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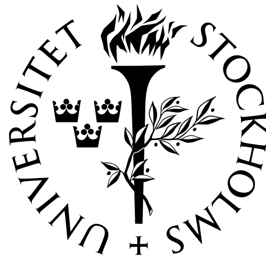


# MITOCHONDRIAL DNA VARIATION IN THE ARCTIC FOX

**- POPULATION HISTORY, GENE FLOW AND SPECIES IDENTIFICATION**

Love Dalén
Ekologi

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# Mitochondrial DNA Variation in the Arctic Fox

## Population History, Gene Flow and Species Identification

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-Licentiate thesis-
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**Paper III:** Dalén L, Götherström A, Angerbjörn A. 2003. Identifying species from pieces of faeces. Conservation Genetics, *in press*.

# Mitochondrial DNA variation in the arctic fox
## - population history, gene flow and species identification

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**Abstract**
The arctic fox (*Alopex lagopus*) is a cold adapted species that is capable of long distance migrations. These characteristics make the arctic fox different from temperate species in terms of what can be expected on its response to historic fluctuations in climate and the degree of genetic differentiation among populations. In this thesis, I investigate how the glacial cycle has affected the distribution and amount of mitochondrial DNA (mtDNA) variation in the arctic fox, and how populations throughout the Arctic are differentiated from each other. A particular emphasis is put on whether there is a gene flow from Siberia to the endangered Fennoscandian population. The nucleotide diversity in the arctic fox was low and a mismatch analysis suggested that arctic foxes expanded rapidly in population size at the onset of the last Ice Age, possibly from an interglacial refugium. There was little phylogeographic structure among current populations, no difference in haplotype diversity between previously glaciated and unglaciated regions, and the overall population differentiation was relatively low. This implies that there is a current gene flow between most populations. The number of haplotypes and amount of haplotype diversity in Fennoscandia, together with a relatively low $\Phi_{ST}$ value, point to that there is a current gene flow also between Siberia and Fennoscandia. Finally, I show that by utilizing the observed mtDNA variation in arctic foxes, it is possible to separate faeces from arctic foxes, red foxes and wolverines. Identification of arctic fox faeces is useful both in the management of the Fenno-scandian arctic fox population and in obtaining samples for future genetic analyses.

## INTRODUCTION

A widely used definition of ecology is "the scientific study of the interactions that determine the distribution and abundance of organisms" (Krebs 2001). Although ecological studies often focus on how biotic and abiotic factors affect the distribution of species, another perspective involves the interactions that determine the distribution of genetic variation within species. Such processes include historical changes in the distribution and abundance of a species as well as current gene flow among populations (Avise *et al.* 1987).

Among the most important historical factors that have influenced the distribution of genetic variation in organisms are the glacial cycles (Taberlet *et al.* 1998, Hewitt 2001). Glaciations lasting some 100 000 years (*i.e.* Ice Ages) have come and gone over the last 2.4 million years (Hewitt 1999), with relatively short, warm periods in between. The cold periods have forced temperate species in continental North America and Europe to endure repeated

isolations in southern refugia, whereas the warmer interglacials have allowed them to expand northwards to recolonise previously glaciated regions (Hewitt 1996).

Gene flow among populations counteracts population differentiation, both when the differentiation is caused by genetic drift and by natural selection (Hartl 1988). It is however important to note that gene flow is not synonymous to migration, since individuals may migrate from one population to another without breeding, and will consequently not contribute to the other population's gene pool. Gene flow is important in conservation biology, as gene flow into endangered populations can reduce the level of inbreeding depression and loss of genetic variation caused by genetic drift (Hedrick & Kalinowski 2000).

The majority of phylogeographic studies on terrestrial mammals have been made on temperate and tropical species that do not display long distance migrations (*i.e.* thousands of kilometres), for example bears (Taberlet & Bouvet 1994), elephants (Nykaana *et al.* 2002), moose (Hundertmark *et al.* 2002) and hedgehogs (Seddon *et al.* 2001). An arctic species capable of long distance migrations, such as the arctic fox, may be expected to display less population differentiation and could very well have responded differently to the glacial cycles.

*The arctic fox*

The arctic fox (*Alopex lagopus*) is a small circumpolar canid that inhabits the arctic tundra. It is well adapted to arctic conditions (Fuglei and Øritsland 1999) and tolerates temperatures below -40$^{o}$ C without having to increase its metabolic rate (Scholander 1950). The worldwide population size is approximately 330 000 - 930 000 individuals (Angerbjörn *et al.* 2003). The species as a whole is therefore not threatened, although some populations are (*e.g.* the Fennoscandian population). Arctic foxes have been divided into two different ecotypes, ”lemming” foxes that mainly feed on lemmings (*Lemmus* & *Dicrostonyx sp.*) and ”coastal” foxes that feed on birds and carrion (Bræstrup 1941). ”Lemming” foxes are found in North America, Eurasia and East Greenland, whereas ”coastal” foxes inhabit habitats where there are no lemmings, such as Iceland and Svalbard. Arctic foxes of the ”lemming” ecotype have different life history strategies than those belonging to the ”coastal” ecotype, where the former migrate more frequently and have larger litter sizes than the latter (Tannerfeldt & Angerbjörn 1998). Arctic foxes are capable of unusually long migrations: there are reports of arctic foxes travelling more than 2000 km over the polar sea ice (Garrott and Eberhardt 1987). How frequent these long distance migrations are, and how they affect the differentiation of arctic fox populations is however unknown.

*The Fennoscandian population*

The arctic fox is considered one of the oldest species in Fennoscandia, having followed the retreating ice edge at the end of the last Ice Age. Today, it inhabits the mountain tundra in Sweden, Norway, Finland and the Kola Peninsula. Arctic foxes used to be a common sight in the mountains and the population size in Sweden alone probably exceeded 4 700 individuals during peak years (Tannerfeldt 1997). However, at the end of the 19$^{th}$ century the Fennoscandian population started to decline as a result of heavy hunting pressure. As the population became smaller and smaller during the first decades of the 20$^{th}$ century, fur prices skyrocketed which in turn caused the hunting to become even more intense. In Sweden, the population size after this demographic bottleneck was estimated as 100-150 adults (Lönnberg 1927). Despite being protected by law in Sweden (1928), Norway (1930) and Finland (1940) the population did not recover (Löfgren & Angerbjörn 1998). Today there are *c.* 100 adults in Sweden, Norway and Finland altogether. The population density of arctic foxes on the Kola Peninsula is uncertain but is probably somewhat higher (*pers. obs.*).

It is likely that there are several reasons why the Fennoscandian population has failed to recover. The main reasons seem to be interspecific competition from an increasing number of red foxes on the mountain tundra as well as food shortage caused by irregularities in the lemming cycle (Tannerfeldt *et al.* 1994, 2002). Additional possible explanations include disease, inbreeding and low genetic variation.

## GENERAL METHODS

*Laboratory methods*

DNA can be extracted from tissue samples using a wide variety of methods, where various buffers are used for tissue lysis, normally followed by digestion with proteinase K. The DNA is then often extracted from the resulting solution using phenol/chloroform, or guanidium thiocyanate and silica. Several commercial kits are also available, which usually are based on the latter method. DNA can also be extracted from faeces. Such DNA can however be quite problematic to work with since faeces contain substances that inhibit the polymerase chain reaction (PCR). Furthermore, faecal DNA is often degraded and occurs in small numbers (Wayne *et al.* 1999). This puts special demands on the extraction method and the precautions taken against contamination.

Once extracted, the DNA can be amplified in a PCR. We have used four different primers: one reverse primer (*H3R*) that binds to most mammals, and three forward primers that bind to arctic foxes (*Pex1F*), red foxes (*Vul1F*) and wolverines (*Gulo1F*). For arctic foxes, we have amplified a 332 base pair (bp) segment of the control region (synonymous to the hyper-variable region 1, HVR1) in the mitochondrial DNA (mtDNA). This region evolves at a rapid pace and is therefore suitable for intraspecific phylogeographic studies (Avise *et al.* 1987). The region between the primers is 292 bp long. The length of the sequences reported in Paper 1 (294 bp) is due to a part of the reverse primer having been mistakenly included in the consensus sequences.

Sequencing was performed using a CEQ 2000XL automated sequencer (Beckman Coulter), or at a commercial laboratory (Kiseq, Stockholm). Each amplification product was sequenced for both the heavy and light strands.

*Statistical analyses*

A number of statistical methods have been developed to deal with gene sequence (*e.g.* mtDNA sequence) data. At the most detailed level is the nucleotide diversity (Nei 1987) which is based on the number of nucleotide differences among haplotypes (when several individuals have identical sequences, each unique variant is called a haplotype). Since this measure is an estimate of the number of mutations that have occurred since the divergence of a common ancestor, it can be used to infer population history. For example, the distribution of pairwise differences among sequences, the mismatch distribution (Rogers & Harpending 1992), can be used to test hypotheses on historical changes in population size. The nucleotide diversity, in combination with a rate of molecular divergence for that particular mtDNA region and the generation time for the species in question, can also be used to estimate the effective population size (Wilson *et al.* 1985).

The evolutionary relationships between the haplotypes can be illustrated in a phylogeny or a minimum spanning network (MSN). When such a tree or network is combined with information on the geographic distribution for each haplotype, a phylogeography is obtained (Avise *et al.* 1987). Phylogeographies can yield information about, for example, past fragmentation and range expansions (Avise *et al.* 1987, Templeton 1998). The spatial distribution of mtDNA haplotypes among populations can also be used to estimate population

differentiation, for example through $\Phi_{ST}$ statistics, and to determine the geographical structuring of the genetic variation (Excoffier *et al.* 1992). The $\Phi_{ST}$ value is a measure on the proportion of genetic variation that can be explained by differences among populations.

The frequency of each haplotype can be used to calculate a population's haplotype diversity (H), which is a measure of genetic variation (Nei 1987). The level of haplotype diversity can normally be expected to be lower in populations inhabiting recently colonised regions than in regions that have been inhabited for a long time (Hewitt 1996), unless there is a high gene flow between the populations. A comparison of the haplotype diversity in previously glaciated and unglaciated regions can thus yield information on the level of gene flow among populations.

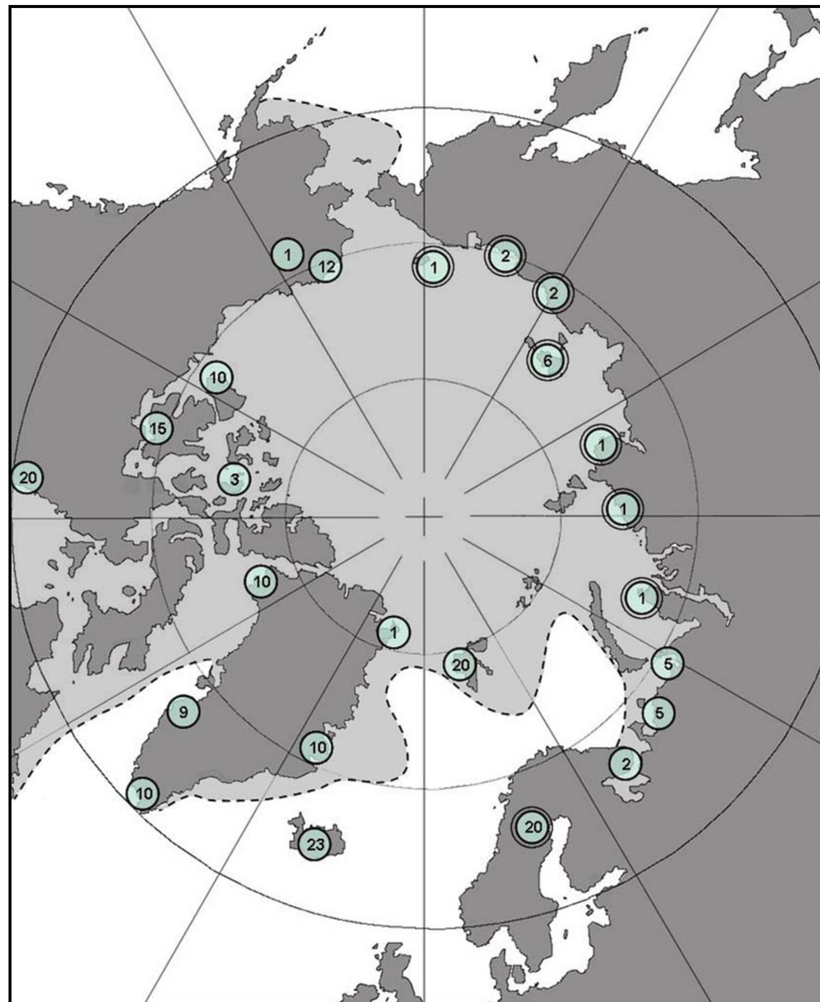


**Figure 1.** Sample sites in the Arctic (double circles indicate sample sites from Paper 1). Each number indicates the number of arctic foxes sampled at each location (Paper 2). The light grey area inside the dashed line illustrates the extent of polar sea ice in January (data from EOSDIS NSIDC Distributed Active Archive Center).

## PAPER I

In this paper we address the question of whether the Fennoscandian arctic fox population is genetically isolated from the much larger Siberian population. At a first glance, the constantly ice-free coasts of Norway and the northern Kola Peninsula along with the taiga forest stretching as far north as the White Sea seem to constitute formidable geographic barriers. On

the other hand, arctic foxes are documented long-distance travellers (Pulliainen 1965, Eberhardt & Hanson 1978, Garrott and Eberhardt 1987). Based on the small size of the Fennoscandian population and its history with a bottleneck a hundred years ago, we tested three different hypotheses. The first hypothesis was that the Fennoscandian population has been isolated from the Siberian population, both before and after the bottleneck a hundred years ago. The second hypothesis was the opposite, that there has been a gene flow between the populations both before and after the bottleneck. The third hypothesis was that the Fennoscandian population has become isolated as a result of the bottleneck. These hypotheses generate different predictions on what can be expected on the monophyly, the amount of genetic variation and the number of haplotypes in the Fennoscandian population. For the first hypothesis, we expected Fennoscandian haplotypes to be monophyletic compared to the Siberian haplotypes, that we would find very few haplotypes (possibly only one as in Scandinavian wolverines and lynx; Walker *et al.* 2001, Hellborg *et al.* 2002) and that the haplotype diversity would be significantly lower in Fennoscandia than in Siberia. For the second hypothesis, the expectations were the opposite. For the third hypothesis, the Fennoscandian haplotypes would not be expected to be monophyletic due to gene flow prior to the bottleneck, but there should be very few haplotypes and low haplotype diversity in Fennoscandia due to genetic drift after the bottleneck.

We sequenced a part of the mitochondrial control region (referred to as HVR1 in Paper 1) from 17 Swedish and 15 Siberian arctic foxes (*see* Fig. 1 for sampling locations). Three haplotypes were observed in Sweden, which were the same as the three haplotypes observed in Norway in a previous study (Strand *et al.* 1998). It therefore seems that there are three haplotypes in Scandinavia. In Siberia, we observed seven haplotypes, of which six were only found in Siberia and one was shared with the Fennoscandian population. The haplotype diversities were equal between the two populations and a phylogenetic analysis showed that the three Swedish haplotypes were not monophyletic. The genetic distance between the two populations, as measured by $\Phi_{ST}$ statistics (Schneider *et al.* 2000), was 0.17. Taken together, these results suggest a gene flow between the two populations, both before and after the bottleneck.

This study also gave rise to new questions. First of all, if the three Scandinavian haplotypes are the result of migration from Siberia, as we proposed, why did we not find more than one of these haplotypes in Siberia as well? Second, what does a $\Phi_{ST}$ value of 0.17 correspond to compared to the relationship between other arctic fox populations in the world?

## PAPER II

To resolve questions concerning the species' history and patterns of gene flow, we analysed mtDNA control region variation in arctic foxes from their entire distribution range. Regarding the population history, we did not expect to find the same patterns as have been observed in more temperate species. The main reason for this was that we anticipated that the arctic fox, being a cold adapted species, had been affected by the Ice Ages in a different way compared to temperate species. Based on the frequent long distance movements in arctic foxes, we hypothesised that there is a gene flow between populations connected by land or the polar sea ice (*i.e.* all populations except Iceland, *see* Fig. 1).

We analysed 191 arctic foxes from Fennoscandia, Svalbard, Iceland, Greenland, Canada, Alaska and Siberia (Fig. 1). The overall nucleotide diversity was relatively low ($\pi$ = 0.009) and a significantly negative $F_S$ value (Fu 1997) together with a mismatch analysis (Rogers & Harpending 1992) suggested that the arctic fox has gone through a rapid expansion in population size, which presumably was preceded by a demographic bottleneck.

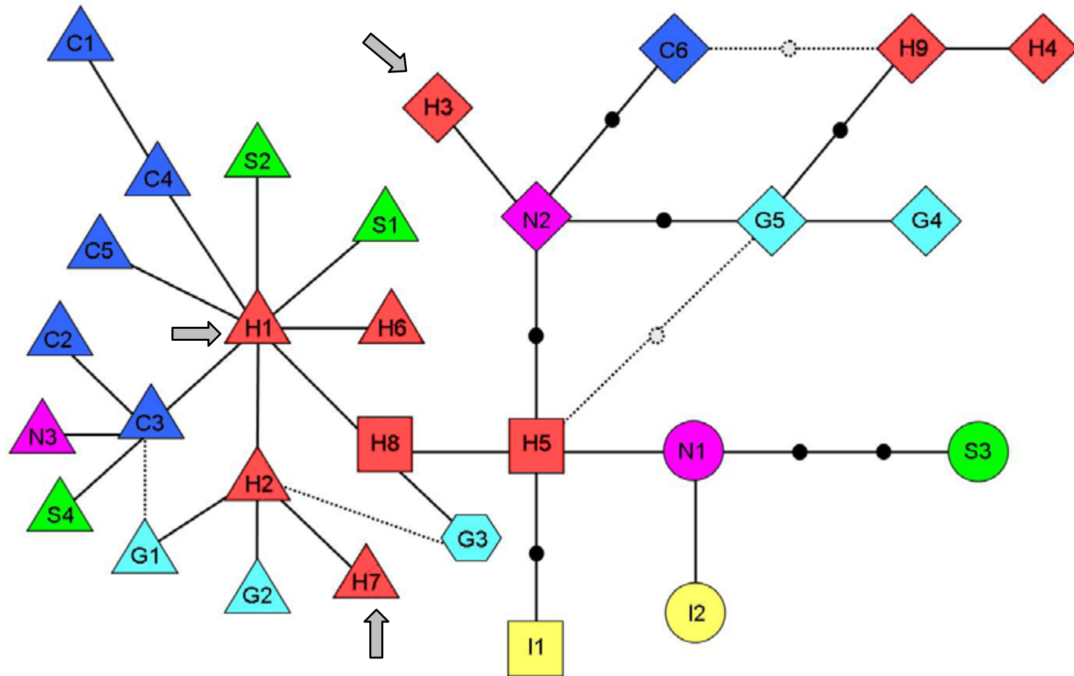


**Figure 2.** The Minimum Spanning Network (MSN). Haplotypes are named (and coloured) after geographic origin: Holarctic (H), Nearctic (N), Canada (C), Siberia (S), Greenland (G) and Iceland (I). Each branch represents one mutational step; missing haplotypes are represented by a dot. Equally parsimonious branches are shown with dashed lines. The shape of the haplotypes illustrates the second nesting level in the Nested Clade Analysis. Haplotype G3 was not nested until the third nesting level. Arrows indicate the three haplotypes found in Scandinavia.

The time of this expansion, as suggested by the mismatch analysis, was 118 000 years before present. Although the confidence interval was large, this time coincides with the end of the last interglacial, which was approximately 5°C warmer than at present (Funder *et al.* 1998). These results indicate that the previous interglacial had a negative impact on arctic foxes, and we therefore propose that arctic foxes respond to the glacial cycles in the opposite way to temperate species (*i.e.* contracting during interglacials and expanding during glaciations).

Several of the haplotypes had a Holarctic distribution and we found no phylogeographic structure (Fig. 2). Furthermore, there was no difference in haplotype diversity between previously glaciated and unglaciated regions, and the $\Phi_{ST}$ values among populations were predominantly low. It therefore seems that there is a gene flow among all sampled populations, with the exception of Iceland (which, as expected, had the highest $\Phi_{ST}$ values). We could however not find a correlation between geographic and genetic distances among the populations. Instead, it seems that there is a particularly high gene flow between arctic fox populations of the "lemming ecotype" (Fig. 3). One possible explanation for this pattern is that "lemming" foxes migrate longer and more frequently than "coastal" foxes, and that there is a reduced fitness in migrants from one type of habitat to the other.

This study also shed further light on the proposed gene flow between Siberia and Fennoscandia (Paper 1). The two haplotypes that previously were unique to Fennoscandia were in this extended study also observed in Siberia, which was to be expected if the haplotypes in Fennoscandia were the result of a gene flow from Siberia. The $\Phi_{ST}$ value between Siberia and Fennoscandia (0.12 in this study) is not particularly high compared to the genetic distances among other arctic fox populations. Thus, these two observations further support the conclusion that there is a gene flow between Siberia and Fennoscandia.

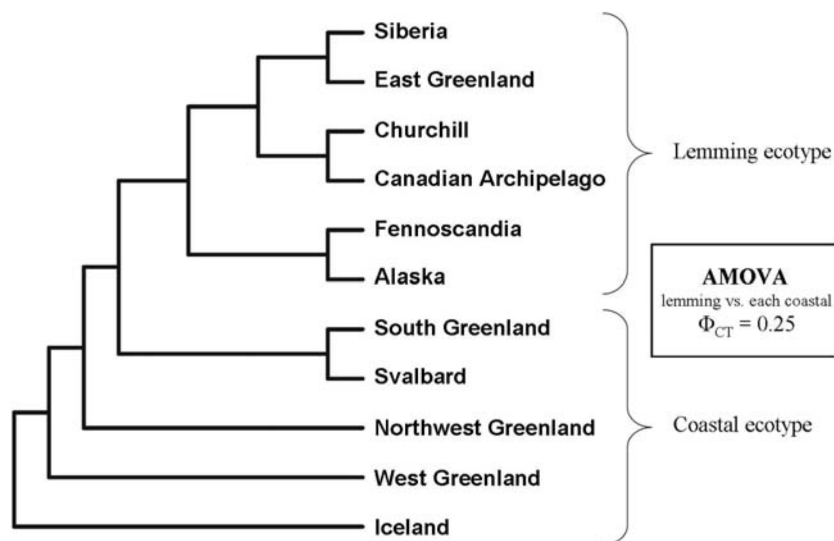


**Figure 3.** Population tree based on $\Phi_{ST}$ values, illustrating the most probable geographical structure in the analysis of molecular variance (AMOVA). The results suggest that there is high gene flow between populations belonging to the "lemming" ecotype, whereas gene flow seems to be lower between populations of the "coastal" ecotype as well as between the two ecotypes.

## PAPER III

Carnivores are usually shy to humans and occur in low densities, which make ecological studies complicated. Endangered carnivores are, more or less by definition, even more problematic. In such cases, faeces can be a very useful source of information, as they can be used for diet analyses and to confirm the presence of the species in a particular area (Kohn & Wayne 1997). Faecal samples can also be used as a source for DNA, giving information about for example population structure (*e.g.* Waits *et al.* 2000) and population size (*e.g.* Kohn 1999). One requirement, however, for these kinds of analyses is that the faeces is correctly assigned to the species in question.

The arctic fox in Fennoscandia is a good example of a species where faeces can be used as a source of information. Arctic fox faeces are also easy to find in the open tundra habitat and on dens. From a conservation perspective, there is a need to confirm the presence of arctic foxes at specific dens in order to direct conservation actions. Furthermore, future microsatellite analyses on the population structure within Fennoscandia will require the use of faeces as a source of DNA. Unfortunately, arctic fox faeces are very similar to faeces from the sympatric red fox and wolverine. To be able to separate faeces from arctic foxes, red foxes and wolverines, we have developed a molecular method based on interspecific mtDNA sequence differences between the three species.

The method is based on the use of three species-specific forward primers, one for each species, and a reverse primer that binds to all three species. The species-specific primers are designed to bind at different distances from the general reverse primer in a PCR (Fig. 4). Thus, the simultaneous application of all four primers on a DNA extract of unknown origin will result in PCR-products of different length depending on which species the faecal sample originates from. This method is very rapid (less than six hours) and circumvents some of the problems associated with earlier methods developed for species identification (*e.g.* Paxinos *et al*. 1997, Palomares *et al*. 2002).

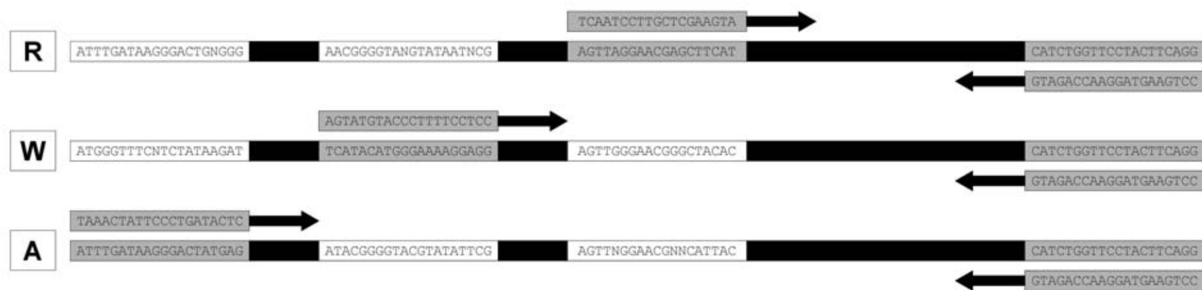


**Figure 4.** Illustration of the species identification method. One of the three species-specific primers will react with the general primer. The resulting fragment size depends on whether red fox (R), wolverine (W) or arctic fox (A) DNA is present in the extract. Intra-specific variable sites in the template are shown as (N).

Using this method, we analysed 128 faecal samples of unknown origin collected throughout Fennoscandia. Of 99 successful samples, 40 were from arctic foxes, 57 from red foxes and two from wolverines. Some samples did not work, probably due to DNA degradation prior to collection, as indicated by the fact that samples collected in summer failed more often than those collected in winter ($\chi^2 = 15.8$, $P = 0.0001$). The results from these analyses have been used to implement conservation actions such as red fox control and supplemental feeding of arctic foxes, as well as to investigate seasonal changes in the spatial distribution of red and arctic foxes (Dalén *et al*. *in prep.*).

## CONCLUDING DISCUSSION

Is there a gene flow from Siberia into Fennoscandia? The number of haplotypes in Fennoscandia is relatively high, both compared to large carnivores in Scandinavia and other arctic fox populations. Similarly, the haplotype diversity in Fennoscandia is "normal" compared to other arctic fox populations (*see* Table 1 in Paper 2), and the $\Phi_{ST}$ value between Siberia and Fennoscandia is not particularly high compared to the genetic distances among other arctic fox populations. Taken together, these results point to that there is a gene flow between Siberia and Fennoscandia. How large this gene flow is in terms of migrants per generation is difficult to assess using mtDNA markers. Further genetic analyses using microsatellite markers will hopefully shed more light on the question of gene flow from Siberia to Fennoscandia.

Is there a Fennoscandian population? That is, do the arctic foxes on the Kola Peninsula actually belong to the same population as the arctic foxes in Sweden, Norway and Finland? It seems that there are additional haplotypes on the Kola Peninsula (Paper 2, Strand *et al*. 1998). This could be due to that the Kola Peninsula is an intermediate area in a "stepping stone" model (Crow & Kimura 1970), but an alternative explanation is that the arctic foxes on the Kola Peninsula belong to the Siberian population, and thus that the main geographic obstacle is the forest between Russia and Finland, rather than the White Sea. If the latter turns out to be the correct scenario, the situation for the arctic foxes in Scandinavia and Finland is more serious than previously thought.

Are there any arctic foxes left in Finland? There has not been a confirmed reproduction in Finland since 1996 (Kaikusalo *et al.* 2000), and the most recent confirmed observation of a wild arctic fox was in 2000, which was run over and killed by a car. During the last three years, we have analysed 40 faecal samples from suspected arctic foxes using the method

described in Paper 3. Not a single one has been from an arctic fox, until last month (October 2003), when we finally could confirm that a faecal sample from an arctic fox had been found close to Lake Inari in northern Finland.

Although the general patterns of how temperate species respond to the glacial cycles can be predicted by the expansion/contraction model (Hewitt 1996), there seems to be little phylogenetic congruence among temperate species (Taberlet *et al.* 1998). That is, temperate species have changed the distributions fairly independently of each other (but not independently of the glacial cycles). One evolutionary consequence of such phylogenetic incongruence is that there would have been less opportunity for long-term coevolution between species (Sullivan *et al.* 2000). The results presented in Paper 2 suggest that arctic species may respond differently to the glacial cycles compared to temperate species (*see also* Flagstad & Røed 2003). Whether there is a higher degree of phylogenetic congruence in arctic species compared to temperate species is unknown, but if there is, it could explain the higher proportion of specialist predators in the Arctic compared to more temperate regions.

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# Paper I

*I några fall har man till och med gått så långt att man uttalat den förhoppningen, att fjällräv från andra håll skulle kunna invandra till trakter, där den förut funnits, men nu ej längre finnes.*

Einar Lönnberg, 1927

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# Is the endangered Fennoscandian arctic fox (*Alopex lagopus*) population genetically isolated?

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**Abstract**

The arctic fox population in Fennoscandia is on the verge of going extinct after not being able to recover from a severe bottleneck at the end of the 19th century. The Siberian arctic fox population, on the other hand, is large and unthreatened. In order to resolve questions regarding gene flow between, and genetic variation within the populations, a 294 bp long part of the mitochondrial hypervariable region 1 was sequenced. This was done for 17 Swedish, 15 Siberian and two farmed foxes. Twelve variable nucleotide sites were observed, which resulted in 10 different haplotypes. Three haplotypes were found in Sweden and seven haplotypes were found in Siberia. An analysis of molecular variance showed a weak, but significant, differentiation between the populations. No difference in haplotype diversity was found between the populations. A phylogenetic analysis revealed that the three Swedish haplotypes were not monophyletic compared to the Siberian haplotypes. These results indicate a certain amount of gene flow between the two populations, both before and after the bottleneck. Restocking the Fennoscandian population with arctic foxes from Siberia might therefore be a viable option. © 2002 Elsevier Science Ltd. All rights reserved.

*Keywords:* Genetics; Phylogeography; *Alopex lagopus*; Threatened; Conservation

## 1. Introduction

The arctic fox [*Alopex lagopus* (L.)] is a small carnivore inhabiting the tundra region of the northern hemisphere, including Svalbard, Iceland, Greenland, North America, Siberia and Fennoscandia. In Fennoscandia, arctic foxes inhabit the mountain and tundra regions of Sweden, Norway, Finland and the Kola Peninsula. The major prey species for most arctic foxes are lemmings (*Dicrostonyx* spp. and *Lemmus* spp.) (Elmhagen et al., 2000). These lemmings fluctuate in numbers on a 3–5 year basis, and the arctic fox population follows these cycles closely (Angerbjörn et al., 1995). As a result of this, the arctic foxes in areas with cyclic lemmings have adopted a reproductive strategy with a large litter size, up to 19 young (Ovsyanikov, 1993; Tannerfeldt and Angerbjörn, 1998). However, only a few of all cubs survive their first year and the adult mortality is ca. 50% (Tannerfeldt et al., 1994). Although both sexes migrate and some individuals have been found as far as 1000 km from their natal sites (Pulliainen, 1965), the Fennoscandian population has been regarded as relatively isolated from other arctic fox populations, owing to the ice-free coasts of Norway and parts of the Kola Peninsula. The taiga forest in western Russia, stretching as far north as the White Sea, also constitutes a geographic barrier between Fennoscandia and Siberia.

In Siberia, no geographical barriers seem to exist for arctic foxes. Even for lemmings, genetic studies have revealed only two geographical barriers in all of Siberia: the Lena and Kolyma rivers (Fedorov et al., 1999). Arctic foxes, however, would have no problems crossing these rivers, at least in winter. Given the homogeneity of the Siberian tundra and the extensive migrations of arctic foxes, the foxes in Siberia could therefore be regarded as a single population.

At the end of the 19th century, the Fennoscandian population declined drastically as a result of heavy hunting pressure. Between the end of the 19th century and 1926, Swedish arctic foxes are believed to have declined from ca. 4700 to 100–150 adults and were therefore protected by law in Sweden in 1928 (Lönnberg,

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1927; Hersteinsson et al., 1989; Tannerfeldt, 1997). Despite this, they failed to increase in numbers. In the last 20 years the situation has deteriorated even further due to an absence of lemming peaks since 1982 (Angerbjörn et al., 2001). Today there are ca. 40 adult individuals in Sweden, 40 in Norway and 10 in Finland. In the Kola Peninsula the situation is uncertain but a rough estimate is ca. 40 adults. Thus, the Fennoscandian population is composed of a mere 100–130 adults, in contrast to the hundreds of thousands of arctic foxes in Siberia (Nasimovich and Isakov, 1985). The low population size in the last 100 years has most likely led to inbreeding. Whether this in turn has led to inbreeding depression is unknown, but a study made by Nordrum (1994) on farmed arctic foxes indicated that inbreeding might result in reduced litter size. From a conservation perspective, the questions regarding inbreeding in the Fennoscandian population are important to resolve. However, only one genetic study has been made on wild arctic foxes, of which only the main results have been published (Strand et al., 1998). Apparently, three haplotypes were found in foxes from Norway and four haplotypes were found on the Kola Peninsula.

When performing intraspecific genetic studies it is important to choose a genetic marker suited to the question at hand. For phylogeographic and phylogenetic studies, mitochondrial DNA (mtDNA) displays properties that are almost ideal (Avise et al., 1987). In the mitochondrial genome, the hypervariable region 1 (HVR 1), also known as control region 1, evolves at a rapid pace. For that reason, HVR 1 has been frequently used as a genetic marker in several intraspecific studies on mammals (Lyrholm and Gyllensten, 1998; Randi et al., 1998, 2000; Slade et al., 1998; Wilson et al., 2000). However, it is important to note that results from mitochondrial studies only reveal the mitochondrial genetic structure, and that this is not necessarily the same as nuclear genetic structure (Taberlet et al., 1995; Waits et al., 2000).

The purpose of this study was to investigate the genetic relationship between Siberian and Fennoscandian arctic foxes, using mtDNA HVR 1 sequences. Given the low population size over the last 100 years in Fennoscandia compared to the large population size in Siberia, there are three different scenarios regarding isolation. (1) The Fennoscandian and Siberian populations have been isolated from each other both before and after the bottleneck event. (2) The Fennoscandian and Siberian populations have not been isolated, either before or after the bottleneck event. (3) The populations became isolated after the bottleneck event.

For the first alternative, a number of predictions can be made: (a) The Swedish arctic foxes should display fewer haplotypes than the Siberian population. In computer simulations of ideal stable sized populations with a variance of five in progeny numbers, all individuals invariably stemmed from a single female ancestor less than $2n$ generations earlier, where $n$ is the number of founding females (Avise et al., 1987). The high variance in lifetime reproductive success (Tannerfeldt and Angerbjörn, 1996) and the fluctuating population dynamics (Angerbjörn et al., 1995) of the Fennoscandian arctic foxes could be expected to reduce that time, possibly to the extent that only one haplotype should be expected in Sweden. (b) If more than one haplotype is found in Sweden, these should be monophyletic compared to Siberian haplotypes. (c) The haplotype diversity (Nei, 1987) should be significantly lower in Sweden than in Siberia.

The second alternative is the direct opposite of the first alternative. Thus, a rejection of all predictions stated above would indicate a lack of isolation both before and after the bottleneck event.

The final alternative is that the degree of isolation may have changed as a result of the bottleneck event. It seems unlikely that the populations were isolated before the bottleneck but not after. The opposite, however, might be plausible as a result of lowered survival in migrants from Siberia to Sweden, especially if the Kola Peninsula is regarded as being an intermediate area in a "stepping stone" model (Crow and Kimura, 1970). In this case, the Swedish haplotypes would not be expected to be monophyletic, but the haplotype diversity and the number of haplotypes should still be low due to isolation for 100 years.

## 2. Materials and methods

### 2.1. Samples

Between 1989 and 1998, fieldworkers and researchers in the Swedish Arctic Fox Project (Angerbjörn et al., 1991, 1995; Tannerfeldt and Angerbjörn, 1996) have caught and ear-tagged a total of 176 arctic foxes. A by-product of the ear tagging procedure is a circular piece of ear tissue ca. 4 mm in diameter. These pieces of ear tissue have either been dried or suspended in 95% ethanol and stored at −80 °C. During capture many foxes defecate, and samples from such faeces have also been collected and stored at −80 °C. Additionally, muscle tissue has been retrieved from animals found dead. Since capture and tagging take place at dens where usually several siblings are tagged, randomly choosing from the total sample could be regarded as being biased. Instead, 20 dens were randomly chosen and from each of these one individual was sampled for the analysis. A blood sample from a suspected farmed fox caught in Skellefteå was also collected, as well as a muscle tissue sample from a farmed fox kept at Tovetorp zoological research station.

In 1994, an expedition along the Siberian coast was organised by the Swedish Polar Research Secretariat

(Hedberg et al., 1999). During this expedition, we collected skin and tissue samples from 20 arctic foxes found dead on the tundra (Fig. 1), and stored them at −80 °C. For a complete listing of the individuals used in the study, see Table 1.

### 2.2. Extraction

Two to three mg of the tissue samples, or 100 mg of faeces, (dry weight) were digested in 100 μl PCR-buffer and 0.1 mg protienase K at 56 °C for 3 h with sporadic agitation. When extracting from faeces, 50 μl of 100 mM *N*-phenacylthiazolium bromide was also added prior to incubation in order to dissolve cross-bindings in DNA and proteins, induced by reductive carbohydrates (Vasan et al., 1996). After incubation, 500 μl of lysis buffer (Götherström and Lidén, 1996) was added to the solution which was subsequently incubated at 60 °C for 1 h. Following centrifugation at 12,000 rpm for 5 min, 600 μl lysis buffer and 40 μl silica suspension were mixed with the supernatant. The remaining extraction followed the protocol of Götherström and Lidén (1996).

Table 1
Individuals used in the study[a]

| Haplotype | ID number | Sample year | Tissue | Origin | Sample site |
|---|---|---|---|---|---|
| Hol 1 | 7 | 1998 | m | SW | 1 |
| Hol 1 | 11 | 1994 | m | SI | 8 |
| Hol 1 | 12 | 1994 | m | SI | 4 |
| Hol 1 | 13 | 1994 | s | SI | 7 |
| Hol 1 | 14 | 1994 | s | SI | 9 |
| Hol 1 | 21 | 1994 | s | SI | 6 |
| Hol 1 | 24 | 1994 | ds | SI | ? |
| Hol 1 | 25 | 1994 | ds | SI | 9 |
| Hol 1 | 26 | 1994 | ds | SI | 8 |
| Hol 1 | 27 | 1994 | ds | SI | 5 |
| Hol 1 | 36 | 1998 | e | SW | 1 |
| Hol 1 | 37 | 1990 | e | SW | 1 |
| Hol 1 | 41 | 1998 | e | SW | 1 |
| Hol 1 | 45 | 1995 | f | SW | 1 |
| Hol 1 | 46 | 1998 | f | SW | 1 |
| Sib 1 | 16 | 1994 | s | SI | 10 |
| Sib 2 | 17 | 1994 | m | SI | 6 |
| Sib 3 | 8 | 1994 | m | SI | 6 |
| Sib 4 | 19 | 1994 | s | SI | 3 |
| Sib 5 | 20 | 1994 | s | SI | 7 |
| Sib 6 | 23 | 1994 | m | SI | 6 |
| Swe 1 | 33 | 1995 | e | SW | 1 |
| Swe 1 | 38 | 1989 | e | SW | 1 |
| Swe 1 | 40 | 1990 | e | SW | 1 |
| Swe 2 | 1 | 1993 | m | SW | 1 |
| Swe 2 | 5 | 1996 | m | SW | 2 |
| Swe 2 | 6 | 1995 | m | SW | 1 |
| Swe 2 | 34 | 1996 | e | SW | 1 |
| Swe 2 | 35 | 1995 | e | SW | 1 |
| Swe 2 | 39 | 1994 | e | SW | 1 |
| Swe 2 | 42 | 1996 | e | SW | 1 |
| Swe 2 | 43 | 1996 | e | SW | 1 |
| FF 1 | 32 | 1996 | m | FF | – |
| FF 1 | 44 | 1900 | b | FF | – |

[a] Haplotype numbers as in Table 3. Tissue used in the extraction: muscle (m), skin (s), dry skin (ds), ear tissue (e), faeces (f) and blood (b). Origin of each individual: Sweden (SW), Siberia (SI) and farmed fox (FF). Haplotypes are named after origin: Holarctic (Hol), Sweden (Swe), Siberia (Sib) and farmed fox (FF). Sample sites are as in Fig. 1.

### 2.3. Amplification and sequencing

Two mammalian specific primers (H1F and H3R) were used to amplify a ca. 350 base pair (bp) long fragment of HVR 1 in the mitochondrial control region (Table 2). When a sequence had been obtained, this was used to design a specific primer, Pex1F, which in turn was used in combination with H3R in order to amplify an approximately 320 bp long fragment of HVR 1.

Amplifications were performed in 25 μl reactions using the polymerase chain reaction method (PCR), and

Table 2
Primer sequences[a]

| Primer | Sequence |
|---|---|
| H1F | 5′-GCCATCAACTCCCAAAGCT-3′ |
| H3R | 5′-CCTGAAGTAGGAACCAGATG-3′ |
| Pex1F | 5′-TAAACTATTCCCTGATACTC-3′ |

[a] The two mammalian specific primers H1F and H3R, and the arctic fox specific primer Pex1F.

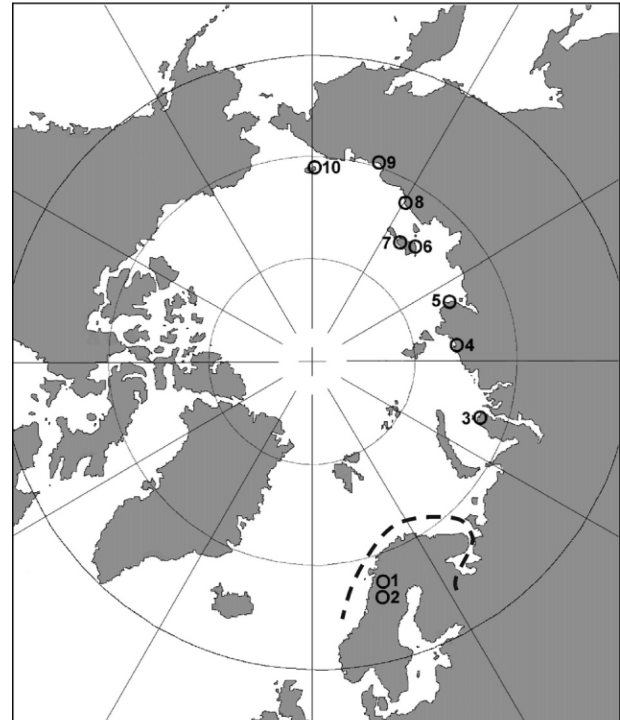


Fig. 1. Sample sites in Sweden and Siberia: (1) Vindelfjällen ($n=16$); (2) Stekenjokk ($n=1$); (3) Northern Yamal Peninsula ($n=1$); (4) NW of Taymyr Peninsula ($n=1$); (5) NE of Taymyr Peninsula ($n=1$); (6) Faadeevksy Island ($n=4$); (7) Kotelny Island ($n=2$), (8) Indigirka ($n=2$), (9) Kolyma Delta ($n=2$), (10) Wrangel Island ($n=1$). The dotted line represents the presumed genetic barrier.

included genomic DNA, 0.2 mM of each nucleotide, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 μM of each primer, 10 × PCR Gold Buffer and 0.75 units of AmpliTaq Gold polymerase (Perkin-Elmer Cetus). The cycling parameters for the PCR-reaction were: 93 °C denaturation for 10 min, followed by 35 cycles of 94 °C denaturation for 20 s, 50 °C annealing for 30 s, and 72 °C extension for 20 s, followed by a single 7-min final extension at 72 °C. 5 μl of the PCR-product was electrophoresed on a 1.2% agarose gel in 1 × TAE buffer in order to investigate the success of the amplification. The amplifications that were considered successful were subsequently purified using the QIAquick Spin PCR purification kit (Qiagen). Some samples were either lost during purification or displayed too low concentration, and had to be reamplified for 25 cycles. During extraction and amplification, blanks were used as contamination control.

Both the heavy and the light strand of the purified amplicons were sequenced using the Big Dye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions. The resulting sequences were aligned using the MacVector/AssemblyLIGN software-hardware package (International Biotechnologies, 1989), followed by visual verification. Identical sequences were assigned to haplotypes, which were named after origin: Sweden (Swe), Siberia (Sib) and farmed fox (FF). Any haplotypes found in both Sweden and Siberia were named Hol (from Holarctic).

### 2.4. Sequence analysis

Population genetic analyses were computed using the software Arlequin (Schneider et al., 2000). Phylogenetic trees were assembled in PAUP 4.0b3a (Swofford, 1999). The phylogenetic trees were rooted with a red fox [*Vulpes vulpes* (L.)] sequence obtained from genebank (accession #AF098155). Maximum parsimony trees were assembled using a heuristic search with TBR-swapping and addition of ten random sequences (2000 permutations). Maximum likelihood trees were assembled using the quartet puzzling algorithm (10 000 permutations). Branch support is given as reliability values (Strimmer and von Haeseler, 1996).

Haplotype diversity ($H$) and nucleotide diversity ($\pi_n$) were calculated according to Nei (1987):

$$H = (n/n-1)\left(1-\sum_{i=1}^{k} p_i^2\right)$$

where $n$ is the number of gene copies in the sample, $k$ is the number of haplotypes, $p_i$ is the frequency of the $i$th haplotype, $d_{ij}$ is an estimate of the number of mutations having occurred since the divergence of haplotypes $i$ and $j$, and $L$ is the number of bases.

Geographical structuring, measured with $\Phi_{ST}$ statistics (a measure analogous to $F_{ST}$), was calculated in an AMOVA (analysis of molecular variance; Excoffier et al., 1992), using the distance method of Tajima and Nei (1984). Significance of variance estimates was obtained with a randomisation procedure (1000 permutations). Maternal gene flow ($N_f m_f$) between populations was calculated from the equation:

$$\Phi_{ST} = 1/(2N_f m_f \alpha + 1)$$

where $\alpha = (n/(n-1))^2$ and $n$ is the number of populations exchanging migrants (as in Slade et al., 1998).

The purpose of the AMOVA analysis was to investigate if it is possible to define the arctic foxes in Sweden as belonging to a population genetically differentiated from the Siberian arctic foxes. The maternal gene flow ($N_f m_f$) gives a theoretical value for how many females migrate between the two populations. These measures may help in assessing whether the Fennoscandian population should be regarded as an evolutionary significant unit, and therefore if restocking from Siberia is a viable option.

## 3. Results

Alignment of the heavy and light strands resulted in a 294 bp long consensus sequence, where base number one corresponds to base number 16 465 according to the numbering introduced by Arnason and Johnsson (1992). This was successfully done for 34 individuals of which 15 were from Siberia, 17 were from Sweden and two were farmed foxes. Twelve variable positions were found in this region. Based on those variable positions all 34 individuals were assigned to one of 10 different mtDNA haplotypes (Table 3). Three haplotypes were found in Sweden whereas seven haplotypes were found in Siberia. The two farmed arctic foxes displayed a unique haplotype (FF 1). The most common haplotype, Hol 1, was found in 15 of the samples (47% of the wild foxes) in both Siberia and Sweden. The other haplotypes were unique to one or other of the two populations. In the Siberian and Swedish samples, 10 substitutions and three indels were observed. Of the substitutions eight were transitions and two were transversions. For a complete listing of all haplotypes found and their observed frequency in each population, see Table 4. The nucleotide composition (heavy strand) in the total sample was A: 28.21%, G: 14.73%, C: 26.16% T: 30.94%.

The haplotype diversity (H ± S.D.) was estimated as 0.657 ± 0.138 in Siberia and 0.662 ± 0.0652 in Sweden (Fig. 2). The nucleotide diversity ($\pi_n$ ± S.D.) was 0.56 ± 0.39% (Siberia) and 0.78 ± 0.50% (Sweden).

Analysis by AMOVA revealed a significant differentiation of the Siberian and Swedish arctic foxes

($P = 0.01$), although only less than one fifth of the variation could be attributed to differences between the populations ($\Phi_{ST} = 0.174$). The number of females exchanged between the populations per generation was estimated from the $\Phi_{ST}$ values obtained in the AMOVA. This gave a value of 0.59 female migrants per generation. Since arctic foxes do not display the properties of an ideal population (for example random mating), this is likely to be an overestimate.

A maximum parsimony analysis of the ten haplotypes, where all uninformative characters were excluded and all informative characters were equally weighted, resulted in 57 trees of equal length (17 steps). Only one branching event was supported by bootstrap analysis. In order to obtain a better resolution on the phylogenetic tree, a maximum likelihood analysis was performed, using the quartet puzzling algorithm. In the tree obtained by this method (Fig. 3) the Swedish haplotypes were separated from each other by strong reliability values, and were not grouped together in a monophyletic group. This means that each haplotype found in Sweden is more closely related to a Siberian haplotype than to any of the other Swedish haplotypes, indicating that the Fennoscandian population has not evolved independently from the Siberian.

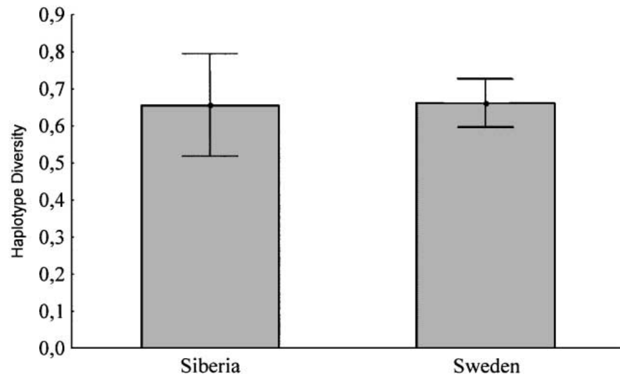


Fig. 2. No significant difference in haplotype diversity was found between Siberia ($n = 15$) and Sweden ($n = 17$). The error bars represent standard deviation.

Table 3
Mitochondrial DNA haplotypes[a]

| | Variable sites | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | 15 | 22 | 23 | 58 | 131 | 134 | 139 | 212 | 219 | 220 | 271 | 278 |
| Hol 1 | C | – | – | T | T | C | T | T | C | T | T | T |
| Sib 1 | • | – | – | A | • | • | • | • | • | • | • | • |
| Sib 2 | – | – | – | C | A | • | C | C | • | C | • | C |
| Sib 3 | – | – | – | • | • | • | • | C | • | C | C | C |
| Sib 4 | • | – | – | • | • | • | • | • | • | C | • | • |
| Sib 5 | • | A | A | • | • | • | • | • | • | • | • | • |
| Sib 6 | – | – | – | • | • | • | • | • | • | • | • | C |
| Swe 1 | • | – | – | • | • | • | • | • | T | C | • | • |
| Swe 2 | – | – | A | • | • | T | C | • | • | • | • | C |
| FF 1 | – | – | – | C | A | • | C | • | • | C | | C |

[a] Haplotypes as defined by variable sites. The haplotype Hol 1 is written in its entirety with two deletions (–). Subsequent matching nucleotides are indicated by dots.

Table 4
Estimated haplotype frequencies[a]

| | Sweden | | | Siberia | | | Genebank accession # |
|---|---|---|---|---|---|---|---|
| Haplotype | *f* | S.D. | *n* | *f* | S.D. | *n* | |
| Hol 1 | 0.35 | 0.1 | 6 | 0.60 | 0.1 | 9 | AF365959 |
| Sib 1 | – | – | – | 0.07 | 0.1 | 1 | AF365961 |
| Sib 2 | – | – | – | 0.07 | 0.1 | 1 | AF365962 |
| Sib 3 | – | – | – | 0.07 | 0.1 | 1 | AF365963 |
| Sib 4 | – | – | – | 0.07 | 0.1 | 1 | AF365964 |
| Sib 5 | – | – | – | 0.07 | 0.1 | 1 | AF365965 |
| Sib 6 | – | – | – | 0.07 | 0.1 | 1 | AF365966 |
| Swe 1 | 0.18 | 0.1 | 3 | – | – | – | AF365967 |
| Swe 2 | 0.47 | 0.1 | 8 | – | – | – | AF365968 |
| FF 1 | – | – | (2) | – | – | – | AF365960 |

[a] Estimated frequencies (f), standard deviations (S.D.) and number of individuals (*n*) for each haplotype.

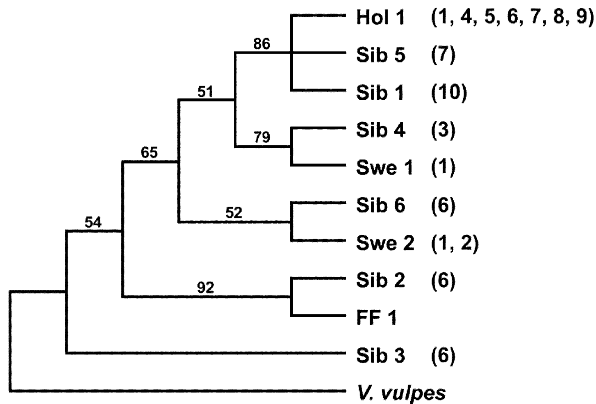


Fig. 3. The maximum likelihood tree was constructed using the Quartet Puzzling algorithm. Reliability values are given at the nodes. The three Swedish haplotypes are separated by strong reliability values. Branches with reliability values below 50% are collapsed. Sites where each haplotype was found are given in parantheses, numbered as in Fig. 1.

## 4. Discussion

The results from the AMOVA indicate a significant, but weak, genetic differentiation between Siberian and Swedish arctic foxes. Although the level of maternal gene flow should be interpreted with caution, since this is a very rough estimate that does not necessarily correspond to the present migration rate between the two populations, it still seems that there may be a certain amount of gene flow between the Siberian and the Fennoscandian populations. This is not what would be expected if the Fennoscandian population is, and has been, isolated from the Siberian population.

Scenario (1) led to the prediction of few haplotypes in Sweden. How many haplotypes should be expected is difficult to ascertain. Given a starting population of some 100 females, the high variance in lifetime reproductive success and the fluctuating population size of Fennoscandian arctic foxes, the time until all females stem from one single ancestor through genetic drift could be expected to be less than 200 generations (Avise et al., 1987). The probability of finding three haplotypes that have survived from a pre-bottleneck number of, for example, 10–20 haplotypes might therefore be expected to be small, but perhaps not unreasonably small. Since the three Swedish haplotypes differ from each other at two, five and seven nucleotide sites respectively (Table 3), it is unlikely that these haplotypes have arisen through mutation during the post-bottleneck period. Thus, the three haplotypes observed in Sweden are either the result of recent immigration from Siberia or they are pre-bottleneck haplotypes that have survived to the present day. This leads to the next prediction.

If the haplotypes observed in Sweden are pre-bottleneck haplotypes, and the Fennoscandian population is, and has been, isolated from the Siberian population, then the three haplotypes were expected to be monophyletic compared to the Siberian haplotypes. The maximum parsimony tree only had bootstrap support for one branching event. This branch included one of the Siberian and the farmed fox haplotypes, making it difficult to draw any conclusions from this tree. The maximum likelihood tree using the quartet puzzling algorithm, however, gave a higher resolution (Fig. 3). In this tree, the three Swedish haplotypes were separated from each other by strong reliability values. The quartet puzzling method has been used in other studies on mtDNA (Leonard et al., 2000; Randi et al., 2000) but has also received criticism for not always obtaining the highest likelihood tree (Cao et al., 1998). Nonetheless, neither of the trees support the prediction of monophyly in the Swedish haplotypes. If they were pre-bottleneck haplotypes, this would indicate that the populations were not isolated from each other before the bottleneck event. The other possibility is that the observed haplotypes in Sweden were the result of recent immigration from Siberia, and then it is reasonable to assume that there was gene flow prior to the bottleneck as well. This, in turn, leads to the final prediction.

The two populations may have become isolated from each other as a result of the bottleneck event and following fragmentation in the Fennoscandian population. If this were the case, the Swedish arctic foxes would be expected to display relatively low haplotype diversity. Since there is no difference between the observed haplotype diversities (Fig. 2) this indicates that there may still be a certain gene flow between the populations. However, this conclusion should be interpreted with caution since nothing is known of the nuclear genetic variation in Fennoscandian arctic foxes and since the observed haplotype diversities may be misleading because of the small sample size.

In the study mentioned earlier (Strand et al., 1998), the three haplotypes found in Norway seem to correspond to the three found in our study from Sweden. The haplotype that is referred to as Hol 1 in this study was in the Norwegian study also found in both Siberia and Fennoscandia. It therefore seems likely that only three haplotypes exist in Scandinavia. Another finding in the Norwegian study was low nucleotide diversity in Siberia, which is confirmed by this study. The most interesting result of the Norwegian study was perhaps the observation of four haplotypes on the Kola Peninsula. Two of these haplotypes have not been observed elsewhere in Fennoscandia. That finding, in combination with the results in this study, suggest that there may be a contact zone at the Kola Peninsula between the Fennoscandian and Siberian populations.

An additional result from this study is that we have confirmed that it is possible to extract DNA from pieces of ear tissue (of which there are samples from nearly 200 Swedish arctic foxes) and even from faeces. This presents an opportunity to track specific individuals, perform

tests on paternity and to investigate the relationship between individual genetic variation and, for example, litter size. It may also be possible to estimate population sizes (Kohn et al., 1999).

In conclusion, this study has shown that arctic foxes in Sweden and Siberia seem to be separated into two genetically identifiable populations. However, the relatively high haplotype diversity, high number of haplotypes and low $\Phi_{ST}$ value in Sweden in combination with the apparent lack of monophyly in the Swedish haplotypes indicate that there is, and historically has been, a certain amount of gene flow between the Fennoscandian and Siberian populations.

These results have implications for the future management of the arctic fox population in Fennoscandia. The population does not seem to constitute an evolutionary significant unit. Although this means that a useful conservation argument has been lost, it also implies that it may be possible to restock the Fennoscandian population with arctic foxes from Siberia. Such a restocking could help counter the effects of inbreeding depression and low genetic variation. Therefore, the genetic variation and effects of inbreeding need to be investigated further using nuclear genetic markers. Another issue of concern is the possibility of hybridisation between wild and farmed arctic foxes. We have shown that, using genetic techniques, it is possible to identify whether an animal is an escaped farmed fox or a wild arctic fox. We have not found any evidence that hybridisation between farmed and wild arctic foxes has taken place, although this needs to be confirmed with paternally inherited markers. More farmed foxes also need to be sampled.

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# Paper II

*I was not a little surprised yesterday morning when I suddenly saw the track of an animal in the snow. It was that of a fox...*
*What in the world was that fox doing up here? Why do they leave the coasts? That is what puzzles me the most. Can they have gone astray?*

Fridtjof Nansen, polar ice pack 85$^{O}$N, 1895

# Population History and Genetic Structure of a Circumpolar Species: the Arctic Fox

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**Abstract**
The circumpolar arctic fox, *Alopex lagopus*, thrives in cold climate and has a high migration rate involving long distance movements. Thus, it differs from many temperate taxa that have been subjected to cyclical restriction in glacial refugia during the Ice Ages. We investigated population history and genetic structure through mitochondrial control region variation in 191 arctic foxes from throughout the Arctic. Several haplotypes had a Holarctic distribution and no phylogeographic structure was found. Furthermore, there was no difference in haplotype diversity between previously glaciated and unglaciated regions. This suggests a current gene flow among the studied populations, except Iceland, which is surrounded by year-round open water. Arctic foxes have often been separated in two ecotypes, "lemming" and "coastal". An analysis of molecular variance suggested a particularly high gene flow among populations of the "lemming" ecotype. This could be explained by their higher migration rate and a reduced fitness in migrants between ecotypes. A mismatch analysis indicated a sudden expansion in population size 118 000 BP, which coincides with the last interglacial. We propose that the arctic fox has been affected by glacial cycles in the opposite way to temperate species, with interglacials leading to short-term isolation in northern refugia.

**Keywords:** *Alopex lagopus*, mitochondrial DNA, phylogeography, gene flow, bottleneck, ecology.

## Introduction

The Quaternary cold periods are considered to have had strong influence on the geographic distribution and genetic variation of organisms worldwide. In continental Eurasia and North America, repeated glaciations caused multiple cycles of isolation in southern refugia and resulted in increased intraspecific genetic divergence (Taberlet *et al.* 1998, Hewitt 2001). Several mammal species display phylogeographic patterns predicted by the expansion/ contraction model with, for example, a high divergence between phylogroups from different refugia and genetic signatures of late Pleistocene expansions in population size (Hewitt 1996). However, in highly mobile species gene flow during interglacials could lead to an admixture of genotypes from different refugia (Cruzan & Templeton 2000). Furthermore, the impact of glaciation would have been different in species that were well adapted to cold climates than in temperate species (Hewitt 2001). Arctic species will not have been in southern temperate refugia and should thus not display the expansions/ contractions associated with them. Arctic species

may, however, have gone through range changes and they could have had different glacial and/or interglacial refugia.

The arctic fox (*Alopex lagopus*) is well adapted to arctic conditions (Fuglei and Øritsland 1999) and tolerates ambient temperatures below –40° C in winter fur without having to increase its metabolic rate significantly to keep the body temperature constant (Scholander 1950). Its diet is composed of a variety of vertebrates (Audet *et al.* 2002), but two ecotypes are generally recognised: "lemming foxes" that mainly feed on lemmings (*Lemmus spp.* and *Dicrostonyx spp.*) and "coastal foxes" that mainly feed on eggs and birds and carrion from the marine system (Bræstrup 1941). The difference between a highly fluctuating food source (lemming) and one that is more stable (coastal) has led to a number of different life history strategies, where "lemming foxes" undergo an enormous reproductive output during lemming peaks compared to "coastal foxes" (Tannerfeldt & Angerbjörn 1998). Furthermore, there are significant differences in migration patterns between the two ecotypes, where "lemming foxes" migrate further than "coastal foxes" (Angerbjörn *et al.* 2003a).

Arctic foxes have a circumpolar distribution and inhabit the tundra regions of North America and Eurasia as well as most arctic islands, including Greenland, Iceland and Svalbard (Audet *et al*. 2002). Several studies suggest a high migration rate in arctic foxes, and that they are capable of long (>1000 km) movements over the polar pack ice (*e.g.* Eberhardt & Hanson 1978). Several subspecies have been proposed for arctic foxes, for example *A. l. fuliginosus* (Iceland), *A. l. groenlandicus* (Greenland), *A. l. spitzbergenensis* (Svalbard) and *A. l. ungava* (Canada) (Audet *et al.* 2002). Frafjord (1993) found some latitudinal differences in morphology between populations on a circumpolar scale, but pointed out that more information was needed on the genetic differentiation among arctic fox populations.

In the present study, we analyse mitochondrial DNA (mtDNA) variation in arctic foxes on a circumpolar scale to investigate the genetic structure and population history of the species. Concerning the population history, we do not expect to find the patterns of a rapid post-glacial increase in population size which have been observed in more temperate species, since the wide distribution of arctic foxes during the last Ice Age (Kurtén 1968, Kurtén & Anderson 1980) suggests that arctic foxes were at least as abundant during this period as they are today. Instead, it is more probable that the warm interglacials have had a negative effect on the abundance of arctic foxes. We do, however, expect to see phylogeographical patterns from a post-glacial range expansion in the arctic fox since they must have colonised formerly glaciated areas at the end of the last Ice Age.

Past fragmentation events may be inferred from genetic distance among haplotypes, and their spatial distribution provides information on current gene flow among populations (Avise *et al.* 1987). Based on the high migration rate and long distance movements observed in arctic foxes, we hypothesise that there is gene flow between all sampled populations, with the exception of Iceland, which is not connected to other populations via the sea ice. We therefore predict little phylogeographic structure, low $\Phi_{ST}$ values between all populations except Iceland and that previously glaciated regions should have haplotype diversity similar to those continuously unglaciated. We also examine the long-term effective female population size and compare this to current estimates of the worldwide population size.

## Materials and Methods

DNA samples were collected from 191 arctic foxes from 13 regions throughout the Arctic. The regions sampled were Svalbard (SVA), Iceland (ICE), East Greenland (EG), South Greenland (SG), West Greenland (WG), Northwest Greenland (NWG), Churchill Manitoba (CHU), Cambridge Bay (CMB), Bathurst Island (BAT), Banks Island (BAN), Alaska (ALA), Siberia (SIB) and Fennoscandia (FEN) (Fig. 1). For the statistical analyses concerned with geographical distances, we divided Siberia into East and West Siberia (ES and WS respectively; two samples from Taimyr, which is halfway between ES and WS, were excluded from these analyses along with one sample where it was not clear whether it was from ES or WS). Cambridge Bay, Banks Island and Bathurst Island were in some instances pooled into Canadian Archipelago (CA) in order to increase statistical power (there was no significant genetic differentiation among the regions within each pooling). Thirty-two of the samples are from the previous study by

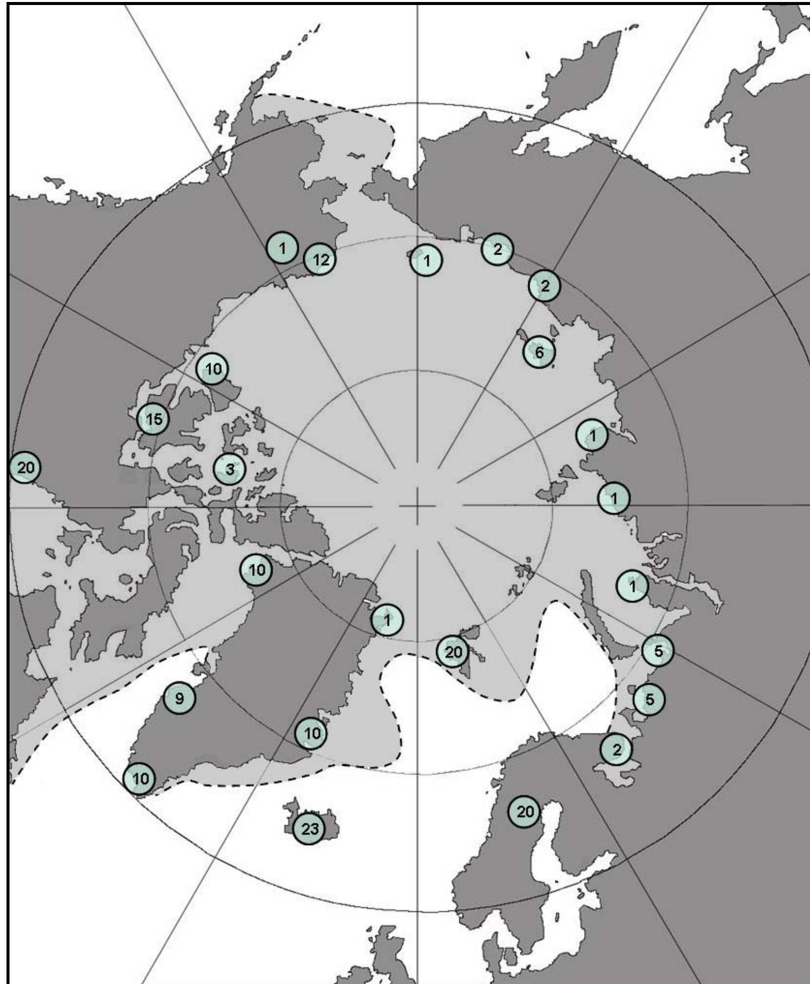


**Figure 1.** Sample sites and number of samples (indicated within each circle) from each location. Populations clockwise from Greenwich Mean Time are: Iceland (ICE), East Greenland (EG), South Greenland (SG), West Greenland (WG), Northwest Greenland (NWG), Churchill (CHU), Bathurst Island (BAT), Cambridge Bay (CMB), Banks Island (BAN), Alaska (ALA), Siberia (SIB), Fennoscandia (FEN) and Svalbard (SVA). The light grey area inside the dashed line illustrates the extent of polar sea ice in January (data from EOSDIS NSIDC Distributed Active Archive Center).

Dalén *et al.* (2002). The samples from Greenland are those previously used for microsatellite analysis by Meinke *et al.* (2001). Tissue samples from Alaska were obtained from the University of Alaska Museum (UAM AF371-AF377, AF379, AF4012-AF4014, AF 4039, AF21094).

Whole genomic DNA was extracted using Qiagen's Dneasy tissue kit (Qiagen). Faecal DNA (n=4) was extracted from *c.* 200 mg of dried faecal matter using the Qiaamp DNA stool mini kit (Qiagen). An approximately 320 bp long fragment of the mitochondrial control region was amplified as previously described in Dalén *et al.* (2002). Sequencing of both the heavy and light strand was carried out using a CEQ 2000 automated sequencer (Beckman Coulter) following the manufacturer's instructions.

Sequences were aligned in BioEdit version 5.0.9 (Hall 1999), checked by eye and assigned to haplotypes, which were named after origin (see Fig. 2). We used the program ModelTest (Posada & Crandall 1998) to evaluate which model of nucleotide substitution gave the best fit to the data. Sequence variability and population pairwise comparisons were computed with the software ARLEQUIN version 2.0 (Schneider *et al.* 2000). Of the nucleotide substitution models supported in Arlequin, the Tamura & Nei (1993) model gave the lowest log likelihood score (with a gamma parameter of 0.7), and this was subsequently used in further analyses. Sequence variability was estimated as haplotype diversity

(H), nucleotide diversity ($\pi$; Nei 1987) and the mean number of pairwise differences (Tajima 1993). Historic demographic expansions of population size were investigated through a mismatch analysis where the distribution of pairwise differences was compared to the expected distribution under a model of sudden expansion (Rogers & Harpending 1992, Schneider & Excoffier 1999). The estimated time of sudden expansion can be calculated from the equation $\tau = 2\mu t$ (Rogers 1995), where $\mu$ is the mutation rate for the sequence and t is the time since expansion (confidence intervals for $\tau$ were obtained from 2000 bootstrap replicates). We also performed Fu's test of selective neutrality with 10 000 bootstrap replicates, where a significant negative $F_S$ value (at $P < 0.02$) would indicate an expansion in population size (Fu 1997). The long-term effective female population size ($N_f$) was approximated using the equation: $N_f = 10^6(p/s)/g$ where p is the nucleotide diversity, s is the rate of sequence divergence and g is the generation time in years (Wilson *et al.* 1985). For the above calculations, we assumed a rate of sequence divergence of 14.2% per million years ($\mu = 2.073 \times 10^{-5}$), a rate that was recently estimated for wolves and coyotes (Savolainen *et al.* 2002), and a generation time of two years.

Using a function implemented in ARLEQUIN, we constructed a minimum spanning network (MSN) based on pairwise differences among haplotypes (including indels). This network was subsequently used in a nested clade analysis (NCA) in an attempt to discriminate between phylogeographic patterns caused by current restricted gene flow and patterns caused by historical events (Templeton 1998). Nesting of the MSN followed the basic rules by Templeton *et al.* (1987). Nesting of ambiguities and intermediate haplotypes was carried out according to Templeton and Sing (1993) and Crandall (1996). Geographical distances between regions were obtained using a distance calculator at http://www.wcrl.ars.usda.gov/cec/java/lat-long.htm (2003-01-28). The null hypothesis of no geographical associations of clades and computation of clade distances (Dc) and nested clade distances (Dn) were carried out using the program GeoDis (Posada *et al.* 2000) with 10 000 permutations. Interpretation of the results obtained in the NCA was obtained using the inference key in GeoDis.

We employed an exact nonparametric procedure (1 000 000 steps in the Markov chain and 50 000 dememorisation steps) to test for differentiation between pairs of populations (Raymond & Rousset 1995). In order to investigate geographical structuring of genetic variation, we used an analysis of molecular variance (AMOVA) with 10 000 permutations (Excoffier *et al*. 1992). We performed six AMOVAs with different hierarchical groupings: [Palaearctic vs. Nearctic], [Palaearctic vs. Nearctic vs. Atlantic islands], [mainland vs. islands], [above 68°N vs. below 68°N], [lemming fox populations vs. coastal fox populations], and [lemming fox populations vs. each coastal fox population]. We then assumed that the most probable geographic structure was represented by the groupings that maximised values of $\Phi_{CT}$ (Vila *et al.* 1999), which is a measure of the proportion of genetic variation among groupings of populations. Population pairwise $\Phi_{ST}$ values (a measure analogous to $F_{ST}$) were generated and tested for significance through 10 000 permutations (Schneider *et al.* 2000). The resulting matrix of $\Phi_{ST}$ values between the different populations was visualised with a UPGMA tree constructed in PAUP (Swofford 1998). To investigate the effect of post-glacial gene flow, we compared the levels of haplotype diversity (H) of populations in formerly glaciated areas with those inhabiting regions not glaciated during the last Ice Age. This was done with a one way ANOVA, as implemented in the software STATISTICA (StatSoft Inc. 1999). For this analysis we excluded samples from BAT due to low sample size. A Mantel Test with 10 000 replicates (Smouse *et al.* 1986) was used to test if there was a correlation between genetic and geographical distances among populations.

## Results

We sequenced 292 base pairs (bp) of the control region for each of the 191 individuals. The sequenced region contained 21 variable sites, which defined 29 different haplotypes (Table 1). All the observed variation was in the form of single base pair substitutions or indels, except for haplotype S3, where a 16 bp deletion was observed (since this region is present in all other haplotypes, as well as in kit and swift foxes (*Vulpes macrotis* and *V. velox*) it is presumably a deletion). This deletion was confirmed by a second amplification and sequence analysis

**Table 1.** Geographical distribution and GenBank accession numbers for arctic fox haplotypes. The number of samples and haplotype diversities (H) are indicated for each geographical region (abbreviations as in Fig. 1).

| | | Geographical Region | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Haplotype** | GenBank # | FEN | SIB | ICE | BAT | CHU | CMB | WG | EG | NWG | SG | ALA | SVA | BAN |
| H1 | AY321121 | 8 | 12 | | | 9 | 6 | 1 | 3 | | 1 | 3 | 7 | 3 |
| H2 | AY321125 | | 1 | 2 | 1 | 4 | | | 5 | 7 | | 2 | 4 | 2 |
| H3 | AY321120 | 9 | 1 | | | | | | 1 | | | | | |
| H4 | AY321124 | | 1 | | | | 1 | | | | | 1 | | |
| H5 | AY321127 | 1 | 1 | 1 | | | | | | | | 2 | | |
| H6 | AY321128 | | | | 2 | | | | | | | | 3 | |
| H7 | AY321129 | 4 | 4 | | | | | | | | 1 | | | |
| H8 | AY321132 | | | 2 | | 1 | 2 | | | | | | | 1 |
| H9 | AY321134 | | | | | | | 6 | | | | 1 | 6 | |
| N1 | AY321136 | | | | | 1 | | 1 | | | | 2 | | |
| N2 | AY321138 | | | | | | 2 | | 1 | | | 2 | | |
| N3 | AY321140 | | | | | | | | | | 3 | | | 1 |
| S1 | AY321123 | | 1 | | | | | | | | | | | |
| S2 | AY321133 | | 1 | | | | | | | | | | | |
| S3 | AY321122 | | 2 | | | | | | | | | | | |
| S4 | AY321126 | | 1 | | | | | | | | | | | |
| I1 | AY321131 | | | 4 | | | | | | | | | | |
| I2 | AY321130 | | | 14 | | | | | | | | | | |
| G1 | AY321135 | | | | | | | 1 | | | | | | |
| G2 | AY321137 | | | | | | | | 1 | | | | | |
| G3 | AY321139 | | | | | | | | | 3 | | | | |
| G4 | AY321141 | | | | | | | | | | 2 | | | |
| G5 | AY321142 | | | | | | | | | | 3 | | | |
| C1 | AY321143 | | | | | 3 | 1 | | | | | | | |
| C2 | AY321144 | | | | | 1 | | | | | | | | |
| C3 | AY321145 | | | | | 1 | 1 | | | | | | | 2 |
| C4 | AY321146 | | | | | | 1 | | | | | | | |
| C5 | AY321147 | | | | | | 1 | | | | | | | |
| C6 | AY321148 | | | | | | | | | | | | | 1 |
| *Sum* | | *22* | *25* | *23* | *3* | *20* | *15* | *9* | *11* | *10* | *10* | *13* | *20* | *10* |
| **H** | | 0.70 | 0.76 | 0.61 | - | 0.76 | 0.84 | 0.58 | 0.76 | 0.47 | 0.84 | 0.91 | 0.76 | 0.89 |

using the two additional primers H1F (5'-GCCATCAACTCCCAAAGCT-3') and P1R (5'-GAGGCATGGTGATAAATCC-3'). The whole deletion was treated with the same weight as substitutions and indels in further statistical analyses. The mean number of pairwise differences between all samples was 2.65 (SD 1.42), and the nucleotide diversity ($\pi$) in the total sample was 0.009 (SD 0.005). Fu's test of selective neutrality gave a significantly large negative $F_S$ value ($F_S = -8.15$, $P = 0.014$).

The distribution of pairwise differences between all individuals did not deviate from the expected distribution under a model of sudden expansion ($P = 0.45$). The extent of divergence was measured as $\tau = 4.889$ (with a 95% confidence interval from 1.674 to 9.298), giving an estimated time of expansion at 118 000 BP (95% confidence interval from 40 000 to 224 000). The observed nucleotide diversity suggested a long-term female effective population size of 32 000 (± 17 000) individuals in the sampling area (*i.e.* the world population).

The minimum spanning network of the different haplotypes revealed no major branching events (Fig. 2). Two haplotypes, H1 and H2, were observed in 42% of all individuals. These two haplotypes, together with several less common haplotypes, had a widespread geographical distribution. The remaining haplotypes were generally site-specific and occurred in low frequencies (Table 1).

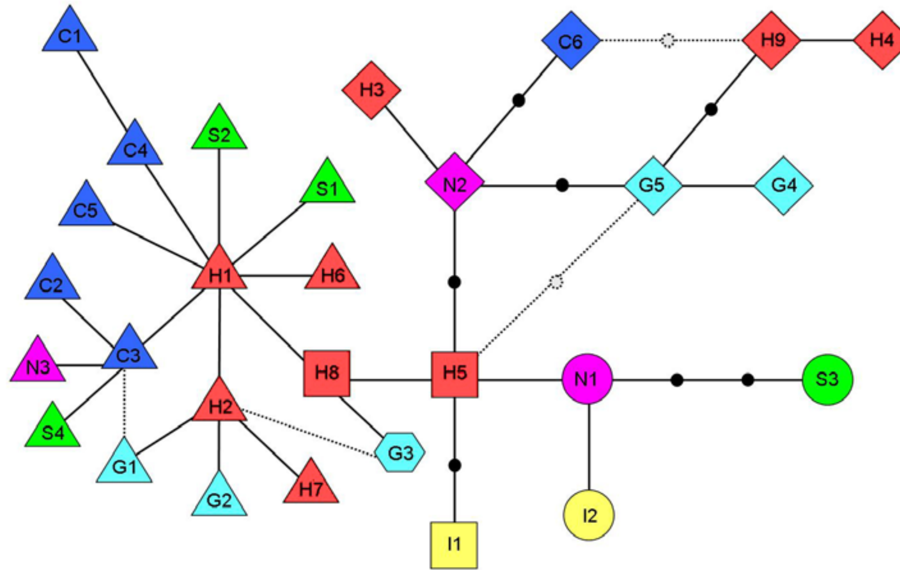


**Figure 2.** The Minimum Spanning Network (MSN). Haplotypes are named (and coloured) after geographic origin: Holarctic (H), Nearctic (N), Canada (C), Siberia (S), Greenland (G) and Iceland (I). Each branch represents one mutational step; missing haplotypes are represented by a dot. Equally parsimonious branches are shown with dashed lines. The shape of the haplotypes illustrates the second nesting level in the Nested Clade Analysis. Haplotype G3 was not nested until the third nesting level.

Haplotypes specific to certain geographical regions did not form monophyletic groups but instead appeared to be randomly distributed in the network (Fig. 2). The Nested Clade Analysis (NCA) did however indicate a significant geographical association for a majority of the nested clades (data not shown). The overall phylogeographical pattern in the NCA was inferred as caused by recurrent but restricted gene flow. This pattern was dominant at the second and third (total network) nesting levels. At the first nesting level the pattern was more complicated with indications of past fragmentations, range expansions as well as restricted gene flow (see Appendix 1 for a complete listing of NCA results).

There was no significant correlation between genetic and geographic distances among populations (Mantel test: r = -0.19, $P$ = 0.90). The exact test of population differentiation indicated that most populations were differentiated although there were exceptions, especially within North America (Table 2). The most probable geographical grouping of populations in the AMOVA was when "lemming fox" populations were grouped against each of the "coastal fox" populations ($P$ < 0.002), where 25.4% of the variation was observed among groups ($\Phi_{CT}$ values for other groupings were all below 3%). The total proportion of variation among all populations ($\Phi_{ST}$) was 30%, and the proportion of variation among populations within groups ($\Phi_{SC}$) was 6.8%. Among the populations, the $\Phi_{ST}$ values were generally low with the exception of Iceland and to some extent West Greenland (Table 2). The haplotype diversity (H) in the different populations varied between 0.47 and 0.91 (Table 1). There was no significant difference in H between previously glaciated and non-glaciated regions (one way ANOVA, n = 13, F = 2.44, $P$ = 0.15), where Banks Island, Alaska and East Siberia were considered as having been unglaciated during the latest Ice Age.

## Discussion

### *Population History*

The earliest historic event we can infer from the mitochondrial DNA variation is that of a sudden expansion in population size. This was presumably preceded by a population bottleneck. The occurrence of a historic bottleneck and subsequent expansion is further supported by the significantly negative $F_S$ value (Fu 1997) and by the low nucleotide diversity (0.009) in the arctic fox. This nucleotide diversity in the control

**Table 2.** Population differentiation test (*P*-values; above diagonal) and cross-wise $\Phi_{ST}$ values for each population (below diagonal). $\Phi_{ST}$ values not significantly different from zero are denoted with a cross (†). Population abbreviations as in Fig. 1.

| Population | FEN | SIB | ICE | CHU | CA | WG | EG | NWG | SG | ALA | SVA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| FEN | | 0.030 | <0.001 | <0.001 | <0.001 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| SIB | 0.116 | | <0.001 | 0.021 | 0.010 | <0.001 | 0.029 | <0.001 | 0.002 | 0.029 | 0.001 |
| ICE | 0.280 | 0.365 | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| CHU | 0.195 | 0.025† | 0.467 | | 0.557 | <0.001 | 0.235 | 0.001 | <0.001 | 0.079 | 0.010 |
| CA | 0.080 | 0.007† | 0.357 | 0.008† | | 0.001 | 0.343 | 0.002 | 0.003 | 0.193 | 0.024 |
| WG | 0.390 | 0.417 | 0.537 | 0.555 | 0.419 | | 0.001 | <0.001 | 0.001 | 0.055 | 0.046 |
| EG | 0.094† | 0.004† | 0.410 | 0.084† | 0.028† | 0.399 | | 0.053 | 0.002 | 0.349 | 0.030 |
| NWG | 0.361 | 0.197 | 0.564 | 0.397 | 0.286 | 0.505 | 0.144 | | <0.001 | 0.004 | <0.001 |
| SG | 0.135 | 0.183 | 0.407 | 0.282 | 0.130 | 0.256 | 0.219 | 0.466 | | 0.004 | <0.001 |
| ALA | 0.043† | 0.074† | 0.204 | 0.179 | 0.052† | 0.210 | 0.076† | 0.272 | 0.063† | | 0.026 |
| SVA | 0.187 | 0.110 | 0.439 | 0.201 | 0.101 | 0.155† | 0.088† | 0.216 | 0.090† | 0.063† | |

region is low compared to many other mammals, for example wolf (*Canis lupus*; $\pi = 0.026$; Vila *et al.* 1999), coyote (*Canis latrans*; $\pi = 0.046$; Vila *et al.* 1999) and moose (*Alces alces*; $\pi = 0.025$; Hundertmark *et al.* 2002). The time of the expansion, as suggested by the mismatch analysis, was estimated at approximately 118 000 BP. Bearing in mind the large confidence interval (40 000 - 224 000 BP), this estimate coincides with the last interglacial which ended at 117 000 BP (Kukla *et al.* 2002).

A similar expansion in connection with the last interglacial was recently observed in a study on reindeer (Flagstad & Røed 2003). Considering that the last interglacial was approximately 5$^{o}$C warmer than at present (Funder *et al.* 1998), it is probable that the arctic foxes (along with other arctic organisms, such as reindeer) were adversely affected during this period. This may, for example, have been through indirect effects such as a northern expansion of red foxes (*Vulpes vulpes*), as it has been proposed that the southern distribution of the arctic fox is limited by red foxes (Hersteinsson & Macdonald 1992). The presence of forest remains from previous interglacials in northern Siberia (Sher 1991) suggests a suitable habitat for red foxes. The arctic fox may therefore have been extinct in continental Eurasia and North America during the last interglacial, persisting only in high latitude islands, and then expanding south as temperatures started to fall some 117 000 years ago. This hypothesis predicts a high current genetic diversity in high latitude islands that were not glaciated during the ensuing Ice Age, since these are the only areas that would have been continuously inhabited by arctic foxes for at least 130 000 years (the low sample sizes from these islands in the present study did not allow us to test this hypothesis). It can also be expected that any sequences recovered from fossil remains less than 100 000 years old would fall within the scope of the mismatch distribution.

During the Ice Age that followed, arctic foxes were widely distributed in Eurasia and Beringia (Kurtén 1968, Kurtén & Anderson 1980). The structure of the minimum spanning network, without distinct phylogroups, indicates a lack of significant geographical barriers during this period (Fig. 2). This is further supported by the lack of evidence of past fragmentation at the higher nesting levels in the NCA (Appendix 1). At the end of the Ice Age it is probable that there was a range expansion into formerly glaciated areas such as Greenland, Iceland, Svalbard and Fennoscandia. Although the second nesting level in the NCA shows some support for a range expansion, it is not as strong as might have been expected. There could be several explanations for this, for example that these areas were colonised by arctic foxes from local refugia (*e.g.* Frafjord & Hufthammer 1994), as has been suggested for other arctic species (Fedorov & Stenseth 2001, 2002). A high post-glacial gene flow could also explain the weak support in the NCA as it may have erased phylogeographical patterns created by an initial range expansion. At

the lowest nesting level, the NCA gives a rather ambiguous picture, possibly due to small sample sizes in the nested clades. There may also be problems with the interpretation of NCA results using the inference key (*see* Knowles & Maddison 2002).

The female long-term effective population size was estimated at 32 000 individuals. Assuming a 1:1 sex ratio and that 40% of all female adult arctic foxes breed during their lifetime (Angerbjörn *et al.* 2003a), this would correspond to an approximate world population size of *c*. 160 000 (± 85 000) adults. This is lower than the census population size of 330 000 to 930 000 adults (Angerbjörn *et al.* 2003b), but is within the margins of what can be expected for a species with a large variance in reproductive success (Bensch & Hasselquist 1999). Thus, we did not find any indication of recent changes in the world-wide population size of the arctic fox as have been reported for other canids (*e.g.* Vila *et al.* 1999).

*Current Genetic Structure*

Although most populations seem to be significantly differentiated from each other, several analyses suggest that there is a restricted current gene flow between the majority of the populations. There is no phylogeographic structure in the minimum spanning network, where presumed ancestral haplotypes are frequent and widespread and newly arisen haplotypes have not yet spread throughout the range of the species. Therefore, the arctic fox appears to be a species with intermediate gene flow and no long-term zoogeographic barriers (category V in Avise *et al.* 1987). A similar lack of phylogeographic structure has previously been observed in fish (*e.g.* Rocha-Olivares *et al.* 2000), and to some extent wolves (*Canis lupus*) (Vila *et al* 1999). The predominantly low $\Phi_{ST}$ values among populations (Table 2) on such a large geographical scale, compared to $\Phi_{ST}$ values of 0.75 in kit foxes, 0.50 in swift foxes (Mercure *et al.* 1993), 0.46 in Mediterranean red foxes (Frati *et al.* 1998) and 0.69 in wolves (Vila *et al.* 1999), also indicate a current gene flow. Yet, low $\Phi_{ST}$ values and poor phylogeographic structuring of haplotypes could also be the result of a post-glacial range expansion. However, the high $\Phi_{ST}$ values between Iceland and the other populations suggest that Iceland is particularly isolated, which is what we expected under the hypothesis that there is current gene flow between all populations except Iceland. The observation of equal haplotype diversities in populations inhabiting formerly glaciated and unglaciated areas further supports the gene flow hypothesis, although it should be noted that colonisation of a formerly glaciated region from several different refugia can also result in a high haplotype diversity (*i.e.* the Phalanx Model; Hewitt 1996). Taken together, these results suggest that there is gene flow among most populations, which is in agreement with previous studies reporting that arctic foxes travel long distances (*e.g.* Eberhardt & Hanson 1978) and illustrate the importance of the polar sea ice for terrestrial arctic mammals.

We could however not find a correlation between genetic and geographic distances, implying that there is no genetic isolation by distance between the populations. There could be a number of explanations for this, such as ice movements, geographical barriers or arctic foxes following polar bears (however, we could find no relationship between arctic fox and polar bear genetic distances; Paetkau *et al.* 1999). A more likely explanation can be found in the relationship between the different populations (Fig. 3) and the geographical structuring of the genetic variation as suggested by the AMOVA. Arctic foxes from East Greenland, Siberia, Churchill, the Canadian Archipelago, Fennoscandia and Alaska form a group of populations that are genetically more closely related to each other than to any of the other populations. This former group consists of populations with "lemming foxes", whereas the latter populations all are of the "coastal fox" ecotype. As indicated by the AMOVA, only 6.8% of the genetic variation can be explained by differences among "lemming fox" populations whereas 25.4% of the variation is explained by differences between the "lemming fox" group and each of the "coastal fox" populations. It therefore seems that gene flow is substantially higher between populations of "lemming foxes" than between the two ecotypes or between "coastal fox" populations. The ecological causes for such a pattern could be that lemming foxes have a higher frequency of long distance migrations (Angerbjörn *et al.* 2003a), and that migrants from one type of habitat to the other have a lower fitness than resident arctic foxes. That "lemming foxes" should migrate longer and more often than "coastal foxes" actually makes evolutionary sense owing to the large-scale spatial synchrony of lemming populations (Krebs *et al.* 2002), which may force foxes

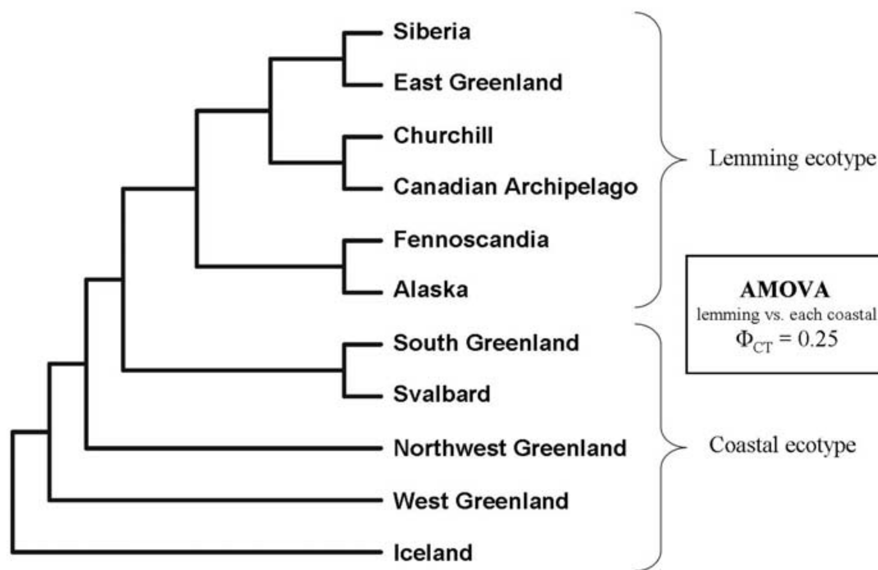


**Figure 3.** Population tree based on $\Phi_{ST}$ values, illustrating the most probable geographical structure in the analysis of molecular variance (AMOVA). The results suggest that there is high gene flow between populations belonging to the "lemming" ecotype, whereas gene flow seems to be lower between populations of the "coastal" ecotype as well as between the two ecotypes.

feeding on lemmings to migrate longer and more frequently than foxes in costal areas where food resources are more stable. The hypothesis that immigrant foxes from a different habitat should have lower fitness than residents was originally proposed by Vibe (1967) as an explanation to the stable difference in fur colour frequency between arctic foxes in Northwest Greenland and Canada, despite an influx of white foxes after lemming peaks in Canada. It has been suggested that different reaction norms in litter size have evolved in fluctuating and stable arctic fox populations respectively (Tannerfeldt & Angerbjörn 1998). The observed pattern might thus be explained if food resource predictability affects selection pressure on reproductive output, giving "lemming foxes" a disadvantage under stable coastal conditions, or by the higher competition for territories in "coastal fox" populations (Angerbjörn *et al.* 2003a).

These results agree well with what is known on the biology of the arctic fox, in particular the extraordinary migration patterns, facilitated by the polar sea ice, and the difference in life history strategies between "lemming" and "coastal" arctic foxes. The generally high gene flow suggested by this study, in particular among "lemming" populations, should also be taken into account concerning the spread of arctic disease, such as rabies.

*Management Implications*

Our samples cover more or less the total distribution of the arctic fox except the populations on the isolated Bering and Mednyi Islands. We found no support for the existence of any subspecies within the sampled area. Furthermore, based on the distribution of mtDNA haplotypes, we have not been able to identify any Evolutionary Significant Units. Iceland may, however, be considered a Management Unit based on its isolation, as indicated by the high $\Phi_{ST}$ values (Table 2). However, Management Units should not be based solely on genetic data. Fennoscandia, for example, is regarded as a Management Unit based on ecological data. In a previous study by Dalén *et al.* (2002) it was suggested that there is a gene flow from Siberia into Fennoscandia, since the haplotype diversity and number of haplotypes in Fennoscandia was higher than expected for a small isolated population. Two observations in this study support that conclusion. First, the $\Phi_{ST}$ values between Fennoscandia and Siberia (0.12) is not particularly high compared to the difference between other populations. Second, the two

haplotypes that had previously only been observed in Fennoscandia were in this study also found in western Siberia, which is to be expected if the haplotypes in Fennoscandia are the result of a current gene flow from Siberia.

On a global level, the results of this study suggest that the high temperatures during the last interglacial may have had a severe impact on the arctic fox as a species. Given the increases in temperature predicted from models on global warming and the negative effect of competition with the temperate red fox (Chirkova 1968, Tannerfeldt et al. 2002), the range of the arctic fox will contract to the north. The local conservation problems for the arctic fox in Fennoscandia today may thus in the near future become a global issue.

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**Appendix 1.**

Results of the nested clade analysis of geographical distances for control region haplotypes in the arctic fox. Clade distances ($D_c$) and nested clade distances ($D_n$) are calculated for each clade within the nested group, and for the average difference in distances between interior and tip clades (I-T). Interior clades are shaded. Significantly large and small values of $D_c$ and $D_n$ are indicated by a superscript L and S respectively. Results from the inference key are given below each nested group and are abbreviated as follows: RGF (restricted gene flow), LDD (long distance dispersal) CRE (contiguous range expansion), PF (past fragmentation), LDC (long distance colonisation).

| Haplotypes | | | 1-Step Clades | | | 2-Step Clades | | |
|---|---|---|---|---|---|---|---|---|
| No. | $D_c$ | $D_n$ | No. | $D_c$ | $D_n$ | No. | $D_c$ | $D_n$ |
| C1 | 540$^{S}$ | 620$^{S}$ | | | | | | |
| C4 | 0 | 657 | | | | | | |
| I-T | -540$^{L}$ | 37 | | | | | | |
| 1-2-3-4No: RGF | | | | | | | | |
| H1 | 2828$^{L}$ | 2668 | | | | | | |
| C5 | 0 | 2702 | | | | | | |
| H6 | 667$^{S}$ | 2130 | | | | | | |
| S1 | 0 | 4381 | 1-1 | 629$^{S}$ | 2540 | | | |
| S2 | 0 | 3060 | 1-2 | 2579$^{L}$ | 2504$^{L}$ | | | |
| I-T | 2411$^{L}$ | 69 | 1-3 | 2041 | 2305 | | | |
| 1-2-3-4No: RGF | | | 1-4 | 0 | 620 | | | |
| C2 | 0 | 1815 | 1-5 | 1843$^{S}$ | 1927$^{S}$ | | | |
| C3 | 620$^{S}$ | 2223 | I-T | 846$^{L}$ | 474$^{L}$ | | | |
| N3 | 1434 | 1357 | 1-2-3-4No: RGF | | | | | |
| S4 | 0 | 4544$^{L}$ | | | | | | |
| I-T | -336 | 258 | | | | | | |
| 1-2-11-12No: CRE | | | | | | | | |
| H2 | 1451$^{S}$ | 1639$^{S}$ | | | | | | |
| G2 | 0 | 886 | | | | 2-1 | 2260 | 2287$^{L}$ |
| H7 | 1311 | 2794$^{L}$ | | | | 2-2 | 2521 | 2583 |
| I-T | 272 | -964$^{S}$ | | | | 2-3 | 1678 | 1914 |
| 1-2-11-12-13Yes: LDC | | | | | | 2-4 | 1838 | 1801$^{S}$ |
| I2 | 0$^{S}$ | 1409$^{S}$ | 1-7 | 1974 | 3990$^{L}$ | 1-6 | 0$^{S}$ | 1311 |
| N1 | 1891 | 2246 | 1-8 | 1694 | 1223$^{S}$ | I-T | 460 | 463 |
| I-T | 1891$^{L}$ | 838 | I-T | -279 | -2767$^{S}$ | 1-2-3-5-6-7-8Yes: RGF & LDD | | |
| 1-2-3-4-9No: PF | | | 1-2-11-17No: *Inconclusive* | | | | | |
| H8 | 1408$^{S}$ | 1946$^{S}$ | 1-9 | 2412 | 2609 | | | |
| H5 | 3322 | 3145$^{L}$ | 1-10 | 0$^{S}$ | 2083 | | | |
| *No tip clades* | | | I-T | 2412$^{L}$ | 527 | | | |
| 1-2-11-17-4-9No: PF | | | 1-2-3-4No: RGF | | | | | |
| H3 | 547$^{S}$ | 1926 | | | | | | |
| N2 | 1465 | 2654 | 1-11 | 2210 | 2403$^{L}$ | | | |
| I-T | 918 | 728 | 1-12 | 0 | 2977 | | | |
| 1-2-3-4-9No: PF | | | 1-13 | 0$^{S}$ | 890$^{S}$ | | | |
| H4 | 3860$^{L}$ | 3568$^{L}$ | 1-14 | 1674 | 1691 | | | |
| H9 | 1184$^{S}$ | 1248$^{S}$ | I-T | -1883$^{S}$ | -1185$^{S}$ | | | |
| I-T | -2676$^{S}$ | -2320$^{S}$ | 1-2-11-12No: CRE | | | | | |
| 1-2-11-12No: CRE | | | | | | | | |

# Paper III

*I en del fall, i spårlöpor eller vid djurkadaver, där kanske både järv, lodjur och räv varit, kan det ibland råda osäkerhet beträffande leverantören av ett spillningsprov, vilket gör en insamling meningslös.*

Bertil Haglund, rovdjursinventeringarna 1960-1966

# Identifying Species from Pieces of Faeces

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**Key words***:* PCR, DNA, primers, *Alopex lagopus, Vulpes vulpes, Gulo gulo*.

## Introduction

Endangered populations are complicated to study due to low densities. However, faeces are frequently used as a source of information in conservation to confirm presence and investigate diet. Moreover, advances in molecular biology permit researchers to analyse DNA from faeces rendering information on populations, home ranges, genetic variation and phylogenetic relationships (Kohn & Wayne 1997).

To complete such studies, however, the faeces need to be correctly assigned to the species in question. This can sometimes be difficult through conventional methods (Reed *et al*. 1997; Farrell *et al*. 2000). Davison *et al*. (2002) showed that even expert naturalists fail to distinguish faeces from martens (*Martes martes*) and red foxes (*Vulpes vulpes*). Several molecular methods have been developed for species identification of faeces. A direct approach involves amplification and sequencing of DNA extracted from the faeces (Höss *et al*. 1992; Farrell *et al*. 2000). This approach is straightforward but time consuming and expensive. Therefore, several studies have instead employed species-specific restriction enzymes (Paxinos *et al*. 1997; Hansen & Jacobsen 1999), but if only one restriction site is used, a failure in the reaction would lead to false positives. In a recent study, faeces from Iberian lynx (*Lynx pardinus*) were identified by use of species-specific primers (Palomares *et al*. 2002). However, in this study a failed amplification could be due to low DNA content of the sample or the faeces originating from another species, thus creating false negatives. Mills *et al*. (2000) analysed hair samples and used a combination of felid-specific primers and several restriction enzymes that solves these two problems.

In this paper we describe a simple six-hour method for faecal samples that handles the problem of false negatives. The method is designed to separate arctic fox (*Alopex lagopus*), red fox and wolverine (*Gulo gulo*), since faeces from these species are difficult to distinguish. The method has been implemented in the management of the endangered Fennoscandian arctic fox. Our method is based on a multiple primer system, from now on referred to as Rapid Classificatory Protocol PCR (RCP-PCR). One of the primers is designed to anneal to all species, whereas the others are species-specific. The specific primers bind at different distances from the general primer. Hence, the use of all primers in a single tube PCR results in fragments of different size depending on which species the faeces originates from (Fig. 1).

We designed species-specific primers *Pex1F* (arctic fox: 5'-TAAACTATTCCCTGATACTC-3'), *Vul1F* (red fox: 5'-TCAATCCTTGCTCG AAGTA-3') and *Gulo1F* (wolverine: 5'-AGTA TGTACCCTTTTCCTCC-3'). The primer *H3R* (5'-CCTGAAGTAGGAACCAGATG-3') was chosen as reverse primer since it binds to most mammals (e.g. humans, elephants, beavers, horses, cows, arctic foxes; *personal observation*). The species-specific primers had a 100% match to 27 red fox and 44 wolverine control region sequences on the genbank

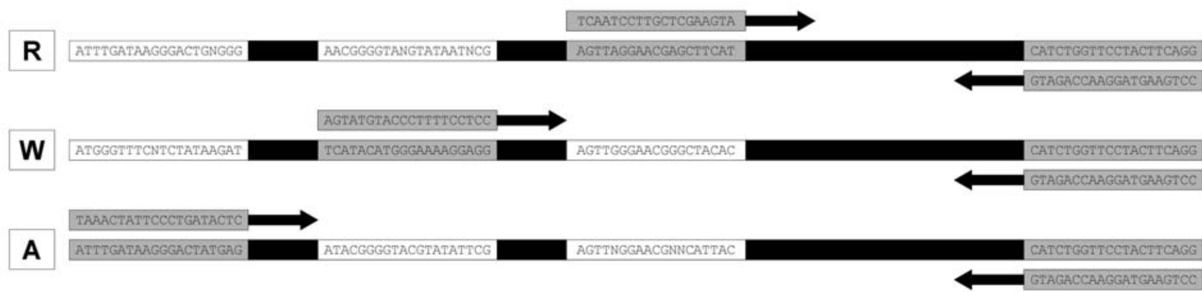


**Figure 1.** Illustration of the concept of RCP-PCR. One of the three species-specific primers will react with the general primer. The resulting fragment size depends on whether red fox (R), wolverine (W) or arctic fox (A) DNA is present in the extract. Intra-specific variable sites in the template are shown as (N).

database. Further, we have sequenced c. 200 arctic foxes from all over their distribution area using *Pex1F*, and never had a failed amplification. It is therefore unlikely that a polymorphism would generate false negatives.

We initially extracted DNA from muscle tissue of two wolverines, three arctic and three red foxes using Qiagen's Dneasy tissue kit (Qiagen). The primers were first tested alone with H3R in a PCR, and then together in a single tube PCR. All reactions yielded single fragments of the expected size when scored on a 1.5% agarose gel.

Faeces with known origin were collected at a zoo (Skansen, Stockholm) and in the field from dens with known inhabitants (Table 1). DNA was extracted using Qiaamp DNA stool kit (Qiagen), and was subsequently subjected to a four-primer single tube 25 μl PCR as follows: 2 μl DNA extract, 0.2 mM of each nucleotide, 2.5 mM MgCl2, 0.1mg/ml BSA, 0.5 μM of each primer, 10 x PCR Gold Buffer and 0.75 units of AmpliTaq Gold polymerase (Perkin Elmer Cetus). The cycling parameters for the PCR-reaction were: 94˚C denaturation for 10 min, followed by 35-40 cycles of 94˚C denaturation for 20 s, 55˚C annealing for 30 s, and 72˚C extension for 20 s, followed by a single 7-min final extension at 72˚C.

We also analysed 128 faeces of unknown origin collected during field surveys all over Fennoscandia. The sampling protocol was designed to minimise the possibility that several samples were taken from the same individual. The samples were gathered in plastic jars containing silica pellets (Wasser *et a*l. 1997) and stored at -80$^{o}$ C pending extraction. We used approximately 200 mg of faecal material for each extraction and analysed as described above. We also sequenced one fragment from each species. Sequences were obtained using a CEQ 2000XL automated sequencer (Beckman Coulter) according to the manufacturer's instructions. All extractions were performed in a physically isolated laboratory, dedicated to DNA extractions. We used one extraction blank for every ten samples extracted.

The sequence from the 332 bp fragment was identical to a haplotype observed in wild Scandinavian arctic foxes (Dalén *et al*. 2002). The 242 bp fragment gave a wolverine sequence. The 100 bp fragment gave a fox sequence. This 100 bp region is identical in some red and arctic fox haplotypes. However, since the sequence obtained did not correspond to any of the arctic fox haplotypes observed in Scandinavia it presumably came from a red fox.

Samples of known origin consistently gave the results expected, as did the sequences from the PCR products. This suggests that RCP-PCR provides an effective method for species identification on tissues of unknown origin. There is a possibility that amplification from non-target species would lead to false positives. However, faeces from non-target sympatric species, such as small mustelids and wolves (*Canis lupus*), are easy to separate by eye from the target species. Furthermore, since this approach allows identification of all potential defecators, it bypasses the problem of false negatives discussed above. Of 128 unknown faeces, 99 were successful (Table 1) but 29 did not yield any PCR-product, probably due to low DNA content as samples collected in summer failed more frequently (40%) than in winter (9%; $\chi^2 = 15.8$, $P = 0.0001$)

We have applied this method in the conservation of the arctic fox in Fennoscandia to

**Table 1.** Results from the RCP-PCR. The values in brackets were muscle samples whereas all other were from faeces.

| Sample | Results from the PCR | | | | |
|---|---|---|---|---|---|
| | Arctic fox (332bp) | Red fox (100bp) | Wolverine (242bp) | Failed | TOTAL |
| **Known AF** | 11+(3) | 0 | 0 | 0 | |
| **Known RF** | 0 | 11+(3) | 0 | 0 | |
| **Known W** | 0 | 0 | 2+(2) | 0 | |
| **Unknown** | 40 | 57 | 2 | 29 | 128 |

implement conservation actions such as supplementary feeding and red fox control.

RCP-PCR is both cheaper and more rapid than methods previously used to identify the origin of unknown faeces. The total time for an analysis, including extraction, was less than six hours. This method can be used for identification of unknown tissues between any species, provided that enough variable sites exist for species-specific primers to be designed.


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