^3 of muscle, liver, kidney or testis) were cut into slices using a scalpel and were washed in 1 × SSC before the addition of 400 µl of 150 mM NaAc (pH 7.0); 1.25 mg ml^{-1} proteinase K; 50 mM DTT and 2% NP40 (non-ionic detergent). The samples were incubated at +37 °C overnight and were then extracted twice with phenol/chloroform. DNA was recovered by two rounds of ethanol precipitation and was subsequently dissolved in TE buffer.

DNA was also prepared from a few serum samples (collected in 1980–1984) from captive animals for which no tissue had been stored. Serum (1 ml) was centrifuged at 13000 × g for 15 minutes and the supernatant was discarded, leaving some 25 µl. To the remaining solution 200 µl of a 5% Chelex suspension was added and DNA preparation was then continued as described in Ellegren (1994).

(*c*) *Microsatellite genotyping*

We employed a set of 12 canine microsatellites selected either on the basis of being highly polymorphic among domestic dogs or being known to reveal polymorphism among wolf-like canids. The markers were AHT125 (Holmes *et al.* 1994), VIAS-D10 (Primmer and Matthews 1993), vWF (Shibuya *et al.* 1994), 109, 123, 172, 173, 204, 213, 225, 250 and 377 (Ostrander *et al.* 1993).

PCR was carried out in 10 µl reactions using approximately 100 ng genomic DNA in 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-Cl; 200 mM dNTP; 0.1% Tween-20; 0.25 U *Taq* polymerase (Dynazyme) and 1–5 pmol of each primer. One primer in each pair was labelled with [γ-^{32}P]dATP (0.25 µCi/pmol primer) using T4 polynucleotide kinase (Amersham). PCR cycling (run in a Techne PHC-3 thermal cycler) comprised 30 rounds of +94 °C for 30 s., +55–60 °C for 30 s. and +72 °C for 30 s., except that the first cycle had a prolonged denaturation step of 3 minutes and a 5 °C higher annealing temperature than used subsequently. The last cycle was followed by an extra 10 minute extension at +72 °C.

PCR amplifications were separated in 6% denaturing polyacrylamide gels and visualized by autoradiography.

(*d*) *Sequence analysis of the mitochondrial DNA (mtDNA) D-loop*

A 257 bp fragment of the mtDNA D-loop was amplified and sequenced from all wild wolves and from animals representing the three different matrilines present in the sample of captive animals (as judged from pedigree records). At least two animals from each domestic matriline were analysed and the sequences gathered from these pairs of individuals were consistently identical.

Three primer sets, defining two overlapping regions, were designed from coyote mtDNA sequence (Robert Wayne, personal communication) and were used in a nested configuration to amplify parts of the D-loop. Primer sequences (5′-3′) were D1: AGAGGGACATTACGAGCAAGG, D2: CCTAAGACTTCAAGGAAGAAGC, D3: TGTAAAACGACGGCCAGTTTGATGGTTTCTCGAGGCATGG, D4: Biotin-CTCCACCATCAGCACCCAAAG, RD3: Biotin-TTGATGGTTTCTCGAGGCAT-GG and RD4: TGTAAAACGACGGCCAGTCTCCACCATCAGCACCCAAAG. D1+D2 represents the outer primer pair while D3+D4 and RD3+RD4 are internal pairs. The first 18 nucleotides of D3 and RD4 correspond to the M13–21 universal primer sequence and served as target for subsequent sequencing reactions. D3+D4 and RD3+RD4 define the same mtDNA fragment, the primer sequences are identical besides the M13-linker. The positions of the 3′-ends of the inner primer pairs correspond to H16403 (D3 and RD3) and L15995 (D4 and RD4) in the human mtDNA genome (Andersson *et al.* 1981).

Ten nanograms of wolf DNA was used in 50 µl amplifications (on a Perkin Elmer 9600 thermal cycler)

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W1:   CTGAAATTCT TCTTAAACTA TTCCCTGACA CCCCTACATT CATATATTGA ATCACCCCTA CTGTGCTATG TCAGTATCTC CAGGTAAACC
W2:   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
W3:   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
W4:   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

W1:   CTTCTTCCCT CCCCTATGTA CGTCGTGCAT TAATGGTTTG CCCCATGCAT ATAAGCATGT ACATAATATT ACATTCTTAC ATAGGACATA
W2:   ---------- ---------- ---------- --G------- ---------- ---------- ---------- -T--C----- ----------
W3:   ---------- ---------- ---------- ---------- ---------- ---------- ---------- -T--C----- ----------
W4:   ---------- ---------- ---------- ---------- ---------- ---------- ---------- -T-------- ----------

W1:   TTAACTCAAT CTCATAATTC ACTGATCTAT CAACAGTAAT CAAATGCATA TCACTTAGTC CAATAAGGGC TTAATCA
W2:   ---------- ---------- ---------- --G------- ---------- ---------- ---------- -------
W3:   ---------- T-----G--- ---------- ---------- -G-------- ---------- ---------- -------
W4:   -C-------- ---------- -T------G- --G------- ---------- ---------- ---------- -------
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Figure 2. Nucleotide sequences from a 257 bp region of the mitochondrial DNA D-loop in grey wolves. Dashes indicate identical positions.

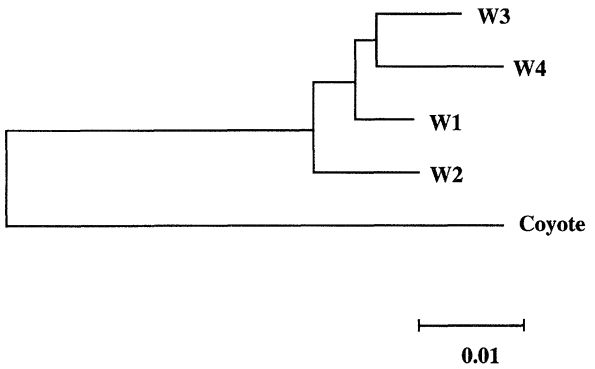

Figure 3. A phylogenetic tree based on mitochondrial DNA D-loop sequences of grey wolves. The tree was rooted by including sequence data for a coyote.

with 5 pmol of D1 and D2. The other PCR ingredients were as indicated above with the exception that the amount of *Taq* polymerase (Perkin-Elmer) was 1 U. The PCR profile was 25 cycles of +94 °C for 15 s., +65 °C for 30 s. and +72 °C for 1 min., with an initial denaturation step of +94 °C for 2 min and final extension step of +72 °C for 10 min. Of this amplification 1 µl was used as template for the nested reaction with the primers D3 and D4 or RD3 and RD4. The conditions for the nested amplifications were the same as for the outer amplification except that the annealing temperature was +69 °C.

Amplification products from the nested PCR were immobilized on a solid support by mixing 40 µl PCR reaction with 300 µg pre-washed streptavidin-coated paramagnetic beads (Dynal) in 40 µl of 10 mM Tris-Cl (pH 7.5); 1 mM EDTA; 2 M NaCl and incubated at +20 °C for 15 min. DNA was made single-stranded by denaturation with 50 µl of 0.1 M NaOH at room temperature for 5 min, the supernatant was removed and the immobilized biotin-containing strand was washed once with the solution indicated above and once with TE buffer. The beads were finally resuspended in 18 µl of 280 mM Tris-Cl (pH 7.5); 100 mM $MgCl_2$.

Dye primer sequencing was performed using an ABI Catalyst robotic workstation (Applied Biosystems Inc.). Primer annealing was achieved by incubation at +65 °C for 5 min. followed by cooling to +25 °C over 8 min. and then to +4 °C. Annealing reactions contained 3.3 µl (A), 1.7 µl (C) or 6.4 µl (G and T) PCR product and 0.4 pmol fluorescently dyed M13–21 JOE (A), FAM (C), 0.8 pmol TAMRA (G) or ROX (T) primer (Applied Biosystems Inc.). After annealing, a 3:1 mixture of the specific nucleotide solution (1 mM each of dATP, dCTP, dc7GTP and dTTP; 5 µM of the specific ddNTP; 50 mM NaCl; 40 mM Tris-Cl pH 7.5) and extension buffer (300 mM citric acid pH 7.0; 318 mM DTT; 40 mM $MnCl_2$) was added (2 µl for A, 1.5 µl for C, 4 µl for G and T) together with 1 (A and C) or 2 (G and T) U of T7 DNA polymerase (Pharmacia) and extension was allowed to continue for 7 min. at +37 °C. The reactions were interrupted by the addition of 40 µl 10 × TE buffer and cooling to +4 °C, and then pooled. The beads harbouring single-stranded templates and complementary extension products were washed once with 1 × TE and finally suspended in 6 µl formamide. The sequencing reactions were run on an ABI 373A instrument and the output data were analysed using the SeqEd software (Applied Biosystems Inc.).

(*e*) *Phenetic analysis*

A phylogenetic tree based on mtDNA sequences was constructed using the neighbour-joining algorithm in PHYLIP (Felsenstein 1993), following correction for multiple hits according to Jukes & Cantor (1969). From microsatellite data, genetic relatedness between pairs of individuals was calculated as allele sharing, i.e. 1− the average proportion of shared alleles over loci (Bowcock *et al.* 1994), using the program MICROSAT (Goldstein *et al.* 1995). Allele sharing provided a

measure of genetic distance that subsequently was applied for a UPGMA (unweighted pair group with mathematical average) clustering of individuals using PHYLIP.

3. RESULTS

We surveyed the genetic relationships among 21 captive and 15 wild Swedish grey wolves through detailed analysis of mtDNA (DNA sequencing) and nuclear DNA (microsatellite genotyping) variability. The free-living animals were collected in 1977–1994 and essentially comprised all known grey wolves found dead in Sweden during this period. Two main groups of individuals, with respect to time and locality, can be identified in the set of wild animals, (i) two wolves from the northernmost part of Sweden shot in 1977 and 1979, respectively (V14 and V15 in figure 1) and (ii) 12 wolves from southern Sweden found in 1984–1994 (V1–V4 and V6–V12). In addition, one wild wolf was killed by car in northern Sweden in 1992 (V5). The captive animals were either descendants from several generations of inbreeding of two Scandinavian (one Swedish and one Finnish) sibling founder pairs, or were the result of recent matings between the former animals and Russian or Estonian zoo imports.

(*a*) *Maternal lineages*

Sequence analysis of a 257 bp fragment of the mtDNA D-loop revealed four different haplotype variants among the wild and captive animals (figure

Table 1. *Microsatellite alleles (arbitrarily designated 1, 2, 3... etc.) found in different groups of wild and captive grey wolves, respectively.*

marker	group of animals: wild 1977–79	wild 1984 onwards	Swedish captive[d]	total number of alleles
AHT125	1[a], 3[a], 4, 5	2[b], 4, 5	2, 4, 5 (3)	5
VIAS-D10	2, 3, 4[a]	1[bc], 2, 3	2, 3, 4	4
vWF	4, 5[a], 7, 8[a]	1[b], 3[bc], 4, 6[b], 7[c]	1, 2, 4, 6, 8	8
109	2[a], 5	5, 6[b]	2, 4, 5, 6 (1, 3)	6
123	3, 4	1[b], 2[b]3	2, 3, (1)	3
172	1, 2[a]	1	1, 2	2
173	1, 3[a], 4	1, 4[c]	1, 2, 3, (4)	4
204	1, 2, 3	1, 2, 3	1, 2, 3, (4)	3
213	2[a], 3[a], 7	1[bc], 4[bc],5[b], 7[c]	5, 8, 11 (6)	9
225	2[a], 3, 4	3[c], 4	1, 2, 4	4
250	3[a], 6	1[bc], 2[bc],6	5, 6, (2, 3)	6
377	1[a], 6	4[bc], 6	3, 5, 6 (2, 4)	6

[a] Indicates an allele found in the north Swedish grey wolves from 1977 and 1979 not present in wolves from southern Sweden 1984 onwards.
[b] Indicates an allele found in south Swedish grey wolves from 1984 onwards not present in the two wolves from northern Sweden 1977 and 1979.
[c] Indicates an allele found in south Swedish grey wolves from 1984 onwards not present in the captive population descending from the two Swedish and Finish sibling founder pairs.
[d] Alleles in brackets are those introduced in the captive population by Russian and Estonian zoo imports.

Table 2. *Expected heterozygosities at 12 microsatellite loci in different groups of grey wolves. Data from the North-American populations are from Roy et al. (1994).*

locus	all wild Swedish	wild 1984 onwards	all captive Swedish	original captives[a]	Vancouver	Kenai	Alberta	N Quebec	NW Territories
AHT125	0.69	0.65	0.61	0.54	—	—	—	—	—
VIAS-D10	0.44	0.32	0.67	0.58	—	—	—	—	—
vWF	0.76	0.69	0.72	0.57	—	—	—	—	—
173	0.41	0.35	0.46	0.21	—	—	—	—	—
109	0.46	0.46	0.79	0.71	0.72	0.67	0.77	0.49	0.71
123	0.64	0.66	0.53	0.48	0.57	0.56	0.50	0.45	0.64
172	0.06	0	0.16	0.11	0.50	0.50	0.52	0.40	0.64
204	0.65	0.66	0.62	0.59	0.53	0.50	0.68	0.54	0.46
213	0.69	0.60	0.64	0.62	0.56	0.71	0.71	0.82	0.86
225	0.53	0.50	0.59	0.64	0.66	0.45	0.64	0.49	0.71
250	0.62	0.59	0.65	0.44	0.53	0.63	0.78	0.72	0.83
377	0.37	0.21	0.70	0.62	0.49	0.53	0.81	0.52	0.87
Mean ± sd[b]	0.53 ± 0.19	0.47 ± 0.21	0.59 ± 0.16	0.51 ± 0.18	—	—	—	—	—
Mean ± sd[c]	0.50 ± 0.21	0.46 ± 0.24	0.58 ± 0.19	0.53 ± 0.19	0.57 ± 0.09	0.57 ± 0.10	0.70 ± 0.10	0.57 ± 0.14	0.73 ± 0.15

[a] Excluding captive animals descending from Russian and Estonian zoo imports.
[b] The mean ± sd of all 12 loci.
[c] The mean ± sd of the eight loci typed in all populations.

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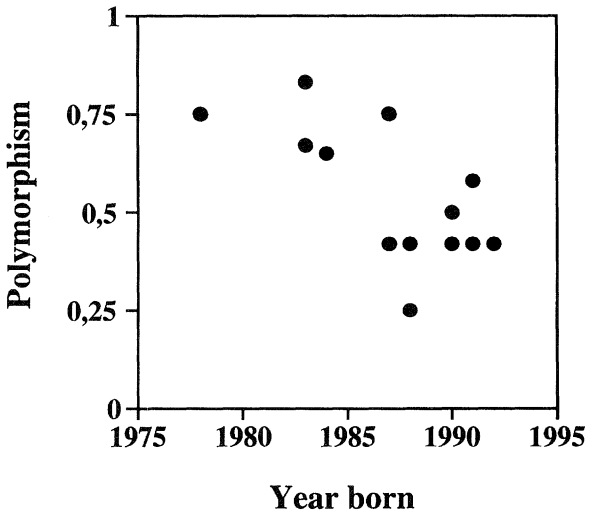

Figure 4. Relationship between polymorphism (proportion of microsatellite loci that were in a heterozygous state) and year of birth among wild grey wolves from southern Sweden. For five animals of unknown age, an age of two years (i.e. the mean of the aged animals) was arbitrarily set in this analysis. The relationship remains significant when excluding these five animals ($r_s = 0.76$, $p = 0.02$).

2). Variant W1 was present in all wild animals sampled in southern Sweden as well as in the animal from northern Sweden in 1992. W1 was also found in the zoo population where it could be identified as representing the maternal lineage originating from the Swedish founder female. In addition, W1 was present in animals descending from an Estonian bitch recently introduced into Swedish breeding.

Variant W2 was only found in the captive population and represented the maternal lineage originating from the Russian female introduced into Swedish breeding in 1983. Variants W3 and W4 were both exclusively found in a single specimen, namely the two wild grey wolves (one of each sex) shot in northernmost Sweden in 1977 and 1979, respectively. This implies that the maternal relatives to these two wolves can be excluded as possible founders of the population in southern Sweden.

Pairwise comparisons revealed 1.6–3.1% (mean = 2.1%) nucleotide sequence divergence between the mtDNA haplotypes. A phylogenetic tree (figure 3), using a coyote sequence as outgroup, constructed with the neighbour-joining method has the three Scandinavian grey wolf sequences on a branch separate from the Russian sequence but the statistical support for the tree is low as judged from bootstrapping (data not shown).

(*b*) *Nuclear genetic relationships among Swedish grey wolves*

Twelve grey wolf homologues to canine $(CA)_n$ microsatellites revealed varying degrees of polymorphism between loci, the number of alleles ranging from two at locus 172 to nine at locus 213, as summed over all 35 animals analysed (table 1). The allelic diversity averaged 3.91 ± 1.44 in all wild animals. Exclusion of the two wolves from northern Sweden in 1977 and 1979 resulted in a significantly lower diversity in the remaining wild animals, 2.75 ± 1.05 (Wilcoxon signed-rank test, $z = 3.0$, $p < 0.001$). The diversity among the captive animals originating from the two Scandinavian sib pairs was 3.00 ± 0.85 (3.92 ± 1.08 including wolves descending from crosses with Russian or Estonian zoo imports). The mean heterozygosity was similar in the free-living and in the captive populations (table 2), i.e. 0.53 ± 0.19 SD among all wild animals, 0.47 ± 0.21 among the animals sampled after 1983 and 0.51 ± 0.18 among the captive animals of Scandinavian origin.

Within the sample of wild wolves from Southern Sweden, polymorphism declined in animals born more recently (figure 4; $r_s = -0.65$, $p = 0.02$). Wolves born at the time of the discovery of the southern Swedish population were heterozygous at about three quarters of the microsatellite loci, a proportion similar to that found among unrelated individuals in the captive population (data not shown). In contrast, wolves born after 1987 were heterozygous at 25–60% of the loci, suggesting allele loss due to genetic drift. There was also a tendency for wolves born recently to show lower allelic diversity than older wolves (2.67 ± 0.89 versus 2.42 ± 0.90, Wilcoxon signed-rank test, $z = 1.6$, $p = 0.06$)

The allelic distributions in different groups of wolves gives some further insights into the relationships within and between the groups. First, since up to five alleles were found at a single locus among the wild wolves from southern Sweden, this group of animals must descend from at least three different founders/immigrants. Second, the fact that the two wolves shot in northern Sweden in 1977 and 1979 showed one or two alleles not found among the southern Swedish animals at almost all loci suggests that the former animals, or their close relatives, were not the founders of the southern population. Third, and importantly, the regular occurrence of alleles unique to the southern wolf population is a strong argument against the zoo population being a possible founder source for that population. Four loci (VIAS-D10, 173, 225, 377) showed one unique allele, two loci (vWF, 250) had two unique alleles whereas one locus (213) displayed no less than three unique alleles in the southern Swedish wolves.

A phenetic analysis of the genetic relationships between all wolves can be performed by calculating the genetic distance for each pair of individuals as allele sharing (Bowcock *et al.* 1994). These proportions ranged from 0.18 to 0.82, the lowest values recorded for some pairs of wild animals as well as some pairs of zoo animals, and the highest for some comparisons of one zoo animal and one wild animal. For this analysis we also genotyped five North-American grey wolves, which consistently displayed distances > 0.8 in comparisons with Scandinavian animals. The relative appearance of the different captive animals in a UPGMA clustering based on these distances correlates closely with known kinship, suggesting that the topology of the tree accurately reflects true genetic relationships

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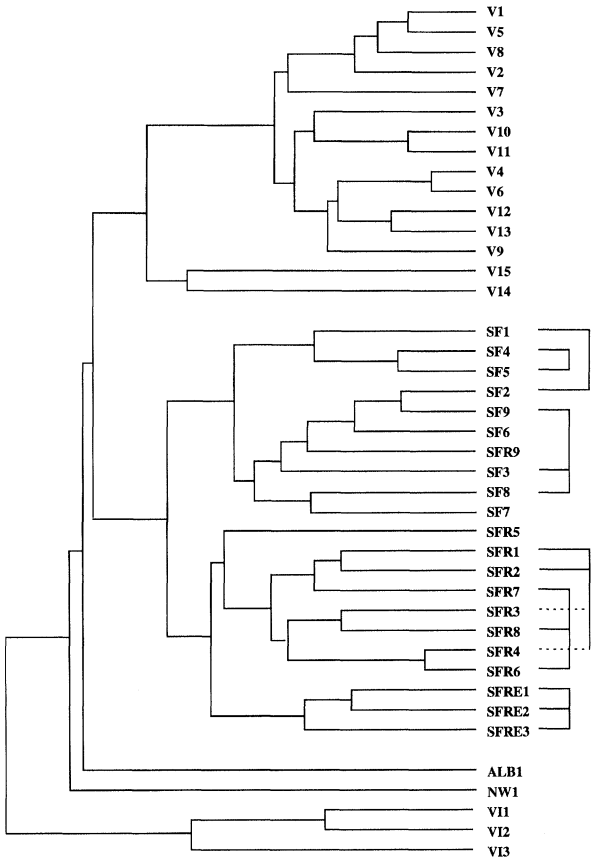

Figure 5. UPGMA clustering of all wolves included in this study based on allele sharing as a measure of genetic distance. V1–15 are wild wolves, SF1–9 are captive wolves originating from two Swedish/Finnish sib pairs, SFR1–9 are captive wolves originating from matings between Russian zoo imports and animals from the Swedish/Finnish lineage, and SFRE are animals that descend from matings between Estonian and Russian zoo imports and animals from the Swedish/Finnish lineage. VI1–3 are North-American animals from Vancouver Island, ALB1 is from Alberta and NW1 is from Northwest Territories. Cases of first-degree relatives are connected with brackets.

among the wolves (figure 5). Note the significant clustering of most cases of first-degree relatives and the general separation (with one exception) of animals from the Swedish/Finnish lineage (SF) and animals descending from matings with the former wolves and Russian (SFR) or Russian/Estonian (SFRE) zoo imports. The early branching of the North-American individuals, including the clustering of all three animals from Vancouver Islands on a separate branch, further validates the accuracy of the tree. Interestingly, the tree clearly separates all wild Swedish grey wolves from all captive wolves. Among the wild wolves, all animals from southern Sweden form a branch separated from that of the two animals from northern Sweden 1977 and 1979, again indicating the latter animals, or their close relatives, were not founders of the southern Swedish population. The wolf found in northern Sweden in 1992 clusters within the southern Swedish animals, consistent with its mtDNA identity to this group of animals. A tree with essentially the same topology was obtained using the neighbour-joining method (data not shown).

4. DISCUSSION

(*a*) *Genetic variability in the free-living population*

This study analysed the genetic relationships among 15 free-living grey wolves from Sweden and compared the genetic profiles of these animals with that of wolves from the captive Swedish zoo population. The fact that the number of wild wolves was limited must obviously be taken into consideration when evaluating the results. However, the material represents a majority of the wild grey wolves found in Sweden during the last two decades, so the data may shed some light on the genetic status of an endangered mammal at the border of its range.

The degree of nuclear variability among the wild wolves from southern Sweden (mean $H = 0.46$) was similar to that among the captive animals known to have been subject of intense inbreeding (Laikre & Ryman 1991). Somewhat higher variability has been reported for grey wolf populations from Alaska, Northern Quebec and Vancouver ($H = 0.57$) and even higher values for Alberta and Northwest Territories populations ($H = 0.70$–0.73; Roy *et al.* 1994). Allelic diversities appear also to be higher in North American populations (3.4–6.4; Roy *et al.* 1994) than in southern Swedish animals (2.75). The interpretation of microsatellite variability requires caution because it is often not appreciated that the degree of polymorphism is affected not only by overall genome variability but, to a considerable extent, also by the intrinsic relationship between polymorphism and number of repeats at simple repeat loci (Weber 1990). Weber's compilation of human $(CA)_n$ polymorphisms revealed that loci with an average number of repeats of 12 had a mean heterozygosity of 0.25, while the corresponding figure for loci with repeats containing some 20 units was 0.70. A similar relationship has been identified in other species, including the dog (Fredholm *et al.* 1995). Mean population heterozygosities will therefore depend significantly on characteristics of the marker set employed, a situation that renders comparisons between studies based on different markers (e.g. studies of different species) complicated. However, since eight of the 12 markers used in this study are the same as those used in the survey of the North American grey wolf populations (Roy *et al.* 1994) and since the average heterozygosities as well as allelic diversities revealed by these eight alone are very similar to the complete set of 12 markers (table 2), we conclude that our sample of grey wolves from southern Sweden has a somewhat lower degree of nuclear variability than that found in different North American populations.

Is the moderate level of polymorphism found in Swedish grey wolves threatening from a genetical point of view? While the fact that the difference in variability between Swedish and North American populations was not extreme and would not indicate an alarming situation, a more detailed analysis challenges this suggestion. The southern Swedish population obviously has a very recent origin and our sample included animals found shortly after the reappearance of wolves in this region as well as animals found in more recent years. Since the population is most likely to have been

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founded by a few individuals, a situation supported by the apparent mtDNA monomorphism, we should expect the initial gene pool to have been limited. Given that the wolves have subsequently been reproductively isolated, genetic drift and inbreeding may have acted to significantly lower the degree of genetic variability. This is corroborated by the significant decline in polymorphism of individual wolves with more recent year of birth (figure 4). (In theory, an alternative explanantion to the observed loss of heterozygosity in wolves born in recent years is that genetic drift could have increased the frequency of microsatellite null alleles. We consider this highly unlikely however since pedigree data from the zoo population gave no indication of a null allele at any of the 12 loci studied.) The DNA data of our sample of animals may hence reflect a process in which this small and isolated population currently loses a significant proportion of its genetic variation.

While inbreeding depression has widely been recognized as threatening to small populations (e.g. Ralls *et al.* 1979), low levels of genetic variation may not necessarily imply an acute risk for populations if the gene pool has been purged from deleterious, recessive alleles (Merola 1994). Moreover, genetic uniformity is not uncommon among carnivores (Merola 1994) and it has previously been postulated that some carnivores would be rather insensitive to the detrimental effects of inbreeding (Shields 1983), although this view has been questioned (Ralls *et al.* 1986). Grey wolves live in small packs with a limited exchange of individuals between years, and it has been suggested that this social structure would promote matings between close relatives (Mech 1970), with the possible result that adaptations to inbreeding would occur. However, the suggestion that the grey wolf is preadapted to frequent inbreeding has been contradicted by observations from the Swedish zoo population (Laikre & Ryman 1991). Here, inbreeding showed a significant negative effect on juvenile weight, reproduction and longevity. Moreover, a hereditary form of blindness, likely to be lethal in a natural population, has been found segregating among the captive Swedish wolves. These observations indicate that deleterious alleles were not uncommon in the Scandinavian population when the two sib pairs founding the zoo stock were gathered (1950–1960). Importantly, these alleles, or others with negative effects on fitness, may still exist in the contemporary Swedish grey wolf population, a situation that suggests that the signs of a recent decline in genetic variability is indeed alarming. Furthermore, while the contemporary population remains reproductively isolated, it is evident that the degree of inbreeding will continue to increase. For the long-time persistence of the grey wolf on the Scandinavian peninsula it therefore seems important that gene flow can be established between these animals and wolves from the much larger Russian population.

(*b*) *The origin of the contemporary Swedish grey wolf population*

At the time of the appearance of wolves in southern Sweden, the Swedish zoo population only harboured animals descending from the Swedish/Finnish founder sib pairs. While the mtDNA data could not exclude animals from the latter group as potential founders of the wild population, the genetic profiles at the 12 microsatellite loci clearly opposed this idea. First, for seven of the 12 microsatellites investigated we found up to three different alleles among the wild wolves that were not detected in the zoo population. While in theory this could be attributed to a sampling bias of the zoo population, it must be emphasized that at the time of the appearance of wolves in southern Sweden, the zoo population had already suffered from several generations of extensive inbreeding. Gene drop simulations have indicated that the mean number of alleles per locus surviving among the captive animals in 1980 was 4.7, 1.8 from the Swedish sib pair and 2.9 from the Finnish sib pair (Laikre & Ryman 1991; gene drops calculate the number of alleles as the number of different chromosomes, not identical by descent, present in a pedigree. Since the captive population was founded by four animals, there was initially eight alleles per locus but these may of course not represent eight different genetic variants). Given that the microsatellites employed in this study showed no signs of extreme hypervariability, we must assume that the actual number of distinguishable microsatellite alleles present in the pedigree in 1980 was considerably less than 4.7. In our sample of nine animals descending from the Fennoscandian founders, born in 1976–1984, we found on average 3.0 ± 0.85 allele size variants per locus, suggesting that most alleles present in the pedigree had been detected. The regular occurrence of microsatellite alleles unique to the contemporary wild population is therefore a strong indication that this population was not founded by animals released from Swedish zoos. The phenetic relationships among wolves as revealed by an UPGMA clustering using allele sharing as a measure of distance similarly distinguish all wild animals from the captive population (figure 5).

What was the origin of the grey wolves appearing in southern Sweden in 1980? The two animals shot in northernmost Sweden in 1977 and 1979, and their close relatives can almost certainly be excluded as founders since they showed (i) different mtDNA haplotypes and (ii) several unique microsatellite alleles as compared to animals from the southern Swedish population. Moreover, (iii) the UPGMA phenogram (figure 5) does not indicate a close genetic relationship between animals from the two geographical regions. One possibility is that the grey wolf was never extinct from the large and fairly inaccessible woodlands and mountain areas in southern Norway, close to the Swedish border. There are verified reports of tracks or signs of wolves in this area as late as in the early 1970s (Bjärvall 1988) and it is possible that some animals survived and started to propagate around 1980 (Larsson 1988). Also, although the north Swedish wolves from 1977 and 1979, or their close relatives, are

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unlikely to have been the founders, it cannot be excluded that other animals migrated into Sweden and Norway during this period of a known influx of wolves from the east. Grey wolves are known to migrate over considerable distances (Mech 1970; Bjärvall 1988). An important finding in this study was that up to five different microsatellite alleles were present at a single locus in the southern Swedish population and, assuming that no mutation has occurred, this would imply that the population was founded by at least three different individuals or that immigration has occurred. Microsatellite mutation rates vary between loci but rarely exceed 10^{-3} (Weber & Wong 1993). Moreover, loci which show hypermutability are also extremely polymorphic (Primmer *et al.* 1996) and again, since none of our markers were hypervariable, mutation events in the few generations that can have passed after propagation seem unlikely. It may be argued that if three different animals had migrated southwards through large parts of Norway and Sweden, it is likely that they would have been recorded, an argument that supports the idea of remaining animals in southern Norway as the source for the contemporary population.

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