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Y chromosome haplotyping in Scandinavian wolves (*Canis lupus*) based on microsatellite markers

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Abstract

The analysis of mitochondrial DNA sequences has for a long time been the most extensively used genetic tool for phylogenetic, phylogeographic and population genetic studies. Since this approach only considers female lineages, it tends to give a biased picture of the population history. The use of protein polymorphisms and microsatellites has helped to obtain a more unbiased view, but complementing population genetic studies with Y chromosome markers could clarify the role of each sex in natural processes. In this study we analysed genetic variability at four microsatellite loci on the canid Y chromosome. With these four microsatellites we constructed haplotypes and used them to study the genetic status of the Scandinavian wolf population, a population that now contains 60–70 animals but was thought to have been extinct in the 1970s. In a sample of 100 male wolves from northern Europe we found 17 different Y chromosome haplotypes. Only two of these were found in the current Scandinavian population. This indicates that there should have been at least two males involved in the founding of the Scandinavian wolf population after the bottleneck in the 1970s. The two Scandinavian Y chromosome haplotypes were not found elsewhere in northern Europe, which indicates low male gene flow between Scandinavia and the neighbouring countries.

Keywords: grey wolf, haplotype, isolation, microsatellites, migration, Y chromosome

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Introduction

The grey wolf (*Canis lupus*) was once spread all over North America, Europe and Asia (Mech 1970), but in the early Middle Ages extermination of wolves began in central and northern Europe (Carbyn *et al*. 1995). Today, wolves have disappeared from most of Europe, USA, Mexico and southern and eastern Asia (Mech 1970). In Scandinavia, wolves were common until the mid-19th century (Wabakken *et al*. 1992). In 1966, legal protection of the wolf was introduced in Sweden, but only a few years thereafter the species was thought to be extinct from the Scandinavian Peninsula (Naturvårdsverket 2000). However, in 1980, a few wolves were seen in southern Sweden, in the county of Värmland, and in southern Norway (Fig. 1; Wabakken *et al*. 1992). These animals formed a stable pack and since 1983 breeding has taken place almost every year in this area, and the population size has increased. The wolf population on the Scandinavian Peninsula was estimated to be about 50–72 individuals in 1998 (Wabakken *et al*. 2001). As there is no regular occurrence in northern Scandinavia, the population may be isolated (Fig. 1). The management situation is complicated since the population, though small, causes large economic losses to sheep and reindeer breeders (Naturvårdsverket 2000). Three alternatives have tentatively been suggested to explain the origin of the population (Ellegren *et al*. 1996; Wabakken *et al*. 2001): immigrants from neighbouring populations (e.g. Finland and Russia), release from captive populations, or survival of a few individuals during the demographic bottleneck.

The current Scandinavian wolf population has low levels of genetic variability. A study by Ellegren *et al*. (1996) showed that all modern Scandinavian wolves analysed ($n = 12$) shared the same mitochondrial DNA (mtDNA) haplotype, and that the heterozygosity at 12 microsatellite

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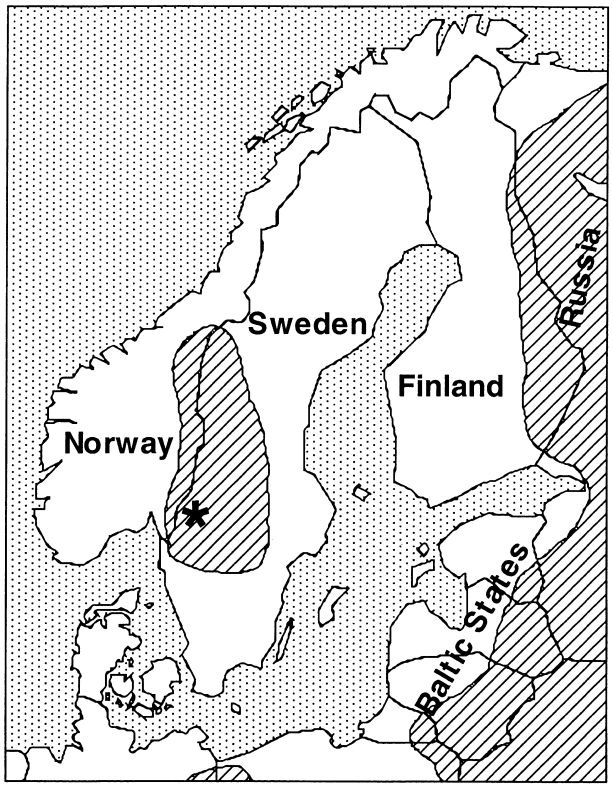

Fig. 1 Present distribution of wolves in Scandinavia and surrounding areas (shaded area). The location of the first appearance of wolves in the early 1980s is indicated with an asterisk (adapted from Wabakken *et al*. 2001).

loci had decreased over time after recolonization. Additional analyses, totalling over 30 individuals, have failed to find additional mtDNA lineages (Vilà *et al*. in preparation). All the current animals may be descendants of the individuals that founded the population in the early 1980s and extensive inbreeding can be expected (Ellegren 1999). Unless genetic contact can be established with more eastern wolf populations, a continuous loss of genetic variability may be inevitable. Inbreeding depression has been reported in a study of captive wolves from Swedish zoos, e.g. reduction in juvenile weight, as well as impaired reproduction and longevity (Laikre & Ryman 1991). Moreover, a hereditary form of blindness frequently appeared in the same captive population as a result of inbreeding (Laikre *et al*. 1993). The founders of the Swedish zoo population were two Swedish and Finnish full-sib pairs captured in 1950–60 (Ellegren *et al*. 1996). The existence of deleterious alleles in the zoo founders suggests that Scandinavian wolves have not been purged of deleterious alleles and therefore that such alleles might also be present in the contemporary wild population. Furthermore, such alleles may currently exist at high frequencies due to founding effects, potentially causing inbreeding depression.

Population genetic studies have been greatly facilitated during the last 10–15 years by the introduction of new DNA-based approaches. Sequence analysis of mtDNA and genotyping of highly variable nuclear microsatellites have become the two standard tools in most genetic surveys in animals, allowing inferences to be made on phylogenetic relationships and maternal gene flow (mtDNA), phylogeographic patterns and levels of genetic variability (mtDNA and microsatellites), and fine-scale population structure (mainly microsatellites). Recent advances in human genome analysis (Lahn & Page 1997) and in human population genetics (Casalotti *et al*. 1999; Hurles *et al*. 1999; Pritchard *et al*. 1999; Su *et al*. 1999; Thomas *et al*. 2000) now suggest that Y chromosome haplotyping may develop to become the third main tool in the next generation of genetic studies of natural populations. Y chromosome analysis has the obvious advantage that it specifically follows paternal gene flow, thus complementing studies of maternal gene flow based on mtDNA. Several features make the Y chromosome a suitable target for population genetics analysis. Although not having the high mutation rate characteristic of mtDNA (Seielstad *et al*. 1999), the Y chromosome offers an essentially inexhaustible source of polymorphisms given that its size is several orders of magnitude larger than that of mtDNA and that most of the chromosome, in contrast to mtDNA, consists of noncoding DNA. Moreover, it represents a single segregating unit, i.e. being a nonrecombining chromosome (with the exception of the very small pseudoautosomal region), and haplotypes composed of a very large number of segregating sites can thus be constructed.

In the case of the Scandinavian wolf population, analysis of Y chromosome haplotypes can, among other things, provide information on the number of founding males. There should have been at least as many male founders as the number of Y chromosome haplotypes present in the population, assuming no mutation. Additionally, the comparison of haplotypes in different populations can be used to trace the origin of the Scandinavian wolf population. It can also be used to reconstruct male migration patterns between populations in northern Europe. Migration of wolves from other populations (in Finland and Russia; Fig. 1) might be the only natural way to restore the genetic diversity in Scandinavian wolves and management decisions are, and will be, taken depending on the existence of possible migration (Naturvårdsverket 2000). To establish a system for Y chromosome haplotyping in wolf populations we report here the development of locus-specific microsatellite markers on the wolf Y chromosome, based on sequence data presented by Olivier & Lust (1998) and Olivier *et al*. (1999). We also describe an initial characterization of their variability in a haplotypic context. Moreover, we apply these markers to the Scandinavian wolf population to address questions about founding and migration pertinent to conservation issues.

Materials and methods

Strategy for the development of locus-specific markers

Olivier *et al*. (1999) identified two different microsatellite sequences (termed MS34 and MS41) from the canine Y chromosome using a random amplified polymorphic DNA (RAPD) approach (Olivier & Lust 1998). When primers for polymerase chain reaction (PCR) amplification flanking these microsatellite sequences were constructed, however, none of the primer pairs revealed locus-specific amplification in dogs. In both cases two fragments, normally of different size, were obtained from amplification of male dog DNA, while no amplification was obtained from females (Olivier *et al*. 1999). We obtained a similar pattern when we used the primers for amplification of wolf DNA (data not shown). Since duplication of DNA sequences is a common event on the Y chromosome (Jobling *et al*. 1996; Lahn & Page 1997; Tilford *et al*. 2001), this observation suggests that both these microsatellites had been duplicated during the evolution of the canine Y chromosome. To test for this possibility and, if correct, to be able to develop locus-specific markers that subsequently could be integrated to allow Y chromosome haplotyping, we cloned amplification products obtained from wolf DNA with the original primers reported by Olivier *et al*. (1999) and screened the clones with single-strand conformation polymorphism (SSCP).

DNA extraction and amplification

DNA was initially extracted from tissue samples from two male wolves from different populations in Europe (Latvia and Spain). A modified phenol/chloroform protocol (Sambrook *et al*. 1989) was used, after digestion with proteinase K. DNA was dissolved in water and the concentration was measured with a fluorometer (Hoefer).

The specific canine Y chromosome primers MS34F plus MS34R and MS41F plus MS41R, developed by Olivier *et al*. (1999), were used to amplify the corresponding fragments initially from the two male wolves. PCR reactions included 1 × PCR buffer II (Perkin Elmer), 0.4 mM dNTPs, 0.5 μM of each primer, 5 mg/mL bovine serum albumin and 0.02 U Ampli *Taq* Gold polymerase (Perkin Elmer). For MS34 3 mM $MgCl_2$ was used and for MS41 2 mM $MgCl_2$. The reactions were run in a total volume of 10 μL with 10–50 ng DNA as template.

The PCR profile for MS34 included an initial denaturation step at 95 °C for 10 min, 14 touchdown cycles with 30 s at 95 °C, annealing at 72 °C for 30 s and decreasing 0.5 °C each cycle, and 1 min at 72 °C, followed by 25 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. An extension step of 10 min at 72 °C was added after the last cycle. For MS41 the PCR profile was a similar touchdown reaction but starting at a lower temperature, i.e. six cycles with annealing at 68 °C and decreasing 0.5 °C each cycle, followed by 32 cycles as above.

Cloning and sequencing

After PCR, the products were purified with QIAquick PCR Purification Kit (Qiagen). Ligation of PCR products into a pUC18 vector was performed at room temperature for 2 h using the 'New improved 2*Rapid ligation' kit (Promega). Plasmids were introduced into competent cells by electroporation at 1800 V. Cells were incubated at 37 °C over night on agar plates containing X-gal and IPTG. To be able to discriminate, in the ligations, between the different Y chromosome fragments containing the duplicated microsatellites, single strand conformation polymorphism (SSCP; Hayashi 1991) analysis was used. Cloned inserts were PCR amplified with the same primers used previously and PCR products were denatured and run in 12% acrylamide gels at 3 W for 16 h at 20 °C. Afterwards DNA was visualized using silver staining (Bassam *et al*. 1991). Clones identified as containing different inserts on the SSCP gel were selected for sequencing and template DNA was prepared with a QIAquick PCR Purification Kit. Sequencing was performed using BigDye terminator cycle sequencing chemistry on an ABI 377 instrument (Perkin Elmer). The corresponding protocols recommended by the manufacturer were used.

Primer design and microsatellite amplification

New forward primers were designed from the obtained sequences (see Results), in order to be able to amplify independently individual copies of the duplicated fragments. These primers were labelled with different fluorescence labels (6-FAM and TET) to allow them to be run together in the same PCR reaction. The four microsatellite loci identified by this procedure were named MS34A, MS34B, MS41A and MS41B, and a corresponding nomenclature was used for the new forward primers.

The PCR mix for MS34A and MS34B included 1 × PCR buffer (Qiagen), 4 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 μM forward primer A, 0.5 μM forward primer B, 1.0 μM reverse primer and 0.02 U Hot Star *Taq* polymeras (Qiagen). The PCR profile was 10 min at 95 °C, followed by 35 two-step cycles with 30 s at 95 °C and 45 s at 72 °C, and was completed with 10 min at 72 °C.

The PCR mix for MS41A and MS41B included 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.4 μM forward primer A, 0.5 μM forward primer B, 0.9 μM reverse primer and 0.02 U Ampli Taq Gold (Perkin Elmer). The PCR profile included an initial denaturation step at 95 °C for 10 min and eight touchdown cycles, with 95 °C for 30 s, 54 °C for 30 s decreasing 0.5 °C each cycle, and 72 °C for 1 min,

followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. An extension step of 72 °C for 10 min was added at the end.

Comparison of populations and data analyses

For population comparisons DNA was extracted from male wolves originating from several populations in northern Europe. Fourteen wild wolves from Scandinavia and 13 wolves from the Swedish zoo population identified as males by the collector and verified by us using amplification of the SRY gene (Meyers-Wallen *et al*. 1995) were analysed. Additionally, 16 male wolves from Finland, 26 from Russia and 31 from the Baltic States were genotyped, to be able to study the relationship between Scandinavian wolves and animals from neighbouring populations. The samples were genotyped on an ABI 377 instrument (Perkin Elmer) and data were analysed using the software programs GENSCAN and GENOTYPER (Perkin Elmer).

Since the four markers analysed are located in the nonrecombining region of the canine Y chromosome (see Results), they can be combined into haplotypes. We used the number of haplotypes in different populations as a measure of genetic diversity. Because mutations in microsatellite loci are normally produced by the addition or deletion of one or two repeat units (Ellegren 2000a), two haplotypes that differ at multiple microsatellite loci or by multiple repeat units are not likely to have recently derived from each other.

Results

In order to isolate locus-specific markers from the Y chromosome microsatellites amplified by two pairs of primers reported by Olivier *et al*. (1999), we cloned PCR products obtained in amplification with these primers on male wolves (see Materials and methods). Two different types of clones, within each individual, were identified for MS34. Both types were about 330 base pairs (bp) in length, and after sequencing they were found to differ by three point mutations and one insertion/deletion of 2 bp in the microsatellite flanking regions (Fig. 2a). They varied also in the number of CA repetitive elements. Similarly, for MS41 two different types of clones were identified within each individual. These differed by two point mutations in the c. 238 bp region amplified, one interrupting the repeat array in the middle of the microsatellite, and one immediately flanking it (Fig. 2b). Again, the number of CA repeats was also different between the types. To allow locus-specific amplification of the different microsatellites, we used the deletion in MS34 and the point mutation immediately adjacent to the microsatellite in MS41 to design, for each marker, two new forward primers to be used with original reverse primers (Fig. 2). These four new primer pairs detecting the MS34A, MS34B, MS41A and MS41B loci all yielded single fragments in amplification of male wolf DNA. Since no amplification was still obtained from female DNA (n = 138), we conclude that four locus-specific microsatellite markers had been developed for the nonrecombining region of the canid Y chromosome.

The four microsatellites were genotyped in 14 males from the wild Scandinavian wolf population, in 13 males from the Swedish zoo population and in 73 males from other north European wolf populations. All markers were polymorphic with two to nine alleles seen among the 100 male wolves analysed (Table 1). In a preliminary analysis of haplotype diversity among north European (including Scandinavian) wolves, 17 different haplotypes were found

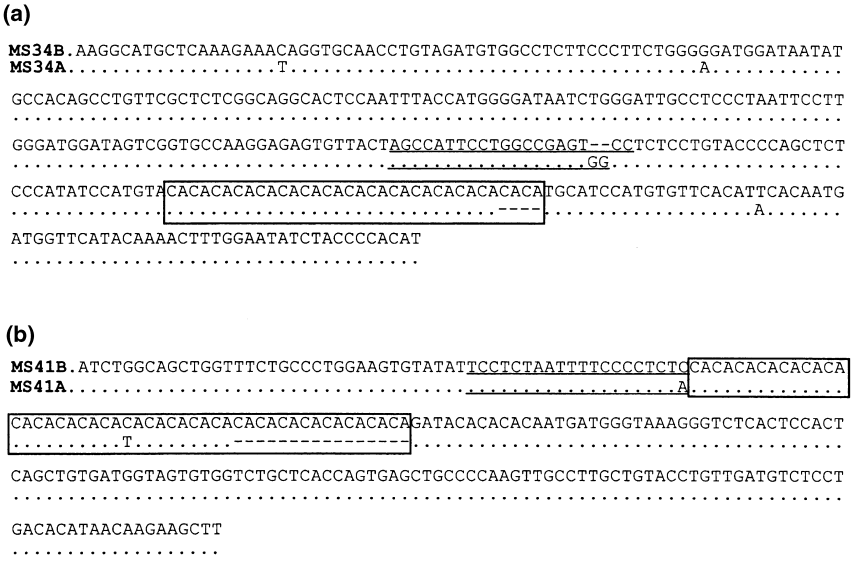

Fig. 2 DNA sequences obtained for MS34 (a) and MS41 (b). Identical positions are indicated with dots, and gaps in the alignment are indicated with dashes. Specific forward primers designed to amplify independently each copy are indicated by underlined sequences. Microsatellite sequences are inside the boxes.

Table 1 Characteristics of four wolf Y chromosome microsatellites

Microsatellite	Primer sequence	No. alleles	Observed length (bp)
MS34A	MS34AF 5′-AGCCATTCCTGGCCGAGTGG-3′ MS34R 5′-GGTCCCCTTTTGCCATAGTGT-3′	4	172–178
MS34B	MS34BF 5′-AGCCATTCCTGGCCGAGTCC-3′ MS34R, as above	5	174–182
MS41A	MS41AF 5′-TCCTCTAATTTTCCCCTCTA-3′ MS41R 5′-CTGCTCGACCCTCTTCTCTG-3′	2	208–210
MS41B	MS41BF 5′-TCCTCTAATTTTCCCCTCTC-3′ MS41R, as above	9	212–228

Table 2 Y chromosome haplotypes found in the North European wolves and allele sizes at four microsatellite loci (in base pairs)

Haplotype	MS34A	MS34B	MS41A	MS41B
A	172	182	208	214
B	172	182	208	220
C	174	178	208	226
D	172	180	208	216
E	178	176	208	216
F	176	176	208	218
G	176	178	208	222
H	174	178	208	228
I	172	180	208	212
J	172	180	208	214
K	178	176	208	218
L	174	178	208	222
M	176	178	210	224
O	172	182	208	216
P	178	174	208	216
Q	174	176	208	220
R	174	178	208	214

Table 3 Distribution of Y chromosome haplotypes across wolf populations in northern Europe

Haplotype	Scand. (14)	Zoo (13)	Finland (16)	Baltic states (31)	Russia (26)
A	3				
B	10				
C	1			3	
D		13			
E			6	5	6
F			5		3
G			2	1	1
H			3	3	2
I				1	
J				4	
K				5	10
L				4	
M				4	1
O					1
P					1
Q					1
R				1	

Table 4 Time distribution of haplotypes A, B and C in the Scandinavian wolf population. Each year corresponds to the date when individual male wolves were killed

Haplotype A	Haplotype B	Haplotype C
		1977
	1984	
	1986	
	1986	
	1989	
	1992	
	1992	
1993		
1996		
	1997	
	1998	
1999		
	2000	
	2000	

(Table 2). Three of these haplotypes (A, B and C) were seen in wild Scandinavian wolves, and a fourth one (D) was found fixed in the Swedish zoo population (Table 3). A and B differed by three repeat units at one locus (MS41B) but were otherwise identical. C differed from A and B at three loci (MS34B, MS41A, MS41B) by a total of nine and six repeat units, respectively (Table 2). A total of four haplotypes were observed in Finland, nine in Russia and 10 in the Baltic States (Table 3). Haplotypes A, B and D were not found outside Scandinavia, but haplotype C was also found in the Baltic States. The haplotypic diversity is lower in Scandinavia than in the other populations. The number of haplotypes found per sample analysed was 0.21 in Scandinavia (0.15 excluding haplotype C, see below), and 0.25, 0.35 and 0.32 for Finland, Russia and Baltic States, respectively.

The temporal occurrence of haplotypes A, B and C in Scandinavian male wolves is shown in Table 4. Haplotype A was found in three animals collected during the period 1993–99, haplotype B was found in a total of 10 animals collected during 1984–2000, while haplotype C was only found in a single individual killed in 1977 in northern Sweden.

Discussion

The isolation and characterization of polymorphic microsatellite markers on the Y chromosome can help explain the recovery of the Scandinavian wolf population during the last decades. One important question is the number of males that founded the population in the 1980s. In this study we found three Y chromosome haplotypes (A, B and C) among modern Scandinavian wolves. Haplotype C, however, was only found in one individual from northernmost Sweden killed in 1977. This was before wolves re-appeared in southern Sweden and this particular animal, and its relatives, were excluded as founders of the extant population based on data from mtDNA and autosomal microsatellite loci (Ellegren *et al*. 1996). Our results therefore suggest that there could have been as few as two males involved in the founding of the current Scandinavian population, given that we only identified two different Y chromosome haplotypes (A and B) in samples from southern Scandinavia collected after 1983. Together with the observation of a fixed mtDNA variant in the population (Ellegren *et al*. 1996), a minimum of three founders is suggested (two males and one female). This agrees with the interpretation derived from the presence of a maximum of five alleles at autosomal microsatellite loci (Ellegren *et al*. 1996). Of course, the observation of two paternal lineages only provides an absolute minimum number of male founders, given that multiple males with identical Y chromosome haplotypes may have been involved. Similarly, the presence of a fixed mtDNA variant in Scandinavia represents a minimum number of founding females because that variant is also very common outside Scandinavia (Vilà *et al*. in preparation).

We cannot distinguish between the scenarios of two males founding the population in the early 1980s and only one early male founder and the other lineage being introduced later. Haplotype A was first seen in a sample from 1993, which might be indicative of a relatively recent recruitment. However, the limited sample size of males from before 1993 precludes strong conclusions on this point.

Related to the question of the number of founders is the origin of founders. If the current Scandinavian wolf population descends from immigrants from northern Europe, e.g. from Finland, Russia, or the Baltic States, we would have expected to find haplotypes A and B also in these populations. However, these haplotypes were not seen in any of our 73 male samples from other north European countries. Assuming that these samples are from wolves of a single panmictic population, for any haplotype present in a frequency of 5% in this population the probability of being missed in a sample of 73 is lower then 0.025. However, our preliminary data using mtDNA sequences and microsatellites suggest that some fragmentation between north European wolf populations exists (Vilà *et al*. in preparation). In light of this, although our results are not evidence of immigration from other North European populations, it is probably premature to exclude other north European wolf populations as the origin of the current Scandinavian population.

The sudden appearance of wolves in southern Scandinavia in the early 1980s, at least 1000 km from the closest regular occurrence of Finnish or Russian wolves, led to speculation about a possible origin from a release from the captive zoo population (see Ellegren *et al*. 1996). According to the pedigree for the Swedish zoo population only one surviving paternal lineage should be expected. Indeed, all 13 zoo males analysed in this study shared the same haplotype (D), not found in the wild population. From this we conclude that the paternal lines in the wild Scandinavian wolf population cannot originate from the Swedish zoo population.

The presence of Y chromosome lineages in the wild population not observed in captive or in neighbouring populations is compatible with the hypothesis that the extant population could have been derived from some Scandinavian male wolves surviving during the bottleneck. However, we cannot provide any direct support for this idea. The analysis of these markers in ancient wolf samples, collected before the population decline in Scandinavia, could help to address the question.

The three haplotyes found in Scandinavia (A, B and C) are not likely to have arisen from each other by mutation in recent times. Assuming that mutations in microsatellite sequences are normally produced by the addition or deletion of one or, rarely, two repeat units (Ellegren 2000a), the differences between these haplotypes correspond to several mutational steps. The average mutation rate for human Y chromosome microsatellites is in the order of 0.2% per generation (Ellegren 2000b; Jobling & Tyler-Smith 2000). If this figure is roughly applicable to other mammals and considering that only a few generations have passed since wolves reappeared in Scandinavia (mean generation time of 3 years; Mech & Seal 1987), it indeed seems unlikely that one of these haplotypes has recently derived from another. A similar reasoning can be made for excluding a recent mutational origin of the haplotype found in the zoo population (D), which differed from the haplotypes found in Scandinavia at two (A and B; totalling two and three repeat units, respectively) or three (C; seven repeat units) microsatellite loci.

The captive and wild Scandinavian wolf populations show similarly low levels of genetic variability at autosomal markers (Ellegren 1999). Our results also indicate relatively low diversity of Y chromosome haplotypes, consistent with the low diversity of mtDNA variants (Ellegren *et al*. 1996; Vilà *et al*. in preparation). This reduced genetic variability could be the result of the demographic bottlenecks. Consequently, the establishment of gene flow with

other populations that could allow the recovery of some variability is important for the long-term survival of the Scandinavian wolf population. Our results indicate that immigration of males might not be taking place and, until more complete genetic evidence is available, effort should be allocated to allow gene flow.

This study represents one of the first reports of Y chromosome microsatellites in a wildlife species and their application for fine-scaled Y chromosome haplotyping in a natural population. We anticipate that more studies of this kind will follow. Since dispersal patterns in many organisms are characterized by pronounced differences between sexes (Greenwood 1980, 1983), the ability to study specifically gene flow in both sexes is highly warranted and will add to the study of the effects of such differences on phylogeographic patterns. Moreover, since variation in male reproductive success is often higher than that among females (a consequence of the prevailing direction of sexual selection, Andersson 1994), the ability to follow patrilines can be particularly important in studies of social structures and mating systems. Y chromosome haplotyping obviously also represents a fast method to exclude possible fathers in paternity studies and to determine multiple paternities in single litters or clutches.

Admittedly, however, the use of Y chromosome haplotyping in population genetic studies of natural populations is still often hindered by lack of sequence information necessary for polymorphism screening. Identification of Y chromosome microsatellites or single nucleotide polymorphisms (SNPs) should thus be given high priority in population genetic studies. The combined use of SNPs and microsatellites in Y chromosome haplotyping should allow both deep-rooted events (slowly evolving SNPs) and more recent population differentiation (fastly evolving microsatellites; see de Knijff 2000; Forster *et al*. 2000) to be followed.

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This study was part of a larger project analysing the genetic diversity of Scandinavian carnivores that is being done at the Department of Evolutionary Biology, Uppsala University. Within this project, led by Hans Ellegren, Anna-Karin Sundqvist (PhD student) and Carles Vilà (assistant professor) focus on wolf genetics. The isolation of male specific DNA sequences was part of Michael Olivier's PhD Research at Cornell University.
