Eeva Jansson

PAST AND PRESENT GENETIC DIVERSITY AND STRUCTURE OF THE FINNISH WOLF POPULATION
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Abstract

Many species and populations have perished as a consequence of human actions. During the last ~200 years, large carnivores have been almost completely extirpated from Western Europe. Large-scale wolf hunting started in Finland around the 1850s, and the population size quickly collapsed. The population was very small until the mid-1990s, when wolves started to regularly reproduce in Finland again. The wolf is an endangered species in Finland, and the biggest threat to the species’ survival is excessive hunting.

In this doctoral thesis study, I inspected the genetic structure and diversity of the Finnish wolf population using neutral genetic markers. Almost 300 wolves from the contemporary Finnish population and over 50 wolves from the north-western Russia were analyzed with genetic methods. Additionally, the genetic history of the population was examined with the help of over 100 museum samples.

The modern Finnish wolf population proved to be genetically as diverse as the non-endangered Eastern European and North American wolf populations. However, the genetic diversity decreased significantly during the study period (1995–2009), and was at its lowest level in the final phase of the examination. In tandem, the inbreeding coefficient rose to a relatively high level. Genetic sub-structures were observed due to social structures within wolf packs. The mean dispersal distances of wolves were approximately only 100 km. The Finnish wolf population is divided into neighbourhoods of related individuals, and their size substantially decreased during the study period. This pattern, together with the growth of the inbreeding coefficient, suggests that lost alpha individuals in wolf packs are replaced by their offspring.

This study demonstrated that Russian and Finnish wolf populations are nowadays genetically differentiated. Gene flow between the populations is low, despite the geographic interconnection. Only a few possible immigrants from Russia into Finland were detected in the study. The effective size of the Finnish wolf population proved to be small, and was mainly below the often-considered critical size of 50. Historical analysis revealed that the Finnish wolf population was formerly genetically more diverse, more continuous with the Russian wolf population, and had a more than 90% larger effective size.

On the basis of this study, the genetic status of the Finnish wolf population is worrying and needs to be monitored. The population should be substantially larger than today and/or the amount of gene flow higher, so that the population viability could be considered secured even in the short term.

Keywords: Canis lupus, conservation genetics, effective population size, gene flow, inbreeding, neutral genetic variation, population bottleneck
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Tiivistelmä


Tarkastelen tässä väitöskirjatyössäni Suomen susipopulaation geneettistä rakennetta ja monimuotoisuutta neutraaleja geenimerkkejä käyttäen. Tutkimuksessa analysoitiin geneettisin mene- telmin lähes 300 sutta nyky-Suomesta sekä yli 50 sutta Luoteis-Venäjältä. Lisäksi populatiota geneettistä historiaa selvitettiin yli 100 museonäytteen avulla.


Susien dispersaalimatkat olivat keskimäärin vain noin 100 km. Suomen susipopulaatio on jakautunut toisilleen suurin olevien yksilöiden naapurustoiksi, joiden koko pieni huomattavasti tutkimuksessa havaittiin vain matalilla.


Tutkimuksen perusteella Suomen susipopulaation geneettinen tila on huolestuttava ja tarvitsee seurantaa. Populaation tulisi olla nykyistä huomattavasti suurempana ja/ta/ta geenivirran määrän korkeampi, jotta populaation elinvoimaisuuden voitaisiin katsoa olevan turvattu edes lyhyellä aikavälillä.

Asiakanat: Canis lupus, efektiivinen populaatiokoko, geenivirta, neutrali geneettinen muuntelu, populaation pullonkaula, sukusiitos, suojelugenetiikka
In memory of Minna.

Thank you for teaching me so much.
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Tornio, March 2013

Eeva Jansson
Abbreviations

\( A \) number of alleles

\( A_R \) allelic richness

\( b \) slope

bp base pair

cpDNA chloroplast DNA

DNA deoxyribonucleic acid

\( F_{IS} \) departure from Hardy-Weinberg proportions within subpopulations, local inbreeding coefficient

\( F_{ST} \) proportion of genetic variation due to differences among populations, index of population differentiation

\( H \) number of haplotypes

\( H_d \) haplotype diversity

\( H_e \) expected heterozygosity

\( H_o \) observed heterozygosity

\( H_R \) haplotype richness

IBD isolation by distance

\( K \) number of clusters

LD linkage disequilibrium

mtDNA mitochondrial DNA

\( m \) proportion of immigrants, migration rate

\( N \) number of, size of an ideal population

\( N_b \) number of breeding individuals, neighbourhood size

\( N_c \) census population size

\( N_e \) effective population size

\( N_m \) average number of immigrants

\( P \) statistic test value

PCR polymerase chain reaction

\( P_R \) private haplotype richness

\( S \) number of polymorphic sites

SD standard deviation

\( \pi \) nucleotide diversity
List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.


*Jansson née Roininen
# Table of contents

Abstract

Tiivistelmä

Acknowledgements 9

Abbreviations 11

List of original articles 13

Table of contents 15

1 Introduction 17

1.1 The grey wolf (*Canis lupus*) ............................................................. 17

1.1.1 Distribution.................................................................................. 17

1.1.2 Habits and habitats ................................................................... 19

1.2 History and current status of the Finnish wolf population .......... 21

1.3 Population viability ................................................................. 24

1.3.1 Small population size and the role of genetic factors .......... 24

1.4 Amount of genetic variation............................................................. 26

1.5 Inbreeding .................................................................................. 26

1.6 Population bottlenecks ............................................................... 27

1.7 Population structure ................................................................. 27

1.8 Population connectivity and gene flow ......................................... 28

1.9 Effective population size ............................................................... 30

1.10 The use of molecular markers in population genetic studies ....... 32

1.10.1 Microsatellites .................................................................. 32

1.10.2 Mitochondrial DNA .......................................................... 33

1.11 Aims of the study ................................................................. 34

2 Material and methods 35

2.1 Wolf samples .............................................................................. 35

2.2 DNA extraction, genotyping and sequencing ................................ 36

2.2.1 Handling of museum samples .............................................. 37

2.3 Genetic analysis .......................................................................... 38

2.3.1 Amount of genetic variation .............................................. 38

2.3.2 Inbreeding ........................................................................ 38

2.3.3 Population bottleneck tests ................................................ 39

2.3.4 Population structure .......................................................... 40

2.3.5 Population connectivity and gene flow ................................. 41

2.3.6 Effective population size ...................................................... 43
3 Results and discussion

3.1 Amount of genetic variation .......................................................... 45
3.2 Inbreeding .................................................................................... 48
3.3 Population bottlenecks ................................................................. 49
3.4 Population structure ..................................................................... 51
  3.4.1 Cryptic population structure – clustering analyses ............... 51
  3.4.2 Limited dispersal – IBD .......................................................... 52
3.5 Population connectivity and gene flow ......................................... 54
  3.5.1 Genetic differentiation between Finnish and Russian wolves ...... 54
  3.5.2 Amount of gene flow and detection of immigrants ............... 55
3.6 Effective population size .............................................................. 57

4 Conclusions.................................................................................. 61

References ..................................................................................... 65

Original articles ............................................................................. 77
1 Introduction

The direct and indirect influence of human activities has significantly elevated the extinction rate of species during the past few centuries, and as a consequence many species and populations are nowadays endangered. This process, commonly referred as the sixth mass extinction (e.g. Barnosky et al. 2011), causes a loss of biological diversity, and is especially striking in large mammals (Cardillo et al. 2005) and other apex consumers due to the large cascade effects of these species on many biological processes and ecosystems (Estes et al. 2011). For instance, recent extinctions of many carnivore populations in Europe and South and North America have caused taxonomically depleted guilds, and many of the remaining populations are likely to be too small to function effectively in the surrounding ecosystems (Dalerum et al. 2009). Scientific knowledge of the threatened populations can – if and when adopted in practice – provide us with the necessary means to manage the remaining populations so that they will be viable in the future.

1.1 The grey wolf (Canis lupus)

The grey wolf (Canis lupus Linnaeus, 1785; hereafter ‘wolf’) is a charismatic and controversial species, and one of the most researched wild animals (Mech & Boitani 2003; Mech 2012). Since the 1990s, molecular markers have widely been adopted to answer important questions of the species’ biology, history and evolution, and numerous molecular studies concerning wolves have already been published (among the first studies, e.g., Kennedy et al. 1991; Lehman et al. 1992; Roy et al. 1994; Ellegren et al. 1996).

1.1.1 Distribution

The wolf is the largest wild species today belonging to the Canidae family and is native to Eurasia, North America and North Africa. In recent historical times, the wolf had the largest distribution area of all terrestrial mammals (Fig. 1; Boitani 2000).
Fig. 1. World-wide distribution area of the grey wolf. The distribution in 2003 ("Present") is shown in the darkest grey, and lighter grey areas indicate the historical distribution range. (The map is freely available at Wikimedia commons, from where it was downloaded on 27 August 2012. The original colours were converted to greyscale.)

In Western and Central Europe, the wolf has been a part of the carnivore community for at least ~500 000 years, and its direct precursors, *Canis mosbachensis* and *C. etruscus*, already existed about 1 and 1.8 million years ago, respectively (Croitor & Brugal 2010). Wolves spread to the northern parts of Europe after the last glaciation and the retreat of the ice sheet approximately 10 000 years ago.

Today, wolf populations are commonly restricted to the wilderness and remote areas (Mech & Boitani 2004). The largest continuous distribution areas are found in North America and Asia (Fig. 1; Boitani 2003). Most wolf populations in Western Europe are nowadays rather small and isolated (Randi 2011). The largest population in Western Europe is located in the Iberian Peninsula, comprising about 2500 wolves (IUCN 2012), but with no connection to other populations (Sastre *et al.* 2011). However, Eastern European wolf populations (Dinaric-Balkan, Carpathian and Baltic) are larger and more connected to each other and via Russia to the Asian distribution area (Mech & Boitani 2004; Linnell *et al.* 2008). Currently, wolves in Europe might number approximately 16 000–20 000 individuals (including the European part of Russia; Linnell *et al.* 2008).

Wolves were abundant in Europe until the early 19th century (Wayne *et al.* 1991). The disappearance of wolves from many areas and the extensive world-
wide population fragmentation during the last ~200 years coincided with the expansion of human populations, as the encroachment of human-occupied land has reduced the number of suitable habitats for wildlife. Besides clear cuttings of large forest areas, habitat fragmentation and the consequent shortage of available prey (Fernández & Ruiz de Azua 2010), western wolf populations have vanished due to active persecution. The other three large predators native to Europe, the bear, lynx and wolverine, have also disappeared from many western countries (e.g. Enserink & Vogel 2006). Despite extensive predator removal programmes that were implemented from the 19th century onwards, including in Eastern Europe (Pulliainen 1980; Flagstad et al. 2003), substantially larger wolf populations survived in eastern European and Balkan countries, and many of them are thriving today (Boitani 2000; Mech & Boitani 2004; Linnell et al. 2008).

The main motive for wolf control was – and still is – economic conflicts with humans (e.g. Woodroffe 2000; Boitani 2000; Bisi & Kurki 2008; Bisi et al. 2010). Wolves prey on a range of game species and livestock (Boitani 2003), and have often been seen as enemies to be feared, persecuted and extirpated (e.g. Mech & Boitani 2004; Bisi & Kurki 2008). The most negative attitudes towards wolf presence are typically among hunters, and people living in areas where wolves have been absent for a long time (e.g. Ericsson & Heberlein 2003; Bisi et al. 2010). Wolf fear and hatred are further increased with prejudices, legends and misinterpretations of its biology (Boitani 2000).

During recent decades, large carnivores have been making a comeback to Western Europe, and expanding their territories into areas where they have been absent for long periods of time (Lucchini et al. 2002; Enserink & Vogel 2006; Linnell et al. 2008). This increase is mainly due to changes in legislation: Wolves are nowadays protected in most European countries (Linnell et al. 2008; Randi 2011) and have naturally spread from the remaining populations as hunting pressure has been reduced. However, wolf conservation and management remain problematic because of widespread illegal or incidental killing (Caniglia et al. 2010; Randi 2011; Liberg et al. 2012), and the fact that many present-day wolf populations are still too small to be viable in the long term (see 1.3).

1.1.2 Habits and habitats

Wolves are ecologically flexible (Mech & Boitani 2003) and have a high dispersal potential of up to several hundreds of kilometres (Wabakken et al. 2001; Valière et al. 2003, Seddon et al. 2006; Ciucci et al. 2009), which explains their large
distribution area. Wolves mostly eat large ungulates such as moose, deer, elk, and wild boar, but the source of nourishment may vary opportunistically from any smaller prey item to livestock, carcasses or even garbage (Mech & Boitani 2004). Occasionally, wolves may also feed on fruits or other plant material (Mech & Boitani 2003). In fact, wolves can survive anywhere having enough of something suitable to eat, and where they are not killed by humans (Boitani 2000). As top predators, wolves have a large impact on ecosystems with effects on prey species and vegetation (Smith et al. 2003; Hebblewhite et al. 2005; Sergio et al. 2008; Estes et al. 2011; Ripple & Beschta 2012).

Except for their varying diet composition, wolves are highly adaptable to various environments and occupy a wide range of habitats from warm deserts to cold tundra (Wayne et al. 2004). As a part of such adaptation, wolves display substantial phenotypic variation in body size, weight and colouration, for instance (e.g. Weckworth et al. 2005; Musiani et al. 2007), and several subspecies of Canis lupus have been described (Boitani 2000; Mech & Boitani 2004).

Social behaviour is a common feature amongst all modern larger representatives of Canidae (Croitor & Brugal 2010). Strong social bonds and a strict hierarchy are peculiar to wolf packs. Wolves are social animals that live within their territories in packs commonly consisting of a family with one breeding pair (alpha wolves) and their offspring from one or more litters (Mech & Boitani 2003). Thus all members of the pack are usually related, apart from the alpha pair. Pack members co-operate in hunting, reproducing and territorial defence (Boitani 2000). Typically, a wolf pack consists of 2–15 individuals, and 1 to 8 cubs are born to a breeding pair annually. In Finland the mean litter size is $4.3 \pm 1.3$ pups (Kaartinen et al. 2010). Large packs are extremely rare in Europe as a consequence of human-mediated control (Boitani 2000). In North-America, however, much larger (up to 42 members; Fuller et al. 2003) and more socially diverse packs (e.g. with subordinate breeders: vonHoldt et al. 2008; Stenglein et al. 2011) have been found. The natural kin-based social organization of wolf packs may be interrupted by disturbance (Brainerd et al. 2008). For example, intense harvesting of wolves in established packs has been found to increase the adoption of unrelated individuals (Grewal et al. 2004; Jędrzejewski et al. 2005; Rutledge et al. 2010).

Wolves require large territories and home ranges, and population densities therefore remain rather low, even in saturated habitats. In Finland, the mean territorial size is $1372 \pm 514 \text{ km}^2$ (Kaartinen et al. 2005). Home range sizes vary considerably and depend on habitat characteristics, prey availability and
population density, among other factors (Fuller et al. 2003). Occupied territories are maintained through howling, scent-marking and direct killing (Mech 1974). In Finland, wolves leave their natal pack typically as yearlings around the time when new cubs are born (from March to May; Kojola et al. 2006), in order to find their own mates and territories (Mech & Boitani 2003). Compared with the high dispersal capability of wolves, typical dispersal distances have often been detected to be rather modest (e.g. vonHoldt et al. 2008; Scandura et al. 2011), and in Finland they are around 100 kilometres (based on radio and GPS transmitters; Kojola et al. 2006). Short mean dispersal distances and the high stability of already established wolf packs may lead to local genetic differentiation (i.e. cryptic population structure; e.g. Scandura et al. 2011; Randi 2011). In general, demographically stable wolf populations seem to be characterized by very limited dispersal and gene flow, whereas long-range dispersal is more common during population expansion and re-colonization phases (Randi 2011).

1.2 History and current status of the Finnish wolf population

The Finnish wolf population represents the most north-western edge of a large, Eurasian distribution area (Boitani 2003; Fig. 1). The Finnish wolf population has generally been considered to be continuous with the Russian Karelian population (Boitani 2003; Linnell et al. 2008). Indeed, the Finnish wolf population did follow the abundance trends in the Karelian population (Pulliainen 1965; 1980) until quite recently (Kojola & Määttä 2004).

At the beginning of the 19th century, wolves inhabited the whole area of Finland (Pulliainen 1980). As in other countries in Western Europe, active hunting to exterminate wolves from Finland started around the 1850s (Ermala 2003; Bisi et al. 2010), and led to a rapid population decline. At first, approximately 300–400 wolves were killed annually in organized hunts (Ermala 2003; Fig. 2), and by the beginning of the 20th century wolves were only present in eastern and northern Finland (Pulliainen 1980; Boitani 2003; Ermala 2003), and as a result, the number of hunted wolves heavily declined (Fig. 2).
Fig. 2. The number of recorded wolf kills in Finland in 1845–2010 (redrawn from Ermala 2003, data for 1991–2010 acquired from the Finnish Game and Fisheries Research Institute and added to the graph). The figure is included in paper (IV).

It is not known with certainty whether the Finnish wolf population ever vanished altogether, but nevertheless it remained very small for a long time, and presumably experienced severe demographic bottlenecks at least during the 1920s and 1970s (Pulliainen 1965; 1980). It has been argued that in the 1920s the population even went extinct due to an outbreak of distemper (Mäensyrjä 1974).

The wolf became protected in Finland outside the reindeer husbandry area in 1973 (Anonymous 2005), but until 1995 it was listed as a normal game species, and the population was controlled by hunting (Bisi et al. 2007). After the accession of Finland into the European Union, the legislation concerning the conservation status of the wolf was tightened, and according to the EC Habitats Directive, the wolf is now strictly protected (Annex IV), except within the reindeer herding area, where hunting is possible (Annex V). These changes in legislation probably contributed to the population growth (Anonymous 1996) and wolves have regularly reproduced again in Finland from the mid-1990s onwards (Kojola & Määttä 2004; Fig. 3).
Fig. 3. The minimum population size ($N_c$) and number of breeding pairs ($N_b/2$) in the Finnish wolf population during 1997–2010. The number of shooting licenses and recorded annual deaths are also shown and follow the same scale as $N_c$. The figure was originally published in paper (III).

After the initial recovery, the Finnish wolf population grew rapidly (Fig. 3) and expanded into new areas (Kojola et al. 2006; 2011). During the peak year of 2006, 25 breeding pairs were detected and the minimum population size estimate was ~250 individuals (Fig. 3). However, after 2006, the trend turned to the opposite direction and the population quickly decreased. This is striking, because the wolf is listed as an endangered species in Finland (Rassi et al. 2010) and a special shooting license is always required for hunting. The annual legal harvest is at most ~15% of the population census size (Kojola et al. 2011; Fig. 3), and no disease epidemics – that could also have explained the heavy decline – have been reported. This strongly suggests that illegal killing might explain the recent population decline (Kojola et al. 2011), but no formal studies on this subject have been conducted in Finland (but see Liberg et al. 2012 for a similar case study on the neighbouring Scandinavian population). According to the latest estimate from
the Finnish Game and Fisheries Research Institute (February 2013), there are now only 120–135 wolves in Finland.

1.3 Population viability

Large carnivores are important ecosystem components, but prone to extinction due to often small and fragmented populations, slow growth rates and large area requirements (Cardillo et al. 2005). Additionally, carnivores have suffered a high level of human persecution, both historically and recently (Woodroffe 2000; Dalerum et al. 2009). Although the wolf as a species worldwide is considered to be of least concern (IUCN 2012), and has a high reproductive potential compared to many other large carnivores, wolf populations in many European countries are considered to be well below the threshold of a viable population (Boitani 2000; see also Traill et al. 2010 and Flather et al. 2011).

Population viability is a complex concept comprising two interacting components: the demographic and the genetic. Demographic models often determine viability as the probability of extinction within a certain number of years, and therefore set levels on the minimum viable population size (MVP) (e.g. Boyce 1992). The genetic viability concept includes, besides short-term survival, a long-term evolutionary perspective: In the short time frame, genetic diversity provides a buffer against environmental fluctuations, whereas in the long term, genetic diversity provides the raw material for natural selection and the ability to adapt to the changing environment (e.g. Frankham 2005; Laikre et al. 2009). Knowledge of the genetic properties of populations of conservation concern, such as effective sizes and connectivity with other populations, is therefore of considerable relevance in management and should not be omitted, for instance, in assessment of the favourable conservation status of populations (Laikre et al. 2009). Genetic information may also provide insights into the demographic structure and history of a population, which are valuable for longer-term conservation planning (e.g. Carmichael et al. 2007; Yang & Jiang 2011; Thalmann et al. 2011).

1.3.1 Small population size and the role of genetic factors

The study of small population size represents one of the foundations of conservation biology (Bouzat 2010). This is because population size is the major
determinant of population well-being and extinction risk (Reed et al. 2003), and small populations are in many ways more prone to genetic erosion.

Extinction is a demographic process caused by deterministic and/or stochastic factors. Deterministic factors, such as habitat destruction and overexploitation inevitably threaten populations when realized, whereas stochastic threats are random, unpredictable changes in environmental, demographic and genetic factors (Allendorf & Luikart 2007, pp. 10–11). Despite their randomness, the abundance and/or the impact of stochasticity is somewhat bound to population size. Stochasticity will become elevated in small populations and may lower the population fitness and increase the risk of extinction (Frankham 2005; Allendorf & Luikart 2007; Palstra & Ruzzante 2008). Genetic stochasticity includes random genetic change via drift (see 1.4) and increased inbreeding (see 1.5), which leads to the loss of genetic variation and an increase in the frequency of harmful allelic combinations (Allendorf & Luikart 2007, p. 10). It is noteworthy that the strength of natural selection is also conditional: natural selection becomes less effective in small populations, and may be swamped by genetic drift as a major evolutionary force (e.g. Allendorf & Luikart 2007, p. 186). If the selection coefficient, \( s \), is smaller than \( 1/N_e \) for an allele, it will act as if it were selectively neutral (Li 1978). Thus, slightly deleterious mutations can accumulate over time in small populations and slightly beneficial alleles can be lost.

Small population size may also increase the risk of hybridization when the probability of finding a mate of the same species is limited (e.g. Allendorf & Luikart 2007, p. 428). Several cases have been reported of introgression from dogs (e.g. Vilà et al. 2003b; Muñoz-Fuentes et al. 2010; Hindrikson et al. 2012) or other canids (e.g. coyotes; Fain et al. 2010; Rutledge et al. 2011) to wolf population gene pools. In conservation, interspecific hybridization is generally thought to be harmful because it can result in the disappearance of a distinct taxon and/or loss of specific adaptations (e.g. Muñoz-Fuentes et al. 2010). Additionally, hybrids may behave abnormally, which can complicate the protection of the endangered species in question (Fain et al. 2010).

In the following chapters, some of the central population genetic concepts related to conservation and small population size are presented. The study of genetic variation (1.4), inbreeding (1.5), population bottlenecks (1.6) and internal structure (1.7), together with population connectivity and gene flow (1.8) and effective population size (1.9) form the body of this work, and all or most of the subjects are covered in papers I–IV.
1.4 Amount of genetic variation

The amount of genetic variation in populations is dictated by the interplay of local population size, gene flow, natural selection, and ultimately the underlying mutation rate (e.g. Frankham 1996). The mutation rate in general is very low, and in practice its role in a short time frame is very small. Therefore, population size (or more precisely, effective population size; see 1.9) and gene flow (1.8) have a more central role, for instance, in conservation and population management. Genetic diversity is acknowledged as a central part of biological diversity and important for population viability in the short and long term (Laikre et al. 2009).

Natural populations are finite in size, which restricts the amount of genetic diversity (i.e. the number of alleles, heterozygosity level and polymorphism) in a population at a certain time. Additionally, genetic drift causes random changes in allele frequencies between generations due to sampling error (Allendorf & Luikart 2007, p. 118), and neutral genetic variation is expected to be lost at a rate inversely proportional to effective population size (e.g. Frankham 1996). On the contrary, gene flow among populations is efficient in counteracting the loss of genetic variation (see 1.8), and in the long term genetic diversity is best preserved in large populations and/or in populations characterized by recurrent gene flow.

1.5 Inbreeding

Inbreeding is often considered one of the major genetic threats in small populations (e.g. Hedrick & Kalinowski 2000; Frankham 2005; O’Grady et al. 2006), where mating between related individuals is inevitable over time. The effect of heterozygosity loss on fitness reduction is often slow and associated with environmental stress, whereas the growth of inbreeding and the accompanying inbreeding depression have an immediate impact (Frankham 2005). Inbreeding depression refers to the lower fitness of inbred individuals due to shared ancestry, and can be explained with two genetic mechanisms: an increase in homozygosity and, by implication, the expression of recessive deleterious alleles, and/or a reduced frequency of heterozygote genotypes with better fitness (e.g. Keller & Waller 2002; Allendorf & Luikart 2007, pp. 307–308).

Inbreeding depression is often very difficult to detect in nature (e.g. Allendorf & Luikart 2007), but its negative effects have been confirmed in wild wolves as decreased over-winter survival of inbred pups (in Scandinavia; Liberg et al. 2005). Increased congenital bone deformities have also been detected in inbred
and isolated wolf populations (Scandinavia, Isle Royale; Räikkönen et al. 2006, 2009). In a captive wolf population, one form of recessive hereditary blindness was confirmed to be expressed frequently due to inbreeding (Laikre et al. 1993).

Usually, wolves strictly avoid mating with close relatives (e.g. Smith et al. 1997; vonHoldt et al. 2008; Rutledge et al. 2010), but in cases where a breeding individual is lost close to the mating season and no unrelated mate is available, close inbreeding might occur (Liberg et al. 2005). A recent study by Geffen et al. (2011) demonstrated that the rate of inbreeding in social canids might be related to the proximity of close relatives and that pack members are excluded as mates, but that mating outside natal packs would be random (i.e. indiscriminate with respect to relatedness). Thus, the active avoidance of inbreeding in wolves can be somewhat questionable.

1.6 Population bottlenecks

When a population undergoes a bottleneck, i.e. a significant reduction in its effective size, it loses its genetic variation due to random drift, when only a small fraction of individuals survive and reproduce in the following generations. Inbreeding is also likely to increase as a consequence of a bottleneck (see above). Equivalent situations to a population bottleneck are the founding or re-establishment of a population from a larger source population; only a proportion of individuals contribute to the new gene pool (Allendorf & Luikart 2007, p. 127). The genetic outcome of demographic bottlenecks may not always be straightforward, however, and chance, selection and population history can play large roles (Bouzat 2010).

A major population size reduction often leaves signals in the genetic pool of a population, and may therefore be subsequently detectable by analysing post-bottleneck patterns of genetic variability. The principles of the statistical testing of population bottlenecks are discussed in detail in chapter 2.3.3.

1.7 Population structure

The genetic structure of any population is a complex interplay of past historical events, species behavioural traits, geographical features limiting gene flow and complex ecological processes shaping it (Pilot et al. 2006). Among historical factors, the last glaciation is known to have had profound effects on the genetic architecture of extant species via postglacial re-colonization dynamics (e.g.
Hewitt 2000), which in wolves can be detected by the presence of two geographically distinct mitochondrial haplotype groups between south-western and eastern Europe (Pilot et al. 2010). More recent historical events and especially the large anthropogenic influence during the last few centuries have caused major shifts in the demography, distribution and genetic structures of many populations (e.g. in wolves, Flagstad et al. 2003; in arctic foxes, Nyström et al. 2006; in western gorillas, Thalmann et al. 2011). Knowledge of historical population structures prior to large anthropogenic influences is important, given that it can be used as a baseline for today’s management goals. In endangered populations, connectivity among the remaining and often very fragmented populations is of special interest, because of the homogenizing effect of gene flow (see 1.8).

 Besides historical changes and connectivity to nearby populations, the presence of cryptic (i.e. hidden) substructures within populations must be taken into consideration. Such structures are often linked to behavioural traits and species ecology including philopatry, dispersal capability, sociality, mating models and food preferences (e.g. vonHoldt et al. 2010; Scandura et al. 2011; Edelaar & Bolnick 2012; Pilot et al. 2012). Different anthropogenic factors may also largely affect the demography and spatial dynamics of wild populations (e.g. Stronen et al. 2012).

### 1.8 Population connectivity and gene flow

The study of population connectivity (demographic cohesion) and gene flow is one of the key issues when defining the current status and evolutionary potential of a population (Waples & Gaggiotti 2006; Palstra & Ruzzante 2008; Lowe & Allendorf 2010). Natural populations are distributed over geographical space with varying degrees of connectivity between regions or subpopulations, ranging from total isolation to panmixia.

Genetic connectivity primarily depends on the absolute number of dispersers among populations, and can be defined as the degree to which gene flow affects evolutionary processes within subpopulations (Lowe & Allendorf 2010). Therefore, the evolutionary population concept emphasizes reproductive interactions between individuals (Waples & Gaggiotti 2006), i.e. the genetic elements of the population processes occur at larger spatial and temporal scales than demographic ones. Consequently, maintaining the evolutionary potential of a population is a long-term conservation issue that requires much larger numbers of
individuals than the short-term maintenance of local populations to avoid demographic extinction (e.g. Linnell et al. 2008; Laikre et al. 2009; Hansen et al. 2011).

Gene flow, even at very low levels seems to be efficient at countering inbreeding and its harmful effects (e.g. Vilà et al. 2003a; Adams et al. 2011), and may maintain the genetic cohesiveness of species by providing selectively advantageous alleles (Rie seberg & Burke 2001). Additionally, gene flow will override the effects of random genetic drift when the migration rate, $m > 1/4 N_e$ (Wright 1931).

It is often difficult to define population limits. Even in situations in which the movement of individuals across regions or assumed/known subpopulations is closely monitored (like the movement of wolves across the border between Finland and Russia; Fig. 4 below) and frequent, the degree to which this movement translates into genetically effective dispersal is often not well established (e.g. vonHoldt et al. 2010).

![Fig. 4. Number of wolves crossing the Finnish–Russian border counted by border guards in 1995–2010. Redrawn from Pulliainen 2011.](image)

29
Different genetic methods can be used to determine the degree of reproductive cohesion between subpopulations. In the absence of true panmixia, a population will be genetically subdivided and allele frequencies will differ significantly (if enough loci and individuals are sampled; Waples & Gaggiotti 2006). These existing genetic differences between (sub-)populations and individuals within them provide the basis to test the origin of individuals and the amount of gene flow. The methods used in this study for estimating gene flow and population differentiation are described in chapter 2.3.5.

As wolves are highly mobile, high rates of gene flow that reduce genetic differentiation among local populations could be expected (Pilot et al. 2006; Randi 2011). Dispersal patterns and distances are, however, affected by many variable factors such as population densities, food availability, social structures, anthropogenic influence and individual preferences (e.g. Pilot et al. 2006; Croteau 2010; Edelaar & Bolnick 2012). Several recent studies have discovered distinct, larger-scale hierarchical population units within grey wolves that correspond with geographic and ecological differences among populations (Carmichael et al. 2001, 2007; Geffen et al. 2004; Weckworth et al. 2005; Musiani et al. 2007; Muñoz-Fuentes et al. 2009), so dispersing wolves tend to stay close to their natal area or choose habitats resembling them (e.g. Pilot et al. 2012). Several recent genetic studies have shown that even on a local scale, wolf populations might be genetically structured and the amount of gene flow restricted (vonHoldt et al. 2010, Scandura et al. 2011; Stronen et al. 2012). A number of reasons exist for such short-distance genetic subdivision, among them human-caused population fragmentation (Stronen et al. 2012) and other anthropogenic factors (vonHoldt et al. 2010) together with the high spatial stability of existing packs (Scandura et al. 2011).

1.9 Effective population size

The concept of effective population size, $N_e$, introduced for the first time in 1931 by Sewall Wright, is one of the most fundamental parameters in evolutionary genetics and conservation biology, because it influences the rate of inbreeding and loss of genetic variation (Waples 2002). In interaction with systematic forces, such as natural selection, mutation and migration, the effective size determines the amount and distribution of genetic variation present in a population (Wang 2005).
Random genetic processes in a population occur at rate inversely related its effective size, which is the size of an 'ideal' population (Wright 1931), with e.g. random mating, an equal sex ratio, non-overlapping generations and a constant population size (Waples 2005). Populations with small effective sizes are greatly affected by genetic drift, and the loss of adaptive potential, and are more prone to inbreeding depression (Keller & Waller 2002; Frankham 2005). Besides directly affecting the rate of genetic change, $N_e$ also determines the relative importance of migration and selection; these forces are deterministic in large populations but can be overwhelmed by random processes in small ones (Waples 2005). Therefore, knowledge of $N_e$ and the different factors affecting it besides the population census size is important in wildlife management and conservation (Luikart et al. 2010).

As natural populations deviate from assumptions of the ideal population in many ways, effective sizes are often much smaller than census sizes (Palstra & Ruzzante 2008). Consequently, $N_e/N_c$ ratios are often relatively low (reported medians: ~0.5, Nunney & Elam 1994; 0.11, Frankham 1995; and 0.16, Palstra & Ruzzante 2008) and may also be unstable if the important factors influencing $N_e$ are not stable. In particular, population size fluctuation, an unequal sex ratio and variance in reproductive success are expected to have a considerable effect on $N_e$ (Frankham 1995). Accurate estimation of $N_e$ with demographic methods (see Allendorf & Luikart 2007, pp. 151–158) requires extensive, often hard-to-gather knowledge on the life history parameters and reproductive biology of a study population. They are not applicable, for instance, for elusive or nocturnal species or for the study of historical populations. Additionally, demographic methods may often overestimate the true $N_e$, because they seldom include all important factors, such as variance in reproductive success, which can reduce the effective size compared to the census size (Luikart et al. 2010).

Several genetic methods have been developed to estimate $N_e$ (for reviews, see e.g. Leberg 2005; Wang 2005; Palstra & Ruzzante 2008; Luikart et al. 2010), which apply different measures of genetic change, and extend to different time frames and on different geographic scales. The most widely used and evaluated concepts of $N_e$ are inbreeding ($N_{eq}$) and variance ($N_{eq}$), which are concerned with the rate of loss of heterozygosity (or the increase of homozygosity) and the change in allele frequencies through time (i.e. drift), respectively (Wang 2005; Luikart et al. 2010). The effective population size and its changes in the Finnish wolf population were estimated throughout this study, and the principles of $N_e$ estimation with genetic methods are presented in 2.3.6.
1.10 The use of molecular markers in population genetic studies

Molecular markers are used to study genetic factors and their manifestation in nature. The rapid development of various techniques and methods to extract, amplify and analyse genetic variation in organisms since the late 1960’s (for the first genetic studies see e.g. Lewontin & Hubby 1966; Harris 1966; Selander & Yang 1969) has in many ways revolutionized our knowledge of genetics and biology in general. All molecular markers can be divided into three conceptually different classes: protein variants (allozymes), DNA sequence polymorphisms and DNA repeat variations (Schlötterer 2004).

Genetic variation can also be divided according to whether it influences individual fitness into neutral and non-neutral variation. Neutral genetic markers are not subject to natural selection, and therefore do not intrinsically provide information about adaptive genetic variation, unless $N_e$ is very small, when the heritability of quantitative traits is also likely to respectively decrease (Willi et al. 2006, see e.g. Marsden et al. 2012 for an example, where neutral and adaptive diversity were correlated in an endangered canine population). Neutrally evolving genetic markers, on the other hand, can be used for instance, to decipher the demographic changes and genetic structures of populations (e.g. Flagstad et al. 2003; Marsden et al. 2012), in individual identification and family relationship studies (e.g. łądżewski et al. 2005; vonHoldt et al. 2008) and in phylogenetic studies (e.g. Pilot et al. 2010).

Wolves and dogs have the same genetic make-up, consisting of 78 chromosomes (38 autosomes and two sex chromosomes). Because the divergence of dogs from wolves, i.e. the domestication process, is in evolutionary terms very recent (≤ 16 300 years ago; Pang et al. 2009; but see Druzhkova et al. 2013), the genomes of these two species have not greatly diverged, and the large number of polymorphic genetic markers developed for dogs are also directly usable for wolves.

1.10.1 Microsatellites

Microsatellites are relatively short (~75–300bp; Allendorf & Luikart 2007) DNA strands with a tandemly repeated sequence motif of one to six base pair(s) in length (Schlötterer 2000). Since their discovery in the early 1980s, they have become widely used markers in molecular and wildlife genetic studies due to their abundance, high polymorphism and biparental inheritance (Ellegren 2004).
Microsatellites serve as a versatile tool, for instance, to identify individuals (‘genetic fingerprinting’), to study family relationships, and to assign and cluster individuals according to the population/group of origin. A large body of software has also been developed for microsatellite markers to decipher, for example, the underlying population structures, demographic histories, the amount of gene flow between populations, and to reveal possibly important units for conservation (e.g. Wayne & Morin 2004). Because of their relatively small size, microsatellite sequences are often also applicable to samples with low DNA quality and/or quantity, e.g. those obtained from non-invasive sampling schemes (e.g. Stenglein et al. 2011; Kopatz et al. 2012).

Microsatellites are rather evenly distributed in organisms’ genomes and mostly selectively neutral (but see Li et al. 2002). Because of their neutral nature, the degree of variability of microsatellites depends on the underlying mutation rate (Ellegren 2004). The mutation rate is generally high, ~$10^{-2} - 10^{-6}$ events per locus per generation (Li et al. 2002), and the majority of the changes are single alterations of the repeat number (i.e. follow the stepwise mutation model, SMM) rather than variations in the primary sequence (Li et al. 2002; Ellegren 2004). The main mutational mechanisms of microsatellites are replication slippage and unequal recombination. In this case, highly repeated genome parts are somewhat inaccurately copied and aligned during cell division, and some of these mutations escape the following repair mechanisms (Li et al. 2002).

1.10.2 Mitochondrial DNA

In the first studies concerning the genetic variation in natural populations, mitochondrial DNA (mtDNA) was investigated (e.g. Avise et al. 1979). Mitochondria are energy-producing cell organelles containing their own double-stranded circular DNA, the vast majority of which is inherited maternally (e.g. Allendorf & Luikart 2007). There is a considerable amount of mtDNA in all eukaryotic cells. Due to this abundance and the relatively small size compared to the autosomal genome, mitochondrial DNA is often much better preserved in specimens of poorer quality. Therefore, mtDNA is widely employed in such genetic applications as ancient and museum DNA analysis (e.g. Ramakrishnan & Hadly 2009) and forensics.

Mitochondrial DNA sequences are also suitable for reconstructing phylogenetic trees (i.e. maternal lineages) since there is generally no recombination between mtDNA molecules (Allendorf & Luikart 2007; Galtier et
al. 2009), and phylogenetic relationships are therefore easy to interpret. A part of the mtDNA commonly used in genetic analyses is known as the control region, which does not contain genes and has the highest variability. In dogs and wolves, the total length of the control region is ~1270 bp (Kim et al. 1998).

Mitochondrial DNA is highly variable in natural populations because of its high mutation rate, which can generate signals of the population history over relatively short time frames. Because of the lack of recombination, the whole mitochondrial genome behaves like a single genetic locus (Galtier et al. 2009), and its usefulness in many population genetic studies is thus rather limited. In genetic studies concerning wolves, mtDNA sequences have recently been utilized, for example, to conclude on the phylogeographic history of wolves in Europe from ancient samples (Pilot et al. 2010), and to reveal the spatial genetic structure due to differing ecological factors (Pilot et al. 2006).

1.11 Aims of the study

This thesis presents the first larger-scale genetic study of the Finnish wolf population. Prior to this study, some Finnish wolf samples had been genetically analysed only for reference purposes (Vilà et al. 1999; Randi et al. 2000; Flagstad et al. 2003; Vilà et al. 2003a; Lucchini et al. 2004; Seddon et al. 2006). The aim of this study was to provide a good general insight into the genetic structure and diversity of the present-day Finnish wolf population based on neutral genetic markers, and to determine the current level of genetic cohesion with the neighbouring north-western Russian population. Important questions to be answered were the following:

1. What is the level of genetic diversity and how is it distributed in the Finnish wolf population?
2. What is the inbreeding coefficient and effective population size?
3. Are any substructures or genetic signals of recent bottleneck(s) detectable?
4. How close is the genetic connection with the Russian population (i.e. what are the levels of gene flow and genetic differentiation between these populations)?

The amount and distribution of genetic variation in the Finnish wolf population during historical times were also investigated, and compared to those seen in the present-day population in order to reveal, whether and how the population has changed during a period of over 150 years of active hunting.
2 Material and methods

The material and methods are only briefly described here. Detailed information can be found in the original papers (I–IV). Background information underlying the genetic analyses is given in more detail.

2.1 Wolf samples

Systematic field studies and the collection of wolf samples were started in Finland in the mid-1990s by the Finnish Game and Fisheries Research Institute, through which the present-day samples used in this study were obtained. In total, 298 contemporary Finnish wolf samples were analysed (all or part of them used in papers I–IV). Figure 5 below shows the geographic location for all collected Finnish samples. In addition, samples from neighbouring Russian Karelian (N = 39) and Arkhangelsk Oblast (N = 14) were used (II, III).

Fig. 5. Map showing the geographic location of wolf samples analysed during this study. Black circles correspond to samples from the contemporary Finnish population, grey circles to historical Finnish samples and white circles show approximate collection sites for the contemporary Russian wolf samples.
Sources of DNA for this research included tissue samples (muscle or liver collected after the death of an individual), pelt samples or blood collected on snow, as well as mouth swabs on FTA™ cards (Whatman) or hair samples from living individuals. In addition to the obtained genotypes (see 2.2 below) and data on the location of the samples, other information gathered by field workers and from autopsy reports was applied. This included knowledge of family relations, sex and the reproductive status of the individuals, amongst others. In papers I and IV the collection date was used as a basis of sample division into approximate temporal groups. In paper III, samples were divided into five temporal birth cohorts for the detection of short-term temporal genetic variation. In order to do so, an estimate for the birth year of each individual was needed. Estimation was based on field observations and/or tooth cementum annulation analysis by Matson’s Laboratory (LLC, Milltown Montana).

Besides contemporary samples, 114 samples between the years 1854–1993 obtained from zoological museums in Finland (Oulu, Helsinki and Kuopio) were utilized (IV). This study material mostly consisted of different kinds of bones: teeth, some pelvic bones, vertebrae, pieces of skull bone and femurs. Other types of historical samples included foot pads, nails, pelt samples and dry blood/neural tissue obtained from inside teeth. The geographic locations for museum samples are indicated in Figure 5 above.

2.2 DNA extraction, genotyping and sequencing

DNA was extracted with different methods depending on the material type. The DNeasy® Tissue Kit (Qiagen) or standard phenol-chloroform extraction protocol was used for tissue samples. The Chelex® method of Walsh et al. (1991) or DNeasy® Tissue Kit was employed for pelt and hair samples, whereas mouth swap samples embedded on FTA™ cards were extracted according to the protocol provided by the manufacturer (Whatman). DNA extraction for museum samples (IV) is presented separately in the following section (2.2.1).

In total, 10–17 different microsatellite loci previously developed for canines were used throughout this study. These loci were polymorphic and highly variable in the Finnish and Russian wolf populations \( H_e \) range \(~0.35–0.85\), and in general unlinked and in Hardy-Weinberg equilibrium (I–IV). Significant linkages detected between loci and deviations from the HW equilibrium in paper III reflected demographic and genetic changes, e.g. the reduction in the population census size and the increase in inbreeding.
For historical samples (IV), genetic variation in the mtDNA control region was also investigated. A 431-bp-long target area encompassing the most reported variable sites of this sequence in wolves was chosen and amplified with PCR primers developed for this study. Shorter overlapping fragments (~140–180 bp) were used if no (satisfactory) sequence was obtained with primers covering the whole target area. PCR products were purified and sequenced using the same primers, but with a fluorescent dye incorporated. Excess dye was removed afterwards and the products were run on an ABI 3730 DNA analyser (PerkinElmer Applied Biosystems).

2.2.1 Handling of museum samples

DNA starts to degrade rapidly after the death of an organism, and over time shorter and shorter DNA fragments remain and are available for genetic analysis. The progress of DNA degradation and possibility to extract usable DNA depends not only on the passing of time, but also, for instance, on the sample type (Wandeler et al. 2007; Casas-Marce et al. 2010), ambient conditions (e.g. Martinková & Searle 2006), and preservation methods used on museum specimens (e.g. Zimmermann et al. 2008).

When the amount of target DNA is low and/or of poor quality, several precautions must be taken into account in order to avoid contamination and to ensure the authenticity of the results (see Wandeler et al. 2007 for a review of this subject). In this study, all pre-PCR phases for museum samples (IV) were conducted in a special laboratory dedicated to work on old DNA (see IV for details). DNA extraction from museum bone samples followed a protocol developed by Rohland & Hofreiter (2007) for old bone samples. Other types of museum samples were extracted with a DNeasy® Tissue Kit (Qiagen) with some modifications (see IV for details). In DNA extraction and the following DNA amplification, several negative controls were run throughout the procedure in order to detect possible contamination. Additionally, the consistency of obtained results was confirmed with independent repetitions: For mtDNA sequences the whole target area of each sequence was covered at least twice. Sequences with unique or rare haplotypes were amplified up to three more times in both directions. For microsatellite loci, a heterozygote genotype was not accepted unless each allele had been observed twice, and a homozygote was not accepted unless three amplifications were consistently homozygous. If these requirements were not met after five amplification attempts, half locus or missing data were
recorded. Microsatellite amplification and reading of the genotypes was conducted independently by two persons (Eeva Jansson and Jenni Harmoinen).

2.3 Genetic analysis

As neutral genetic markers were used in this study, the analysis methods and ideas presented below apply to neutral markers, unless otherwise stated.

2.3.1 Amount of genetic variation

The most commonly used measure to describe genetic diversity is heterozygosity, which gives the proportion of heterozygous polymorphic loci at the population level or the proportion of heterozygous individuals for a given locus. Observed heterozygosity \( H_s \) is the realized measure, whereas expected heterozygosity \( H_e \) tells the expected proportions based on allele frequencies in the study population in question (e.g. Allendorf & Luikart 2007, p. 51). The number of alleles \( A \), allelic richness \( A_s \), and private allelic richness \( P_f \) are also commonly given diversity measures, and can be utilized, for example, in bottleneck testing (see 2.3.3) and indirect gene flow indication (see 2.3.5). The amount of allelic diversity is affected by sampling effort, because rare alleles are more likely to be included when the population sample is large. Richness measures are adjusted according to the smallest sample size, so populations with varying numbers of samples are comparable (Kalinowski 2004). The amount of genetic variation based on 10–17 polymorphic microsatellite loci, was observed throughout this study (papers I–IV). In the last paper (IV) where mtDNA sequences were also utilized, sequence variability was quantified as the number of polymorphic sites \( S \), number of haplotypes \( H \), haplotype diversity \( H_d \) and nucleotide diversity \( \pi \). Due to differing sample sizes, haplotype and private haplotype richness were also estimated.

2.3.2 Inbreeding

Inbred individuals and populations have a higher homozygosity and lower heterozygosity level than expected on the basis of the population allelic frequencies. This is because the parents of inbred individuals share common ancestor(s) and are more likely to be autozygous for any given locus (i.e. that allele is identical by descent; Allendorf & Luikart 2007, pp. 307–308). In the
course of this study, we investigated the occurrence of inbreeding on the population level within the Finnish (I, III, IV) and Russian (II) wolf populations. The inbreeding coefficients ($F_{IS}$), i.e. the average departures of genotype frequencies from Hardy-Weinberg expectations within populations (Wright 1931) were calculated together with their 95% confidence intervals with an appropriate resampling scheme. The estimate measures the deficiency or excess of average heterozygotes in a population sample as:

$$F_{IS} = 1 - (H_o/H_e).$$

2.3.3 Population bottleneck tests

Current genetic methods for detecting bottlenecks from single population samples are all based on detecting deviations from expectations under mutation-drift equilibrium, and contrast two different diversity indices, of which one is more affected by genetic drift than the other (Peery et al. 2012). The ‘heterozygosity-excess’ test is based on the principle that while heterozygosity is relatively insensitive to the effects of bottlenecks, allelic variation is easily reduced (Allendorf & Luikart 2007, p. 127). Rare alleles are more easily lost, and even more so when the bottleneck is severe, as the probability of a neutral allele being lost in a bottleneck is:

$$(1 - p)^{2N}$$

Where $p$ is the frequency of the allele and $N$ is the size of the bottleneck (Allendorf 1986). On the contrary, neutral genetic variation measured as heterozygosity will be lost due to drift in each generation by $1/2N$. As rare alleles are more easily lost in bottlenecks and they contribute only a little to overall heterozygosity in a population, after a bottleneck the population generally has excess genetic diversity at selectively neutral loci (i.e. the observed gene diversity is larger than expected from the number of alleles found in the sample of a constant-size population; Cornuet & Luikart 1996; Luikart & Cornuet 1998). As a consequence of rare allele loss, frequency classes based on allele sizes also become shifted from the normal L-shaped distribution, and significant departures can be detected with the ‘mode shift test’ (Cornuet & Luikart 1996).

In addition to a deficiency of low frequency allele classes, population bottlenecks may also generate gaps in the size distribution of microsatellite alleles (Garza & Williamson 2001), because the number of alleles ($K$) is expected to decline faster than the range in allele sizes ($r$) when there are $\geq 5$ alleles in the
locus, as by chance more lost alleles are then of intermediate sizes (Peery et al. 2012). This ratio of $K/r$ is the basis of the so-called ‘M-ratio test’ (Garza & Williamson 2001). The obtained mean ratio across loci from a given dataset is either (i) compared with ratios derived from putatively stable wild populations, or (ii) alternatively with simulated distributions under mutation-drift equilibrium (Peery et al. 2012). Methods based on the principles mentioned above were also used in this study to detect possible population bottlenecks in the current and historical Finnish wolf population, and in the neighbouring Russian populations (I, II, IV).

Immigration and/or mutations can erase the genetic signals of demographic bottlenecks, which may therefore be detectable only for a short period of time afterwards (e.g. Busch et al. 2007). In this case, the signal might still be detectable with non-autosomal genetic markers (mtDNA and cpDNA) as these sequences are uniparentally inherited and haploid, have only $1/4N_e$ of that for autosomal markers such as microsatellites, and are more susceptible to genetic erosion or the consequences of evolutionary forces (Avise et al. 1984). Population bottlenecks often create sweep patterns similar to the ones caused by natural selection. Therefore, neutrality tests originally developed to detect selection, such as Tajima’s $D$ (Tajima 1989) can be applied even for neutral DNA sequences to detect past demographic changes. Neutrality tests for variation in the mtDNA control region were utilized in this study for museum samples (IV).

### 2.3.4 Population structure

Genetic substructures due to the non-random distribution of genotypes are peculiar to natural populations. Indeed, many ecological and evolutionary processes that influence genetic variation are mediated by space (Guillot et al. 2009), and analysis of the spatial genetic structure within continuous populations in their natural habitat can reveal acting evolutionary processes (Hardy & Vekemans 1999). Genetic methods may help us to find underlying genetic structures, but for the correct interpretation of their nature, understanding of the biology, ecology and demographic history of the study species is crucial.

Two common approaches to study genetic substructures within populations are to examine whether isolation by distance (IBD) is present, and/or whether the present genetic variation is divided into distinct, defined clusters (e.g. Guillot et al. 2009). IBD (Wright 1943; 1946) arises from the limited spatial dispersal of individuals, and can be seen as a decrease in the genetic similarity (i.e.
relatedness) among individuals within populations as the geographical distance between them increases. As a result of IBD, a population will be divided into local ‘neighbourhoods’ (Wright 1946), an area from which individuals can be considered to be drawn at random from a panmictic population (Allendorf & Luikart 2007, p. 209–210). Neighbourhood size can be simply defined as:

\[ N_b = 4\pi\sigma^2 D, \]

where \( \sigma^2 \) is the mean-squared dispersal distance and \( D \) the density of the genes (Guillot et al. 2009).

Clustering methods look for homogeneous domains by inferring populations or clusters of individuals that fulfil some genetic criteria (e.g. Hardy-Weinberg equilibrium, linkage equilibrium or specific allelic frequencies), defining them as distinct groups (Guillot et al. 2009; François & Durand 2010). Unlike methods such as \( F \)-statistics (see 2.3.5), which rely on predefined subpopulations, these Bayesian methods examine whether and how many of such substructures (i.e. clusters) could be found from data with or without individual spatial information included (François & Durand 2010).

In this study, the patterns of IBD (I, III) and genetic clusters (I–IV) within the Finnish and Russian wolf populations were investigated. STRUCTURE (Pritchard et al. 2000), the most popular clustering approach, was utilized in all studies (see attached papers for details). In paper I, another Bayesian approach (BAPS; Corander et al. 2003) was also used. The pattern of isolation-by-distance and the size of neighborhood were estimated only within the contemporary Finnish wolf population, however (I, III).

### 2.3.5 Population connectivity and gene flow

Genetic methods offer a practical means to assess population connectivity, because it is often difficult to measure dispersal directly at large spatial scales (Lowe & Allendorf 2010). Most commonly used methods to describe the distribution of genetic diversity between the levels of hierarchy (e.g. individuals, subpopulations and total population) are based on Wright’s \( F \)-statistics (Wright 1931; see also Excoffier et al. 1992). If subpopulations are genetically diverged, the proportion of genetic diversity due to allele frequency differences among populations, \( F_{ST} \) will be > 0 (Holsinger & Weir 2009). This divergence between subpopulations (or any predefined levels of structures) refers to a limited amount of dispersal and gene flow among them, and the effect of genetic drift changing
the allele frequencies. In this study, genetic differentiation was examined between the Finnish and Russian wolf population (II) in order to determine whether the amount of gene flow is limited, but also between temporal samples among the Finnish population (III, IV) to see, if the local $N_e$ is small enough for genetic drift to cause genetic divergence within the studied time frame.

Genetic differences were further investigated by factorial correspondence analysis (FCA; III, IV), which calculates the maximum genetic variation among individuals, generates axes that describe this variation and then plots individuals along these axes according to their genotype. Graphs from such plots offer a convenient way to illustrate genetic differences between populations.

Additionally, indirect and direct genetic methods (reviewed in Lowe & Allendorf 2010) were used to estimate gene flow between Russian and Finnish wolf populations (I, II, III). The average number of migrants, $N_m$, between Russian populations and Finnish population was estimated in papers II and III with the private allele method of Slatkin (1985; Barton & Slatkin 1986), which is based on the assumption that in genetically subdivided populations the logarithm of average frequency of private alleles is approximately linearly related to $N_m$. The proportion of immigrants, $m$, was quantified in papers II and III with a Bayesian method (Wilson & Rannala 2003) that calculates inbreeding coefficients for each population, the joint probabilities of which are then used to estimate recent migration rates (paper II), or in conjunction with temporal $N_e$ estimation (paper III) using the likelihood-based method by Wang & Whitlock (2003).

Assuming that subpopulations are genetically diverged, likely immigrants may be identified directly on the basis of their multilocus genotypes. This can be done, for example, by Bayesian assignment analysis (Rannala & Mountain 1997), which was utilized in papers I and II. In paper I, in which no likely source population samples from Russian Karelia were available, assignment of immigrants was based on a self-classification approach (i.e. individuals with genotypes not fitting the study population are possible immigrants). In paper II, with Russian Karelian population samples included, the migration rate between these two populations was estimated. Each individual’s multilocus genotype was compared to the marginal probabilities from randomly generated distributions, which determines the statistical probabilities belonging to the tested populations.
2.3.6 Effective population size

Methods to estimate $N_e$ can be divided on the basis of the number of population samples required for (i) point and (ii) temporal estimators (single population sample vs. two or more samples of the same population at different sampling times, respectively). Temporal methods are based on the premise that genetic drift in neutral allelic frequencies is inversely proportional to the effective size (e.g. Palstra & Ruzzante et al. 2008), i.e., they measure the harmonic mean of the variance effective size ($N_{eV}$) over the interval between samples (Waples 2005). On the contrary, single-sample methods estimate in general the inbreeding effective size ($N_{eI}$) of the parental generation or recent past (Waples & Do 2008; Barker 2011) from different genetic factors related to $N_e$, including linkage disequilibrium (see Luikart et al. 2010 for a review).

Besides applying to different time frames, and never for the last sampled population itself (Waples 2005), the two type of effective sizes presented above are expected to be roughly the same only when the population has reached an equilibrium state, and if underlying demographic and ecological dynamics have remained constant for a long time (Palstra & Ruzzante 2008). As this is hardly ever the case in natural populations, it is clear that interpreting the estimates from different approaches can be challenging but necessary and valuable for the interpretation of the results. Moreover, several assumptions that accompany genetic $N_e$ estimation methods are often violated and may create biased estimates (reviewed in Luikart et al. 2010). In particular, violating the assumptions of isolation, a stable population size and discrete generations may be difficult or impossible to avoid in many sampling schemes (including this study), and are thus likely to affect the estimation (e.g. Palstra & Ruzzante 2008; Luikart et al. 2010; Barker 2011). Possible biases that might have affected the obtained estimates in this study are discussed in the attached original papers (I–IV).

In the course of this study, effective population sizes (and its changes) in the contemporary Finnish and Russian wolf populations were estimated using several methods. In paper I, samples collected in 1996–2004 were used to estimate the contemporary and historical $N_e$ (late 19th or early 20th century), in paper II single-sample $N_e$ estimates were obtained for the Karelian and Archangel wolf populations, and in paper III contemporary $N_e$ and its variation within the Finnish wolf population during large demographic changes (1995–2010) was monitored, whereas in paper IV, $N_e$ and its temporal variation was estimated from museum samples collected during the last ~150 years. In total, nine methods to estimate $N_e$...
and its changes were used in this study (two single-sample methods, 6 temporal methods and one simulation method for the detection of demographic changes). Due to their large number, these methods are presented in the original papers (I–IV) only.
3 Results and discussion

Papers I and III somewhat overlap, because in both studies the contemporary genetic diversity and population structure of the Finnish wolf population in 1996–2004 were considered. All wolves analysed in paper I were also included in paper III. However, the time frame in paper III was until 2009, and thus it included the demographic crash in the wolf population after 2006. Additionally, wolves in paper III were grouped into temporal cohorts (corresponding approximately to the generation interval of wolves) based on their estimated or known year of birth instead of the sampling date (as was carried out in paper I). Because generational overlap may bias the estimates of genetic characteristics, this division is likely to give more accurate results on the temporal variation in them (e.g. Luikart et al. 2010). Therefore, the results of paper III are prioritized when the two studies overlap.

3.1 Amount of genetic variation

Despite the documented historical demographic bottlenecks in the Finnish and neighbouring north-western Russian wolf populations (e.g. Pulliainen 1965; 1980, Flagstad et al. 2003; Danilov 2005), the amount of genetic diversity measured in the means of heterozygosity and allelic diversity in the different studies was relatively high (Table 1). A varying number of loci (10–17) were used in different periods and geographic areas. Thus, the results presented in the separate papers are not perfectly cross-comparable or comparable with other studies due to the use of different loci. However, in general the levels of heterozygosity were relatively stable (Table 1), and well in line with earlier studies, in which Finnish wolf samples have been used for reference purposes: Flagstad et al. (2003) reported $H_e$ of 0.69 ($\pm$ 0.12) and $H_e$ of 0.72 ($\pm$ 0.09) for the ‘contemporary Finnish wolves’ ($N = 22$) and in Lucchini et al. (2004) with 13 Finnish wolves analysed $H_e$ was 0.69 ($\pm$ 0.13) and $H_e$ was 0.73 ($\pm$ 0.07). The heterozygosity levels among modern Finnish wolves (I, III) resemble those reported in much larger, non-endangered wolf populations (cf. vonHoldt et al. 2010; Sastre et al. 2011).

Some interesting temporal changes of diversity were observed, however: The expected heterozygosity in the modern Finnish wolf population (III) varied from 0.677 to 0.709, being highest among individuals born in 1995–1997 and lowest at the end of study period (2007–2009). Observed heterozygosities followed a
similar trend, and were 0.749 in 1995–1997 and 0.615 in 2007–2009 (Table 1). The decrease in $H_e$ was statistically significant ($P = 0.021$), and was probably due to the population crash after 2006, increased inbreeding (see chapter 3.2) and the low amount of efficient gene flow (3.5) after the population recovery. At the beginning of the study period, the Finnish wolf population census size was small and there were only a few breeding individuals (Fig. 3), but the genetic diversity was still at its highest. Thus, it is likely that the high diversity reflected the genetic diversity of the Russian Karelian source population on which the Finnish wolf population was demographically dependent at that time (e.g. Kojola et al. 2006). Indeed, gene diversity estimates ($H_e$) for the Karelian samples collected in 1995–2000 (paper II) and Finnish samples born in 1997 or earlier (‘Finnish 1995–1997’ in paper III) were identical (0.709).

### Table 1. Combined results from papers II–IV showing the mean diversity indices and inbreeding coefficients ($F_{is}$; see 3.2) within the contemporary Finnish and Russian wolf populations and in the temporal Finnish museum groups. The number of microsatellite loci used and samples analysed ($N$) are shown. The neighbourhood size ($N_b$) estimates presented are covered in chapter 3.4.3.

<table>
<thead>
<tr>
<th>Population sample (paper)</th>
<th>Number of loci</th>
<th>$N$</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$F_{is}$</th>
<th>$A$</th>
<th>$A_o$</th>
<th>$N_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arkhangelsk 1995–2000 (II)</td>
<td>10</td>
<td>14</td>
<td>0.636</td>
<td>0.634</td>
<td>0.051</td>
<td>4.7</td>
<td>4.2</td>
<td>NA</td>
</tr>
<tr>
<td>Karelian 1995–2000 (II)</td>
<td>10</td>
<td>29</td>
<td>0.709</td>
<td>0.656</td>
<td>0.094*</td>
<td>5.7</td>
<td>4.6</td>
<td>NA</td>
</tr>
<tr>
<td>Karelian 1995–2010 (III)</td>
<td>17</td>
<td>37</td>
<td>0.733</td>
<td>0.691</td>
<td>0.074</td>
<td>6.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1995–1997 (III)</td>
<td>17</td>
<td>43</td>
<td>0.709</td>
<td>0.749</td>
<td>−0.044*</td>
<td>6.5</td>
<td>5.9</td>
<td>131.4</td>
</tr>
<tr>
<td>Finnish 1998–2000 (III)</td>
<td>17</td>
<td>51</td>
<td>0.684</td>
<td>0.673</td>
<td>0.028</td>
<td>5.9</td>
<td>5.4</td>
<td>49.8</td>
</tr>
<tr>
<td>Finnish 2001–2003 (III)</td>
<td>17</td>
<td>83</td>
<td>0.695</td>
<td>0.686</td>
<td>0.030</td>
<td>6.5</td>
<td>5.8</td>
<td>71.6</td>
</tr>
<tr>
<td>Finnish 2004–2006 (III)</td>
<td>17</td>
<td>87</td>
<td>0.687</td>
<td>0.693</td>
<td>−0.002</td>
<td>6.2</td>
<td>5.6</td>
<td>76.3</td>
</tr>
<tr>
<td>Finnish 2007–2009 (III)</td>
<td>17</td>
<td>33</td>
<td>0.677</td>
<td>0.615</td>
<td>0.108*</td>
<td>6.0</td>
<td>5.7</td>
<td>31.5</td>
</tr>
<tr>
<td>Finnish before 1920 (IV)</td>
<td>15</td>
<td>12</td>
<td>0.669</td>
<td>0.624</td>
<td>0.113</td>
<td>5.0</td>
<td>3.7</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1920–1959 (IV)</td>
<td>15</td>
<td>6</td>
<td>0.721</td>
<td>0.741</td>
<td>0.079</td>
<td>4.7</td>
<td>4.2</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1960–1979 (IV)</td>
<td>15</td>
<td>22</td>
<td>0.686</td>
<td>0.729</td>
<td>−0.038</td>
<td>5.5</td>
<td>3.6</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1980–1993 (IV)</td>
<td>15</td>
<td>18</td>
<td>0.676</td>
<td>0.622</td>
<td>0.112</td>
<td>5.5</td>
<td>3.7</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1995–2009 (IV)</td>
<td>15</td>
<td>30</td>
<td>0.697</td>
<td>0.712</td>
<td>−0.003</td>
<td>5.9</td>
<td>3.7</td>
<td>NA</td>
</tr>
</tbody>
</table>

$H_e$, expected heterozygosity, $H_o$, observed heterozygosity, $A$, number of alleles, $A_o$, allelic richness.

* Statistically significant ($P < 0.05$) deviations in $F_{is}$ values. NA, not analysed

Genetic diversity measured as the number of alleles and allelic richness did not reveal large temporal changes in any of the studies (Table 1). In the study concerning historical genetic variation (IV), an interesting pattern was
nevertheless observed: Almost 20% of the alleles (21/107) present in museum samples (collected in 1854–1993, *N* = 58) were not found in the pooled modern population sample (1995–2009, *N* = 298), whereas only four alleles (3.7%) were unique to the concurrent population. The high frequency of these ‘ghost alleles’ in museum samples suggests that the historical wolf population was in fact more diverse, and that allelic variation has been lost in demographic bottlenecks (see e.g. Nyström *et al.* 2006; Ugelvik *et al.* 2011 for similar observations from Scandinavian arctic foxes and Danish large blue butterflies, respectively). In order to see substantial changes in the level of heterozygosity, rather special circumstances may be required, such as a long-term small effective size without immigration (see e.g. studies with isolated wolf populations from Scandinavia; Vilà *et al.* 2003 and from Italy; Lucchini *et al.* 2004), because even in the tightest bottleneck with one breeding pair surviving, ~75% of the heterozygosity is expected to remain in the following generation (e.g. Allendorf & Luikart 2007, p. 127). On the other hand, rare alleles are easily lost in a population bottleneck (see 2.3.3), and they are therefore likely to be more sensitive indicators of past demographic changes, even with some continued gene flow.

Mitochondrial sequences are more prone to genetic erosion in conjunction with demographic fluctuations than autosomal markers due to their lower effective size and mode of inheritance (see 2.3.3). Only three haplotypes have been found in the modern Finnish wolf population (Kiiskilä 2006), and these are the same ones that were among the most recent historical wolves collected in 1980–1993 (Table 2 below). In total, eight haplotypes were observed in the historical wolf population. Five of them were rare (see IV for details) and have most likely been lost in the modern population. Because the lost alleles were rare, the frequency of private alleles was considerably higher in the samples collected before 1960, and the number of polymorphic sites has also reduced notably (Table 2). Similarly to nuclear genetic markers, the change in diversity (*Hd*) was smaller (and not significant).
Table 2. Mitochondrial diversity indices of Finnish wolves in different time periods (Table included in paper IV).

<table>
<thead>
<tr>
<th>Temporal sample</th>
<th>N</th>
<th>H</th>
<th>Hᵣ₀</th>
<th>Pᵣ₀</th>
<th>Hₛ</th>
<th>π</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 1920</td>
<td>18</td>
<td>5</td>
<td>2.80</td>
<td>1.13</td>
<td>0.556 ± 0.130</td>
<td>0.0070</td>
<td>15</td>
</tr>
<tr>
<td>1920–1959</td>
<td>6</td>
<td>3</td>
<td>3.00</td>
<td>2.00</td>
<td>0.600 ± 0.215</td>
<td>0.0132</td>
<td>14</td>
</tr>
<tr>
<td>1960–1979</td>
<td>29</td>
<td>4</td>
<td>2.35</td>
<td>0.25</td>
<td>0.554 ± 0.064</td>
<td>0.0108</td>
<td>11</td>
</tr>
<tr>
<td>1980–1993</td>
<td>28</td>
<td>3</td>
<td>2.17</td>
<td>0.18</td>
<td>0.421 ± 0.103</td>
<td>0.0086</td>
<td>10</td>
</tr>
</tbody>
</table>

N sample size, H number of haplotypes, Hᵣ₀ haplotype richness, Pᵣ₀ private haplotype richness, Hₛ haplotype diversity, π nucleotide diversity, S number of polymorphic sites

3.2 Inbreeding

Differences between the expected and observed heterozygosities resulted in a significant positive inbreeding coefficient among Finnish wolves in the period 2007–2009 and among the Russian Karelian wolves collected in 1995–2000 (\( F_{IS} = 0.108 \) and 0.094, respectively; Table 1). By contrast, the inbreeding coefficient was significantly negative in the Finnish population in the course of population recovery in 1995–1997 (\( F_{IS} = −0.044 \)), suggesting active avoidance of inbreeding.

A positive inbreeding signal obtained indirectly from \( F \)-statistics as heterozygosity deficiency could also be due to excess sampling of (closely) related individuals (e.g. Jankovic et al. 2010) or a cryptic population substructure (i.e. Wahlund’s effect; e.g. Allendorf & Luikart 2007, p. 202). However, the average relatedness (r) between sample pairs in the contemporary Finnish wolf populations was confirmed to be generally low (range −0.029 to 0.026; see III for details), and no significant spatial substructures were found within study populations [no subdivision was detected in the Russian Karelian and Archangel wolf populations, (II) and genetic clusters detected in the Finnish population probably reflected family lines with no clear spatial patterns (I, III)]. Therefore, it is likely that the observed significant inbreeding coefficients in this study reflect true changes in inbreeding, e.g. due to population size decline, a low amount of gene flow and disturbances in pack structures (III).

In summary, inbreeding has recently increased in the Finnish wolf population, and the inbreeding coefficient for the individuals born in 2007–2009 was relatively high (\( F_{IS} = 0.108 \); Table 1) and similar to those reported in isolated wolf populations in Europe (e.g. Italy \( F_{IS} = 0.127 \); Verardi et al. 2006; Iberia \( F_{IS} = 0.177 \); Sastre et al. 2011). Due to well-known detriment of inbreeding
depression to fitness and population viability (see Chapter 1.5), the recent trend is worrying, even though no signs of inbreeding depression have yet been detected in Finnish wolves. On the other hand, no such signs have been looked for, either.

### 3.3 Population bottlenecks

Bottleneck tests of the modern (I) and historical (IV) Finnish wolf populations as well as those of the Russian populations (II) gave somewhat inconclusive results (Table 3).

<table>
<thead>
<tr>
<th>Population sample</th>
<th>Heterozygosity excess test</th>
<th>Mode-shift test</th>
<th>M-ratio test</th>
<th>Tajima’s $D$</th>
<th>Fu’s $F_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finnish 1996–1998 (I)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 1999–2001 (I)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish 2002–2004 (I)</td>
<td>*</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish before 1920 (IV)</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Finnish 1920–1959 (IV)</td>
<td>--</td>
<td>+</td>
<td>NA</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>Finnish 1960–1979 (IV)</td>
<td>--</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Karelian 1995–2000 (II)</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Arkhangelsk 1995–2000 (II)</td>
<td>--</td>
<td>+</td>
<td>--</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Indicates a preceding population bottleneck, -- indicates no support for a preceding bottleneck, NA not analyzed, ? preceding bottleneck possible depending on the evolutionary model used, *note the small sample size.*

The heterozygosity excess method suggested a pre-existing bottleneck only among the Finnish wolves in 2002–2004. Moreover, mode shift inspection revealed an allelic frequency distribution typical of bottlenecked populations in the historical small sample ($N = 6$) collected in 1920–1959 and in the modern Arkhangelsk wolf population, whereas $M$-ratio simulations supported $N_e$ decline in the oldest museum samples (before 1920) under most evolutionary scenarios (see IV for details). On the other hand, demographic tests for mitochondrial sequences, which were conducted for museum data only, showed significant

Different moment-based tests often fail to detect bottlenecks, even in cases where large reductions of population size are known to occur (e.g. Busch et al. 2007; Peery et al. 2012). There are many possible explanations for this contradiction: i) a bottleneck may not be severe enough to leave a genetic bottleneck signal, ii) a bottleneck may have happened too long a time ago, iii) immigration may have erased the genetic bottleneck signal, iv) sample size may have been too small, and/or v) the assumed evolutionary parameters may have been incorrect (Peery et al. 2012). In the Finnish and Russian wolf populations, gene flow (see chapter 3.5) could have been the most important factor overpowering the bottleneck signal(s). Evolutionary parameters for the used microsatellite loci are also largely unknown and sample sizes rather modest, which could also have affected the results.

Moreover, methods differ in their sensitivity depending on the timing and severity of the bottleneck (Williamson-Natesan 2005) and on the pre-bottleneck level of genetic diversity (Peery et al. 2012). For instance, the \( M \)-ratio test is likely to detect a population bottleneck better than the heterozygosity excess test when the bottleneck has been severe and long-lasting (Williamson-Natesan 2005), and has more statistical power when the pre-bottleneck genetic diversity was high (Peery et al. 2012). However, when the underlying evolutionary model of the used microsatellites and/or the historical effective population size are not known, simulation studies to determine deviant \( M \)-ratios might be needed (Peery et al. 2012), and the often-used limit ratio of ~0.7 derived from putatively stable wild populations (thus ratio lower than that would indicate a likely bottleneck; Garza & Williamson 2001) might not always apply (e.g. Busch et al. 2007; Ugelvik et al. 2011). Despite a relatively high \( M \)-ratio of 0.794 among the Finnish wolves sampled before 1920, a bottleneck was supported in this group under the most realistic evolutionary scenarios (see IV for details). In other temporal museum groups, with an unlikely bottleneck, the \( M \)-ratios were clearly higher, 0.856–0.910, as was the case in the concurrent Russian populations (0.850 in Karelia and 0.900 in Archangel; II). Therefore, it is possible (but remains to be confirmed) that the \( M \)-ratios of 0.71–0.73 measured in the Finnish wolf population in 1996–2004 (I) would indeed also indicate genetic bottlenecks.
3.4 Population structure

Regional and continental patterns of genetic subdivision are generally found even in the grey wolf, a species with extremely high dispersal abilities (e.g. vonHoldt et al. 2011, Scandura et al. 2011; Stronen et al. 2012). This study demonstrated that the present-day Finnish wolf population did not form a totally panmictic unit within Finland (I, III, IV), but consisted of varying number of clusters, was characterized by IBD (I, III) and was genetically distinct from the neighbouring Russian population (chapter 3.5; II, III).

3.4.1 Cryptic population structure – clustering analyses

The number of detected clusters ($K$) in the modern Finnish wolf population varied temporally ($K = 3–8$) following population size trends (see III for details). The most plausible biological explanation for these clusters and their varying number was that they represent ‘family lines’ (I and III), whose number was smaller than the number of wolf packs (Fig. 3; number of breeding pairs is equivalent to the number of packs) due to the limited number of breeders and shared ancestry of many individuals and wolf packs. Groups of closely related individuals generating clusters in Bayesian population structure analyses are often seen as a problematic artefact (e.g. Rodríguez-Ramilo & Wang 2012), but can in fact be used to reveal underlying population core structures and changes in them in small populations characterized by close kin groups. The detected clusters in this study did not clearly overlap spatially with known wolf pack territories, and in some cases the clusters had a very wide geographic distribution, suggesting frequent migration between wolf packs and non-existing spatial genetic hierarchy (III). The number of family lines might have been overestimated however, because Bayesian clustering methods tend to be upward biased (i.e. include false positives) when substructures are found within populations characterized by IBD (e.g. Frantz et al. 2009; Meirmans 2012; see 3.4.2).

Similar within-population substructures have been detected, for instance, among wolf populations of the Northern Rocky Mountains in North America (vonHoldt et al. 2010) and in the Italian Apennines (Scandura et al. 2011), and they might be a more general phenomenon reflecting the limited number of founders in (re)established populations (this study), the high stability of established packs, and/or short mean dispersal distances (vonHoldt et al. 2010; Scandura et al. 2011; Randi 2011). Anthropogenic perturbations are likely to
reinforce this genetic divergence within and also between populations, if
dispersing individuals are eliminated and hence connectivity is reduced (vonHoldt
et al. 2010; Stronen et al. 2012).

The cluster analysis of the temporal groups (IV) also revealed internal
population structures within the historical Finnish wolf population and changes in
their proportions during the study period. Temporal clustering is possible when
each of the temporal groups represents long time periods, and genetic drift causes
genetic temporal differentiation (e.g. Flagstad et al. 2003). All temporal museum
groups were significantly differentiated ($F_{ST} = 0.033–0.096$; IV), and the pairwise
genetic distances between groups were positively correlated with their median
sampling year (Mantel’s test: $r = 0.838$, $P = 0.048$). However, genetic drift is not
probably the sole cause for the detected subgroups, because the assignment
probabilities of the wolves in different temporal groups to the clusters did not
decrease linearly with the time difference. The biological significance of the three
groups discovered is hardly related to explicit family structures either (due to the
long study period), and remained somewhat unclear. Interestingly, however, one
of these clusters was peculiar to the oldest samples (before 1920), and all
individuals within this group were from Northern Finland (see Fig. 9 in paper IV).
A distinction between the genetic composition of the northern and southern
wolves was also discovered in the study of Scandinavian historical wolves by
Flagstad et al. (2003). Thus, it is possible, that some wolf type typical of Lapland
was lost when wolves from these areas were effectively eliminated because of
their threat to reindeer herding.

3.4.2 Limited dispersal – IBD

Spatial autocorrelation analyses revealed a clear, significant pattern of isolation
by distance within the concurrent Finnish wolf population (I, III), indicating that
individuals close to each other geographically were on average more related than
those further apart from each other. In general, deviations from population mean
kinship estimates were largest in the closest ($<20–100$ km) and average distance
classes ($\sim200–300$ km), but smaller in the long distance classes ($>400–600$ km).
These long distance classes probably represent those few wolves that dispersed
furthest from their natal packs and colonized new, uninhabited areas (I), and are
therefore important for the demography and spatial dynamics of the population
(e.g. vonHoldt et al. 2010; Randi 2011) but irrelevant with respect to the IBD
concept (cf. Vekemans & Hardy 2004). The slope of the distance regression
varied temporally (III; Fig. 6 below): the negative slope of the regression was smaller ($b = -0.007, P = 0.006$) in the temporal groups at the beginning of the study period (1995–1997) than in the later periods and specifically in the last one (2007–2009; $b = -0.028, P = 0$). Consequently, neighbourhood size ($N_b$) estimated from the slope and the average kinship between adjacent individuals (see I) was much larger (131.4) in the temporal group representing the population recovery phase (1995–1997) than in the group at the end of the study period (2007–2009; $N_b = 31.5$; see Table 1 for estimates from other time periods). The average dispersal distance estimated from samples collected in 1996–2004 (I) was 97.2 km, and well in line with a field study by Kojola et al. (2006), in which the dispersal of wolves equipped with radio or GPS transmitters ($N = 60$) was followed, and the median dispersal distance was 98.5 km (range 35–445 km).

**Fig. 6.** Kinship coefficient vs. distance (log scale) between individuals in the first and last temporal group included in this study. Asterisks denote a significant deviation from the population mean (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Figures were included as electronic Appendices in paper III.

The genetic population structures of many animal species are often greatly affected by different social bonds (e.g. Pope et al. 2006; Hoelzel et al. 2007; Gobush et al. 2009). The model of IBD in highly mobile wolves also stem from sociality: established packs are usually very large in size (see 1.1.2) and spatially
stable (alpha wolves do not leave their home territory; Kojola et al. 2006, see also Scandura et al. 2011). Because the mean dispersal distances of young wolves leaving their natal packs are also relatively short (this study; Kojola et al. 2006), the pattern of IBD is not surprising. Short average dispersal distances in relation to high mobility are probably a consequence of several factors. The abundance of prey and available territories in areas near the natal territory favour short dispersal distances (e.g. Taylor & Norris 2007), whereas population expansion (Randi 2011), a high local density and inbreeding avoidance may trigger long-range dispersal events (e.g. Scandura et al. 2011). The clear steepening of the IBD during the heavy decline in the Finnish wolf population (2007–2009; Figs. 3 and 6) was concurrent with the increase in inbreeding (Table 1). One mechanism that could explain this phenomenon is the sudden loss of alpha individual(s) close to a breeding season (Brainerd et al. 2008). Consequently, this would increase the internal turnover rate of wolf packs and the likelihood of close inbreeding, and lead to higher genetic similarity among individuals within short distances.

### 3.5 Population connectivity and gene flow

The maintenance of dispersal between subpopulations is critical for endangered large carnivores with social pack structures and possessing large home territories (Marsden et al. 2012), because very large population sizes may be needed to ensure the viability in the long term (e.g. Reed et al. 2003; Traill et al. 2007, 2010). Accordingly, for the Finnish wolf population, connection with the Karelian wolf population is of key relevance.

#### 3.5.1 Genetic differentiation between Finnish and Russian wolves

Several analyses demonstrated that the Finnish and north-western Russian wolf populations do not nowadays form just one panmictic population, but are genetically differentiated. In the analyses included in paper II, the genetic distances were relatively large: $F_{ST}$ was 0.151 between the Karelian and Finnish wolf populations and 0.176 between the Arkhangelsk and Finnish populations. This amount of divergence is very close to that (0.177) reported by Seddon et al. (2006) in their study on Finnish and Scandinavian wolf populations. The latter is located far away (> 600 km) and has been effectively isolated since the population re-establishment in the 1980s due to the geographical distance and the reindeer herding areas, being almost impenetrable for wolves (e.g. Seddon et al.
Based on AMOVA analysis, a considerable proportion (15.2%) of the total genetic variation was between the wolf populations of Russia and Finland. These three populations were also clearly separated into their own clusters in STRUCTURE analysis (see II for more details). Later inspection of the genetic differentiation between the Finnish and Karelian population (III) did not suggest any major changes: $F_{ST}$ was somewhat lower (0.086) between the contemporary Finnish (2007–2009) and Karelian (2009/2010) wolf populations, but the differentiation was still highly significant and the amount of gene flow between the populations has remained low (see below).

### 3.5.2 Amount of gene flow and detection of immigrants

The fairly high genetic differentiation detected between the Russian and Finnish wolf populations indicates a low number of immigrants between them. According to Wright’s island model (Wright 1951), an $F_{ST}$ value of 0.152 is equivalent to ~1.4 effective migrants per generation between populations (II), whereas the later measured $F_{ST}$ of 0.086 (III) corresponds to ~2.7 migrants per generation. Another indirect method based on private allele frequencies showed a reverse trend and earlier suggested 3.0 migrants per generation ($N_m$) between the Russian Karelian and Finnish wolf populations (II), although when the estimation was repeated at a later stage and with all available wolves in both populations (III), $N_m$ was only 1.09. Nevertheless, both of these methods suggested a low migration rate in the range of ~1–3 immigrants per generation between the populations. Indirect methods may not be very reliable, however, because they assume equilibrium between drift and migration and their assumptions are overly simple for most wild populations (e.g. Whitlock & McCauley 1999). Thus estimation of the proportion of immigrants ($m$) with other approaches might be more robust (Waples & Gaggiotti 2006; Lowe & Allendorf 2010).

The estimates obtained with Bayesian methods also suggested relatively low migration rates between the populations: the first estimate using the Wilson & Rannala (2003) approach suggested an $m$ value of 0.069 ($± 0.032$ SD) from the Karelian into the Finnish wolf population (II), whereas $m$ estimated jointly with $N_e$ (Wand & Whitlock 2003) for the different periods in paper III varied from 0.016 (2001–2006) to 0.090 (1998–2003; see III for details). Based on the assignment of individual genotypes, the self-classification approach (I) suggested that only four wolves were probable first-generation immigrants (3%), whereas in the later study (II) with the Karelian population used as a reference, the overall
migration rate between the population was 2.7% \( (m = 0.03) \), but only one individual was a probable immigrant into Finland. No individual-based assignments were conducted in the latest temporal period, but FC analysis identified one likely immigrant wolf from Karelia into Finland in 2008 (see III for details).

The findings of relatively high divergence and a low amount of migration between the Finnish and Russian wolf populations has significant implications concerning the management of the Finnish wolf population: Contrary to previous conceptions (Pulliainen 1965, 1980; Boitani 2003; Linnell \textit{et al.} 2008) and common assumptions, the connection between the wolf populations seems to be weak on the basis of this study.

There are several possible explanations for the low amount of wolf immigration to Finland (see II for details), of which the dependence on local densities (e.g. Taylor & Norris 2007) during the dispersal phase might be the most important. If local population densities are low and territories are available near the natal pack territory, there is no ‘need’ for long-range dispersal (see 3.4.2). Wolves are not protected in Russia, and in the Karelian population, for example, the number of wolves has fluctuated greatly, a recent estimate being only around 300–350 individuals (see II for more details and references). Thus, the local density in Karelia is likely to be rather low, and might reduce the migration tendency within Karelian wolves. Even a very low amount of immigration could, however, preserve the adaptive variation \( (Nm > 0.1) \) and enable the avoidance of inbreeding \( (Nm > 1) \), but not prevent the differentiation of allele frequencies between subpopulations due to genetic drift. Importantly, immigrants must also breed in order to have any genetic significance in the recipient population (e.g. Lowe & Allendorf 2010). Probable immigrants detected in this study were all killed or found dead near the Finnish–Russian border (see I and III for details). It is possible that some immigrants were not sampled in this study, and that these individuals could have even gained a reproductive alpha status. However, because no significant positive changes in the Finnish gene pool were noticeable and the inbreeding coefficient actually increased among wolves born by the end of the study period (2007–2009; Table 1), it is likely that the amount of effective gene flow is currently lower than the 1–2 wolves per generation required to ensure the long-term survival of the population (Anonymous 2005).
3.6 Effective population size

Single-sample-based estimates of the effective population size in the current Finnish (I, III) and Karelian (II) wolf populations were relatively small (20.4–76.4; Table 4) throughout this study. These approaches are widely used (e.g. Luikart et al. 2010) and they have been proven to be quite accurate when the actual \( N_e \) is small (e.g. Waples & Do 2008; Barker 2011; Hoehn et al. 2012). LD-based estimates of \( N_e \) are prone to biases if other factors than a small effective size, such as the substructure, are behind LD (Luikart et al. 2010). However, they appear to be rather insensitive to the effect of gene flow even with an \( m \) of ~0.10–0.25, and thus may be used in detecting early indications of population fragmentation and decline (England et al. 2010; Luikart et al. 2010) and they may provide reasonably reliable estimates of local \( N_e \) (Waples 2010). The ONeSAMP method (Tallmon et al. 2008) uses seven summary statistics besides LD in its \( N_e \) estimation and might therefore provide more precise estimates (e.g. Luikart et al. 2010). Estimates derived from ONeSAMP seem to be sensitive to sample size, however (Sotelo et al. 2008; Haag et al. 2010). This effect was also confirmed in this study as smaller random data sets from the original sample provided significantly smaller estimates in two out of five cases (see III for details). Thereby, estimates based on small population samples often characteristic of studies of endangered populations, such as those in paper (IV) (Table 4), might also be downward biased.

Table 4. Compiled results for \( N_e \) estimates based on single-sample approaches in this study. Estimates from an LD-based method and from a Bayesian method with their 95% confidence intervals or limits are shown. Roman numerals after each group refer to the original articles.

<table>
<thead>
<tr>
<th>Population sample</th>
<th>N</th>
<th>LD-( N_e ) (^1)</th>
<th>95% CI</th>
<th>ONeSAMP (^2)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karelian 1995–2000 (II)</td>
<td>29</td>
<td>46.7</td>
<td>38.2–115.8</td>
<td>39.9</td>
<td>24.8–80.0</td>
</tr>
<tr>
<td>Finnish 1995–1997 (III)</td>
<td>43</td>
<td>67.2</td>
<td>54.5–85.2</td>
<td>35.8</td>
<td>28.8–52.2</td>
</tr>
<tr>
<td>Finnish 1998–2000 (III)</td>
<td>51</td>
<td>27.1</td>
<td>23.3–31.5</td>
<td>29.3</td>
<td>24.0–45.0</td>
</tr>
<tr>
<td>Finnish 2001–2003 (III)</td>
<td>83</td>
<td>30.3</td>
<td>27.0–34.1</td>
<td>31.7</td>
<td>25.6–47.2</td>
</tr>
<tr>
<td>Finnish 2004–2006 (III)</td>
<td>87</td>
<td>37.4</td>
<td>33.4–42.0</td>
<td>51.5</td>
<td>44.4–76.3</td>
</tr>
<tr>
<td>Finnish 2007–2009 (III)</td>
<td>33</td>
<td>20.4</td>
<td>17.6–23.8</td>
<td>30.4</td>
<td>25.4–45.4</td>
</tr>
<tr>
<td>Finnish before 1920 (IV)</td>
<td>12</td>
<td>20.5</td>
<td>13.5–35.2</td>
<td>13.2</td>
<td>10.8–19.6</td>
</tr>
<tr>
<td>Finnish 1960–1979 (IV)</td>
<td>22</td>
<td>76.4</td>
<td>48.1–159.0</td>
<td>24.3</td>
<td>20.9–32.7</td>
</tr>
<tr>
<td>Finnish 1980–1993 (IV)</td>
<td>18</td>
<td>45.2</td>
<td>30.4–78.4</td>
<td>23.1</td>
<td>18.5–36.3</td>
</tr>
</tbody>
</table>

\(^1\) Waples & Do 2008, \(^2\) Tallmon et al. 2008
More thorough investigation of the more robust LD-based estimates revealed interesting patterns: The effective population size was relatively high during the population recovery phase (1995–1997; $N_e = 67.2$), and the wolf population was characterized by concurrently high genetic diversity, inbreeding avoidance and a large neighbourhood size (Table 1), probably reflecting the genetic composition of the Russian source population at that time (III). After this period, there was probably a population bottleneck, when only a proportion of the individuals reproduced (Fig. 3), and thus estimates for later time periods (1998–2009) are significantly smaller (Table 4).

The estimate of $N_e$ for the Karelian population in 1995–2000 was somewhat smaller and less precise than that for the Finnish wolves born before 1998. This might be, for example, due to the longer sampling interval and because an unaccounted age structure tends to downwardly bias the estimates (Palstra & Ruzzante 2008). Regardless, the estimate for the Karelian population is surprisingly low. In a recent study by Sastre et al. (2011), 47 Russian wolves south-east from our study area in the regions of Tver, Vologda, Smolensk and Kaluga Oblasts (covering an area > 300 000 km$^2$) were genotyped and the population $N_e$ estimated. Their analysis suggested an LD-$N_e$ of 138.0 (CI 75.9–490.4) for the wolf population of this continuous area, which together with the detected high inbreeding coefficient ($F_{IS} = 0.147$, CI 0.07–0.20) might suggest that the Russian wolf population is nowadays somewhat fragmented, contrary to what is commonly assumed. The $N_e$ estimates from the Finnish and Karelian wolf populations obtained in this study support this hypothesis.

Temporal approach estimates revealed a considerable temporal fluctuation of $N_e$ within the Finnish wolf population (Table 5 below; III). When compared to the single-sample estimates (Table 4) and to the estimated number of breeding individuals (Fig. 3), these estimates were in many cases extremely high, and thus biologically implausible. It is likely that a longer time between temporal population samples could have given more accurate and precise results, because the drift signal against background noise would have been stronger (e.g. Palstra & Ruzzante 2008; Waples 2010; Luikart et al. 2010). For the monitoring of endangered populations, the recommended longer sampling interval of many generations is impractical (e.g. Waples 2010), and estimates from a temporal approach are thus of little value. Estimates based on this approach derived from historical population samples can be of considerable value in conservation, however, because they can provide a requisite base estimate of $N_e$ in a previous, undisturbed population.
Effective size estimates for the historical Finnish wolf population based on temporal approaches and using an interval of ~25 generations were relatively large, varying from 86.0 to 176.4 (Table 5; IV). Instead of the local Finnish wolf population, long-term estimates are likely to reflect the genetic behaviour of a larger metapopulation (e.g., Leberg 2005; Palstra & Ruzzante 2008) and provide a harmonic mean of the \( N_e \) between the sampling points (Waples 2005). Because very few of the oldest historical samples were collected prior to the major demographic changes in the 19th century (see IV for details), and many subsequent bottlenecks are likely to have occurred, the obtained \( N_e \) estimates hardly represent that for a preceding undisturbed population. In fact, the Bayesian method (Beaumont 1999; see also Girod et al. 2011) that was used to infer the past demographic history in paper I strongly supported a long-term decline in the Finnish wolf population. The contemporary population (1996–2004) was estimated to be only ~8% of its historical size, and the ancient \( N_e \) (late 19th to early 20th century) was thus about 590.
4 Conclusions

This study confirmed explicit patterns and rapid genetic changes in the contemporary Finnish wolf population. Major findings included:

1. a low level of gene flow between the present-day Russian and Finnish wolf populations and resulting genetic differentiation,
2. a relatively small local effective population size leading to gradual temporal genetic changes in the population,
3. a significant increase in inbreeding during the recent heavy population decline,
4. clear isolation by distance within the population due to the relatively short mean dispersal distances of wolves and kin-based social structure and,
5. genetic deterioration of the contemporary Finnish wolf population compared to the historical population, which was likely to be much larger and more uniform with the Russian population.

On the basis of the results obtained in this study, it is clear that the Finnish wolf population is currently too small ($N = 120–135$) to maintain a self-sufficient and genetically healthy population in the long-term (e.g. Palstra & Ruzzante 2008). The local effective population size should be considerably larger and/or the rate of the effective immigration from the Russian population higher (i.e. the immigrants should reach reproductive status in the Finnish wolf population) in order to secure the population viability.

The question of population viability is not straightforward, however, and exact numeric conservation goals are therefore difficult to provide. The ‘50/500’ rule of thumb (Franklin 1980) has often been used as a guiding principle in conservation, and as such has become somewhat infamous and misused (e.g. Flather et al. 2011; Jamieson & Allendorf 2012). The rationale behind the 50/500 rule is that the effective population size should be larger than 50 in order to avoid extinction in the short term due to the harmful effects of inbreeding depression on demography, whereas $N_e \geq 500$ is necessary to retain the evolutionary potential of a population (e.g. Jamieson & Allendorf 2012). In the management plan for the wolf population in Finland (Anonymous 2005), it was stated that 20 breeding pairs would secure the short-term viability, if the amount of gene flow from Russian Karelia remains at the pre-existing level. On the other hand, over 25 breeding pairs would be needed if the level of immigration were to decrease. Results from this study, showing a low degree of connectivity (I–III), support the
recommendation of at least 25 breeding pairs. So far this goal has only once been reached, during a peak year in 2006 (Fig. 3).

The genetic viability concept is indisputably important and should be integrated into the conservation of species (e.g. Frankham 2005; Laikre et al. 2009; Jamieson & Allendorf 2012). However, minimum conservation goals based on effective size estimates alone are likely to be insufficient (see 1.3 and 1.3.1) – not least due to the fact that $N_e$ estimates themselves are easily biased and often somewhat inaccurate (e.g. Luikart et al. 2010) and that the stability of the $N_e/N_c$ -ratio is not necessarily consistent (e.g. Palstra & Ruzzante 2008; III). For example, based on the LD-$N_e/N_c$ -ratios obtained in this study (III), ~180 wolves would have been theoretically enough to avoid a significant increase of inbreeding in the time period of 1998–2006 ($N_e/N_c = 0.275–0.285$), whereas as many as 521 wolves would have been necessary to fulfil the minimum $N_e$ requirement of 50 during the last study period in 2007–2009 ($N_e/N_c = 0.097$).

By combining field studies and genetic analyses with the knowledge we have on wolf population dynamics, it is possible to recognize the factors that lower the effective population size, and thus adversely affect the population viability. Because alpha wolves have very great significance in the maintenance of the natural population structure and demography (e.g. Brainerd et al. 2008; Treves 2009), they should have the highest conservation priority. Additionally, immigrants are crucial for the long-term survival of the population and thus their recognition, for example using non-invasive genetic methods could be utilized in conservation (e.g. Lucchini et al. 2002). From the management point of view, the focus of genetic analyses should shift from retrospection to real-time monitoring. The field data continuously collected by the Finnish Game and Fisheries Research Institute and the genetic reference data-base collected in conjunction with this study provide a good foundation for genetic monitoring in the future. However, there is currently no ongoing funding for genetic monitoring.

This study provides diverse, new information on the current and historical genetic status of the Finnish wolf population that can be utilized, for example, in future wolf management. Ongoing genetic studies on wolves in our study group by Alina Niskanen and Jenni Harmoinen concerning immune response-related gene variation (AN) and the social-based population structure (JH) will significantly increase and deepen our understanding of Finnish wolf genetics in the near future. New molecular approaches, such as genome-wide SNP (single nucleotide polymorphism) marker scans and high-throughput genome sequencing
technologies are also now readily available, and will most likely be adopted in future studies concerning the Finnish wolf population.
References


Original articles


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Original publications are not included in the electronic version of the dissertation.
593. Tiikkaja, Marjo (2012) Value creation in collaboration between software suppliers and customers: suppliers’ perspective
596. Härkönen, Laura (2012) Seasonal variation in the life histories of a viviparous ectoparasite, the deer ked
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PAST AND PRESENT GENETIC DIVERSITY AND STRUCTURE OF THE FINNISH WOLF POPULATION