Conservation genomics in inbred Scandinavian wolves using bioinformatic methods

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Abstract
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With the recent and unprecedented progress in retrieving DNA sequence information from a large number of individuals of any species, conservation genetic research has entered a new phase. Specifically, it has become possible to study how genomes of endangered species respond to reductions in population size. Using genomic and bioinformatic approaches, in this thesis I investigate the contemporary Scandinavian wolf population founded 40 years ago by only three individuals, after the original population had been extirpated some decades earlier. The origin of the founders has been the subject of controversy, so I aimed to trace their origin using first male-specific Y chromosome sequences, and then whole-genome sequence data. I compared Scandinavian wolves to wolves from the nearby Finnish-Russian population as well as to publicly available wolf and dog samples from around the northern hemisphere, and found that the Scandinavian founders shared Y-haplotypes only with Finnish wolves. Consistent with this observation, when assessing population structure on the genomic scale, founders clustered with Finnish and Russian wolves, and an admixture analysis showed no other ancestries, nor traces of introgression from dogs.

Small populations tend to have less genetic variation than larger populations, which might reduce their adaptive potential and increase the risk for extinction. A common measure used to investigate the genetic health of small populations is the genetic load, which is the fitness reduction of individuals due to accumulation of deleterious variants. I assessed the genetic load in Scandinavian wolves, divided into the components masked load (comprised of deleterious mutations in heterozygous state) and realized load (comprised of deleterious mutations in homozygous state), using both putatively deleterious single nucleotides and structural variants. I found that the realized load increased with every generation of inbreeding but was alleviated after genetic rescue events when new immigrants entered the population. Finally, I searched for the genetic basis of cryptorchidism, a testis condition that results in lowered fertility and is thought to be related to inbreeding depression. The trait is likely highly polygenic and the fact that only one significant association (to a region on the X chromosome) was found can be explained by that the number of available samples was very low, as is inevitable for small populations.

In conclusion, this thesis explores the origin and the genetic health status of a small and recently founded natural population, and gives insights into how patterns of genetic load are affected by inbreeding and genetic rescue.

Keywords: conservation genomics, *Canis lupus*, bioinformatics, Y chromosome, admixture, genetic load, structural variation, GWAS

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To Julie and Lily,
and all the girls of the future

List of Papers

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

I.   Smeds, L., Kojola, I. and Ellegren, H. (2019). The evolutionary history of grey wolf Y chromosomes. *Molecular Ecology*, 28(9):2173–2191.

II.   Smeds, L., Aspi, J., Berglund, J., Kojola, I., Tirronen, K. and Ellegren, H. (2021). Whole-genome analyses provide no evidence for dog introgression in Fennoscandian wolf populations. *Evolutionary Applications*, 14(3):721-734.

III. Smeds, L. and Ellegren, H. (2023). From high masked to high realized genetic load in inbred Scandinavian wolves. *Molecular Ecology*, 32(7):1567-1580.

IV.   Smeds, L., Huson, L.S.A. and Ellegren, H. (2024). Structural genomic variation in the inbred Scandinavian wolf population contributes to the realized genetic load but is positively affected by immigration. *Evolutionary Applications, acceptable pending minor revision*.

V.   Smeds, L., Ellegren, H. and Åkesson, M. A scan for the genetic basis of cryptorchidism in inbred Scandinavian wolves using GWAS and haplotype analysis. *Manuscript*.

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Additional papers

The following papers were published prior and during the course of my doctoral studies but are not part of the thesis.

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Künstner, A., … Smeds, L … Wilson, R.K. [total of 82 authors] (2010). The genome of a songbird. *Nature*, 464(7289):757-762.

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Contents

Abbreviations

A	Adenine
bp	base-pair
C	Cytosine
DNA	Deoxyribonucleic acid
G	Guanine
GERP	Genomic evolutionary rate profiling
GWAS	Genome-wide association study
IUCN	International Union for Conservation of Nature
kya	thousand years ago
Mb	million base-pairs
mtDNA	mitochondrial DNA
NAHR	Non-allelic homologous recombination
NRM	Naturhistoriska riksmuseet (Museum of Natural History)
PCA	Principal component analysis
SNO	Statens naturoppsyn
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SV	Structural variant
T	Thymine
TE	Transposable element
VCF	Variant call format
WGS	Whole-genome sequencing

Introduction

The rise of new species, as well as the extinction of others are continuous evolutionary processes as old as life on Earth, resulting in the remarkable biodiversity present in the world today. The loss of species is a natural process, but the rate of extinction events during the Anthropocene is extreme (Andermann et al., 2020; Ceballos et al., 2015; Vignieri, 2014). The urbanization causing fragmentation of natural habitats, as well as the anthropogenic global warming that continuously change the environment and climate, have resulted in species vanishing with an accelerating pace – for vertebrates conservatively estimated to 22 times faster than the historical baseline (Ceballos et al., 2015). This has led to the claim that we are now entering the sixth mass extinction (Barnosky et al., 2011; Ceballos et al., 2015; Leakey, 1996; Pievani, 2014; Sills et al., 2018). Simultaneously, large conservation efforts are taking place all over the world to save habitats and threatened species that have not yet become extinct (Maxwell et al., 2020). Many species on the brink of extinction suffer from inbreeding depression and lower fitness compared to larger populations, while others seem to be unaffected despite that only a small number of individuals remain. To disentangle the genetic factors behind inbreeding depression and population fitness is an outstanding question in the field of conservation genomics.

This thesis is about a small population that was re-founded after a regional extinction event, and managed to grow despite setbacks such as few immigrants and severe inbreeding. I have combined genomic data and different bioinformatic tools from the fields of evolutionary and population genetics to explore the genetic variation in this population, and use it to answer questions about its origin and how the genetic health has changed over time.

Genetic variation and its acting forces

Evolution is the relentless change of life constantly happening around us. These changes have made it possible for the first simple life form to evolve over billions of years into all the forms of life existing today. One can discuss evolution on all levels and timescales, but the foundation to all these differences are changes within the genome that occur from mutations. In this section I go through how these mutations arise and the different forces that can remove or maintain them.

Mutation, recombination and gene flow

Mutations can occur during DNA-replication, resulting from errors in the synthesis of a new chromosome before cell division (Brown, 2002). Mutations can also be caused by radiation or chemicals, physically damaging or altering nucleotides in the DNA-molecule. DNA-repair enzymes in the cells aim to minimize errors both before and after replication, but sometimes an erroneous sequence is retained. If the mutation occurs in the germline, it can be inherited down to future generations.

Another important process is recombination – the shuffling of genetic material between DNA molecules. In diploid eukaryotes, recombination leads to exchange of sequences between the homologous chromosomes inherited from the two parents. When the homologous chromosomes pair up during the first meiotic division, double strand breaks are induced on one of the homologs and resolved by a repair machinery which uses the unbroken chromosome copy as a template (Baudat et al., 2013). The structure that is formed during this process can be resolved in two different ways, generally referred to as crossover and non-crossover, respectively. In the case of crossover, the two chromosome copies reciprocally exchange large chromosomal blocks with each other, which can result in new combinations of genetic variants in the gametes. A non-crossover recombination event leads to unidirectional transfer of genetic material from one homologous chromosome to the other (called gene conversion) in a limited chromosomal interval and will therefore only locally affect genetic diversity. Recombination increases genetic variation in the population, and has the advantage to both combine favourable alleles with each other as

well as breaking up combinations of deleterious and advantageous alleles (Felsenstein, 1974). A disadvantage is that also favourable combinations of alleles might be separated by recombination (Charlesworth & Barton, 1996). Recombination can also occur between similar regions that are not allele pairs – for example two different copies of a repeat – this is called non-allelic homologous recombination (NAHR) and can lead to larger structural rearrangements of the sequence such as deletions, duplications and inversions (Carvalho & Lupski, 2016). These are referred to as structural variants (SVs), a type of genetic variation studied in this thesis that I come back to below.

On a population level, new genetic material can also arise through gene-flow from immigrating individuals that breed within the population and thereby contribute with new alleles (Morjan & Rieseberg, 2004). These new variants also have arisen from mutations at some point, but in the donor population.

Natural selection and genetic drift

All processes discussed above contribute with new genetic variation to a population, which can either be maintained or disappear due to different evolutionary forces. Long before the rise of modern genetics, Charles Darwin and Alfred Russell Wallace formed the concept of natural selection – that there are differences in survival and reproductive success between individuals due to differences in their phenotypic traits, with individuals carrying more favourable traits contributing more offspring to the coming generation, thereby increasing the frequency of those traits (Darwin & Wallace, 1858). This could either be traits that are better adapted to the environment, or traits that make the carrier more attractive to potential partners (so called sexual selection).

Mutations occur from errors or damages and it is important to note that they do not arise to improve fitness – they occur by chance and their effect can be either neutral, beneficial or deleterious (Gregory, 2009). In the first decades of the 1900s, a common view was that the vast majority of mutations had an impact – either deleterious that would be removed by natural selection, or beneficial that would be positively selected and eventually get fixed in the population (Huxley et al., 2010; Morgan, 1925). The selection theory was challenged in the 1960s when studies on protein sequences showed that there was more variation seen than could be explained with selection, and many mutations did not seem to have an effect at all. In 1968, Motoo Kimura introduced the neutral theory which postulated that most mutations are neutral, and a large part of variation seen between and within species is just the effect of random chance (Kimura, 1968).

This effect, that allele frequencies can fluctuate and become fixed in populations just by stochasticity due to finite population sizes is called genetic drift, and the phenomenon is of great importance in this thesis. The neutral theory of molecular evolution was later refined and updated to the nearly neutral theory by Tomoko Ohta, who argued that also slightly deleterious mutations can become fixed in a population, and that this is dependent on the population size (Ohta, 1973). The neutral and nearly neutral theories raised a debate between selectionists and neutralists that has been active throughout the last 50 years (Jensen et al., 2019; Kern & Hahn, 2018).

Some observations were hard to explain with the neutral or nearly neutral theories, for example that diversity is reduced in regions with low recombination. Maynard Smith and Haigh (1974) provided an explanation for this through linkage. If a neutral mutation is linked to an advantageous allele, it can hitchhike with the favourable allele to fixation by positive selection, referred to as a selective sweep. Reduced diversity in regions with low recombination can also be a consequence of background selection, if a neutral mutation is linked to a deleterious allele and thereby is removed from the population (Charlesworth et al., 1993). In regions with high recombination rate there is a higher chance that the linkage between the neutral and deleterious (or advantageous) mutation is broken, so that the neutral variant can remain longer in the population. Hence, recombination is very important for the efficacy of selection (Peñalba & Wolf, 2020). This thesis focuses on a very small population, and though all above mentioned processes and forces affect and act on it, the most important factor is likely genetic drift.

Genetic variation on different levels

In this section, I go through the two types of genetic variation used in this thesis, point mutations and structural variants. This is followed by parts on haplotypes – combinations of variants inherited together – and the special case of the sex-specific chromosome which does not recombine and thereby form its own haplotype, both which are of relevance for this thesis.

Point mutations

Mutations that affect a single base-pair in the DNA sequence are called point mutations. The DNA sequence is built up of four types of nucleotides called Adenine (A), Cytosine (C), Guanine (G), and Thymine (T). A point mutation that changes one of these nucleotides to another gives rise to a so-called single nucleotide variant (SNV). If the mutation occurs in the germline and the variant is inherited in the population, it is generally referred to as a single nucleotide polymorphism (SNP). There is no clear consensus for these two terms and

how they should be used; some claim 'SNP' should be reserved for variants that are found in a sufficiently large fraction of a population; others use them interchangeably. In this thesis I follow Heng Li's definition (Li, 2021) and use 'SNP' exclusively, since all variants in our studies are germline mutations. SNPs are the basis of the analyses in **Chapter I-III** and **Chapter V**. When a SNP occurs in the coding sequence of a gene, it can either leave the protein unaffected ('synonymous' mutation), change the protein by coding for a new amino-acid ('non-synonymous' mutation) or disrupt the protein by changing the start or the stop codon ('nonsense' mutation). These different types of changes are of importance to **Chapter III**. A point mutation could also be a deletion or an insertion of a single nucleotide, such mutations are usually referred to as small 'indels' (from insertions/deletions) and are not further investigated in this thesis.

Structural variants

Structural variants are mutations that affect a larger part of the DNA sequence, often defined as more than 50 bp in size. This is an arbitrary distinction to distinguish SVs from indels, which comes from differences in detection methods (see Methods below), and has no biological meaning (Mahmoud et al., 2019; Song et al., 2023). Structural variants can be interchromosomal – fissions, fusions and translocations – which are not explored in this thesis. More commonly they are intrachromosomal and they can either change the total length of the sequence or not. SVs are always defined in relation to a reference sequence. Consequently, deletions are segments missing in the focal individual, but present in the reference; insertions are the opposite – segments in the focal individual that are not present in the reference. A duplication is when a segment in the reference exists in two or more copies in the focal individual, and inversions are segments that have a reverse orientation compared to the reference sequence. Inversions can arise after two double-strand breaks. Intrachromosomal SVs are investigated in **Chapter IV.**

Haplotypes

Genetic variants that are located on the same chromosome copy and inherited together form a haplotype. As an example, consider an individual with two polymorphic sites, one A/C and one T/G. In the whole population, these variants may be found in all possible combinations, but in our example individual, the two variants are linked in a certain way, depending on how they were inherited from the parents – for example A and T from the mother and C and G from the father. Due to recombination when the two parental chromosome copies can interchange sequences, the haplotypes in an offspring might be different from its parents. Haplotypes are therefore often studied in smaller blocks that can be identical over a large number of individuals. Many

sequencing technologies and variant callers do not distinguish sequences from the different haplotypes, and many population analyses methods luckily do not need this information – rather the variants are used as separate entities. However, for some analyses haplotype information is crucial. In this thesis, haplotypes are reconstructed for local ancestry analysis in **Chapter II**, and for association analysis in **Chapter V**.

Sex-specific chromosomes

In diploid, sexually reproducing organisms, the two sexes often differ in their karyotypes for one chromosome pair, generally referred to as the sex chromosomes. The homogametic sex has two identical sex chromosomes (called XX in mammals and other systems where females are homogametic, and ZZ in male homogametic systems), while the heterogametic sex has two different sex chromosomes (called XY or ZW). The chromosome that is specific to one sex – I use Y as an example since this thesis is about mammals – always occurs in a pair with the X chromosome and hence cannot recombine with another Y chromosome (there are no individuals carrying YY). Apart from a small region identical to the X needed for proper segregation during meiosis (called PAR, pseudo-autosomal region), the rest of the Y chromosome is inherited as a completely unshuffled haplotype from father to son. Males that share the same Y haplotype have a shared ancestry, and this information can be used to trace the paternal origin of individuals in a population. This feature is utilised in **Chapter I**.

Conservation biology in the genomic era

In the last decades, new sequencing technologies and large consortia aiming to sequence most if not all life on Earth (for example European Reference Genome Atlas (Cartney et al., 2023), The Darwin Tree of Life Project (The Darwin Tree of Life Project Consortium et al., 2022), Vertebrate Genome Project (Rhie et al., 2021), Bird 10K (Zhang, 2015), 10,000 Plant Genomes Project (Cheng et al., 2018)) has opened up new possibilities in the field of conservation genomics. With more reference genomes available and decreasing costs of sequencing, the field has developed from typically using a small number of markers, such as microsatellites or mitochondrial DNA (mtDNA), to thousands of SNP markers or even whole-genome data (Supple & Shapiro, 2018). From a conservation perspective, this can be critical as mtDNA does not necessarily reflect the biogeographic patterns achieved from whole-genome data (Toews & Brelsford, 2012). One example is the yellow-legged frog, where genomic data revealed five distinct phylogenetic clades that had been missed in previous studies based on only mtDNA, and resulted in new recommendations in terms of managements units for this species (McCartney-Melstad et al., 2018).

The concepts of species and populations and how they are defined are human constructions that can be rather blurry and differ among researchers and between fields (Zachos, 2016, 2018). The most famous is the biological species concept, which defines species to be reproductively isolated from each other (Mayr, 1942), but there are many other definitions and they often disagree with each other. For conservation biology however, precision is crucial since the management relies on political decisions and laws that are based on certain definitions and might not be so easily adjusted (Frankham et al., 2012; Groves et al., 2017). For example, if an endangered population is found to be admixed with another population or species, it might not be legally protected (Jackiw et al., 2015; vonHoldt et al., 2018). For species with complex admixture history, whole-genome data is often essential to disentangle the introgression events. This topic is further discussed below and in **Chapter II**.

Another topic that has become increasingly debated with growing whole-genome data access is whether the conservation field should put the focus on functional or neutral diversity (Hoelzel et al., 2019). Some researchers mean

that there has been too much emphasis on neutral diversity, and that future studies on endangered species rather should target functional sites with adaptive potential (Teixeira & Huber, 2021). This idea comes partly from the fact that there is a lack of clear correlation between diversity levels and International Union for Conservation of Nature (IUCN) Red List status (Schmidt et al., 2023; Teixeira & Huber, 2021), and partly from studies of particular species showing exceptionally low diversity for a long period of time, e.g. island fox (Robinson et al., 2016) and vaquita (Morin et al., 2021). Others claim that although conservation efforts targeting specific traits rather than overall diversity could be relevant in certain situations (Kardos & Shafer, 2018), a general deflating of the importance of diversity is detrimental and will worsen the biodiversity crisis (Kardos et al., 2021). This is further supported by recent studies that have showed that the neutral diversity is highly correlated with functional diversity (Kardos, 2023; Mathur et al., 2023).

There is also an increasing support for a modernization of the IUCN Red List criteria so that genetic data is incorporated when extinction risks are assessed (Brüniche-Olsen et al., 2018; Frankham et al., 2014; Petit-Marty et al., 2021; Schmidt et al., 2023; Willoughby et al., 2015). In this thesis, diversity in putatively functional and neutral regions is investigated in **Chapter III** and **IV**.

Small populations

Small populations are for several reasons more at risk of facing extinction than larger populations. When an already small gene pool is depleted by genetic drift (through accumulation of deleterious alleles and/or loss of variants of adaptive potential) and inbreeding, it can lead to a reduced fitness of the population. This can in turn result in fewer offspring in the next generation and a further reduction of the population size, something referred to as the "extinction vortex" (Gilpin & Soulé, 1986). There is no exact definition for when a population is considered small in terms of number of individuals, as it depends on several factors such as the level of genetic variation and demography. In this section I describe more in detail some of the implications related to small populations.

Inbreeding and genetic load

Inbreeding is the mating between relatives (more closely related than average in the population) that results in increased homozygosity in the offspring, when identical chromosomes or part of chromosomes are inherited from both parents. Inbreeding in itself is not necessarily harmful; and there are examples of populations where some individuals are almost completely homozygous with just marginal effects on fitness, like in some selfing plants (Busch, 2005)

and nematodes (Dolgin et al., 2007). However, with heterozygous recessive deleterious variants in the population, inbreeding can lead to exposure of these variants in homozygous form, resulting in negative fitness consequences. Also, in the case of heterozygous advantage (when the heterozygous genotype gives the carrier higher fitness than both the homozygous genotypes), inbreeding will have negative consequences. When inbred individuals have lower fitness than outbred individuals it is referred to as inbreeding depression (Charlesworth & Willis, 2009). The phenomenon is documented in several species, for example red deer (Huisman et al., 2016), killer whale (Kardos et al., 2023), stickleback (Fraimout et al., 2023), and multiple other vertebrates and plants (Crnokrak & Roff, 1999), sometimes leading to extreme consequences such as population extinction (as the Isle Royal wolves; Hedrick et al., 2019; Robinson et al., 2019).

The genetic load is the reduced fitness of a population due to the accumulation of deleterious mutations, in relation to a population with ideal genotypes (Crow, 1970; Muller, 1950). Before the genomics era, load was often estimated using the measure 'lethal equivalents'; corresponding to a set of deleterious alleles that on average would cause one death if they appeared in homozygous state (Charlesworth & Charlesworth, 1987). This could be assessed by comparing offspring survival between cohorts with different inbreeding levels (Ralls et al., 1988). With whole-genome data, load can be approximated by the number or the fraction of deleterious mutations in an individual. However, fitness will be affected differently depending on if the recessive deleterious mutations exist in homozygous or heterozygous form. Bertorelle et al. (2022) suggested to divide the load into two components; the masked load and the realized load. The masked load (sometimes called potential load or inbreeding load) is made up of recessive deleterious mutations in heterozygous form, which will not affect the actual fitness of the carrier, but has the potential to reduce fitness in future generations should there be inbreeding. The realized load is composed of homozygous deleterious variants which will impact the fitness directly. Genetic load using this division is investigated further in **Chapter III** and **IV**. Of course, not all deleterious variants are necessarily fully recessive, but could have a different dominance relationship with the non-deleterious allele, measured as the dominance coefficient h. Deleterious mutations can also have different effects on fitness, which will affect their selection coefficient, s. Both s and h will impact the true genetic load, but none of them can be extracted directly from genomic data (Robinson et al., 2023). A distribution of fitness effects (DFE) can be inferred from the allele frequency spectra of neutral and non-neutral variants obtained from sequencing data (Eyre-Walker & Keightley, 2007; Eyre-Walker et al., 2006), but for this, samples of unrelated individuals are needed. As this could not be obtained from the study population in this thesis, I remained with the assumption that all deleterious mutations are recessive, and did not further explore dominance

and the distribution of fitness effects. This assumption is supported by a previous study showing that more deleterious mutations are more recessive (Caballero, 2006).

Kimura et al. (1963) predicted that smaller populations should have an increased genetic load due to drift. However, they unexpectedly found both that mildly deleterious mutations could contribute more to load than highly deleterious mutations, and that a smaller population can have a smaller load than an infinite population. This phenomenon was later known as purging, when deleterious alleles become exposed due to inbreeding in small populations and can be removed ("purged") by natural selection (Caballero et al., 2017; Crnokrak & Barrett, 2002; Keller & Waller, 2002; Leberg & Firmin, 2008). More recent genomic studies have shown that purging works most efficiently in populations with long-term small population sizes with some levels of inbreeding, and that highly deleterious mutations are purged more often than those that are mildly deleterious (Mathur & DeWoody, 2021; Robinson et al., 2018; Robinson et al., 2022; Wootton et al., 2023). In historically large populations that have gone through recent bottlenecks, purging has not had time to act, and deleterious mutations with low frequencies in the historical population can instead drift to high frequencies after the bottleneck, leading to inbreeding depression (García-Dorado, 2012; López-Cortegano et al., 2016; Pérez-Pereira et al., 2022). Fitness consequences of deleterious alleles are discussed in **Chapter V**.

Genetic rescue

A small population that is affected by a high genetic load and inbreeding depression can be saved by genetic rescue; when new or previously lost genetic material enters the population through immigration and reproduction of new individuals (Fitzpatrick et al., 2020; Hedrick, 2005; Hedrick et al., 2011; Robinson et al., 2021; Tallmon et al., 2004). Genetic rescue can be human-mediated, when individuals are intentionally translocated to save an endangered population, which was the case for Florida panthers (Pimm et al., 2006), or caused by gene flow from natural immigrants, as in for example the Scandinavian arctic fox (Hasselgren et al., 2018). Despite many successful examples of genetic rescue, some researchers mean it is a strategy that should be used with care (Edmands, 2007; Tallmon et al., 2004). Both the number of immigrants and any masked load they carry will affect the success – for example if only a few new immigrants are introduced and they carry a high masked load, those deleterious mutations can end up in homozygous form in coming generations and have a negative effect on the population fitness (Kyriazis et al., 2021). This was the case in the Isle Royale wolf population where a single immigrant reproduced so successfully that in a few generations, all individuals were inbred descendants to him, and it is thought that the

deleterious alleles he carried were causing the population to go extinct (Hedrick et al., 2019; Robinson et al., 2019). Since the masked load is predicted to be higher in larger populations (García-Dorado, 2007), there has been suggestions that in human-mediated genetic rescue, one should rather use individuals from smaller to medium size populations, where deleterious mutations have had a higher chance to get purged by natural selection (Kyriazis et al., 2021). The effect of a natural genetic rescue event is investigated in **Chapter IV**.

Admixture

As already mentioned, gene flow can rescue inbred populations from extinction, but it also results in mixing of genetic material between populations; a phenomenon referred to as admixture (Chakraborty & Weiss, 1988). Admixture is prevalent and can occur in all populations, not only in those that are small. Sometimes genetic material from one population can stay in the other population for a long time, for example due to adaptive variants. This is called introgression – or adaptive introgression, if the introgressed material is thought to have positive fitness effects (Hedrick, 2013). Extensive admixture between populations can however lead to genetic swamping; where all offspring are more or less hybrids, and the parental populations eventually disappear (Levin et al., 1996; Rhymer & Simberloff, 1996; Todesco et al., 2016). This can in the long term lead to hybrid speciation (Mallet, 2007). As well as saving a threatened species from extinction, admixture can have a more problematic outcome, if the two populations are very different and maybe locally adapted to different environments, the hybrids could have lower fitness than the parental populations. This is referred to as outbreeding depression (Templeton, 1986). Another negative consequence is that admixed individuals or potential hybrid species might be excluded from conservation efforts due to outdated policies and laws (Jackiw et al., 2015; vonHoldt et al., 2018). Examples include the red wolf and eastern wolf in North America, two endemic and threatened species that after whole genome sequencing were shown to be substantially admixed with grey wolves and coyotes, and even claimed to be hybrid species of those two (vonHoldt et al., 2016), which put their protective status into risk (Heppenheimer et al., 2018; Mech & Ronald, 2023; Morell, 2016). A large part of the admixture occurring today is thought to be a result of the Anthropocene, due to habitat reduction and both intentional and unintentional species introduction (Ottenburghs, 2021). Another aspect of human induced admixture is when domesticated animals hybridize with their wild relatives (Randi, 2008; Tensen & Fischer, 2023). Potential admixture between wild wolves and domesticated dogs is further investigated in **Chapter II**.

Study system

This thesis revolves around the Scandinavian wolf population and the neighboring wolf populations of Finland and Western Russia. In this section I give a brief history of the Scandinavian population, and discuss known implications related to its conservation, especially those of a political nature.

The Fennoscandian wolf populations

The fall and rise of Scandinavian wolves

Wolves have likely been common on the Scandinavian peninsula for thousands of years, since the ice sheet retreated after the last glaciation. After humans started settling and transitioned from hunting and gathering to farming, and particularly after they started keeping livestock, the wolves became an increasing threat and competitors for resources. Ever since the Middle Ages, the wolf has been portraited as a symbol of evil, a view likely fuelled by the Christian church (Fritts et al., 2003). In Sweden, there are records of wolf hunts going back to the 13th century, and hunting was intensified from the mid 1600s when bounties were issued for every killed wolf. During a 13 year period between 1827-1839, at least 6,790 wolves were killed in Sweden, in all counties except Malmöhus and the Baltic islands of Öland and Gotland (Lönnberg, 1934). Around the year 1900, only around 100 wolves remained on the peninsula, in the 3 northernmost counties. Not until 1966, wolves were legally protected from hunting, but at that time the population was likely already functionally extinct (Ekman, 2010). In 1978, a single reproduction was recorded in Norrbotten, on the border to Finland (Wabakken et al., 2001). However, the whole pack was killed during the following years.

In the early 1980s, there were again wolf sightings, this time a single couple in Nyskoga, Värmland in the southwestern part of Sweden close to the Norwegian border. That they appeared so far from the last wolves seen in Norrbotten as well as from the closest living population in Finland, immediately raised suspicion, and many people doubted natural immigration (Ekman, 2010). The couple was confirmed to have bred in 1983, 1984 and 1985 (Wabakken et al., 2001), after which the female was shot in late 1985, and the male subsequently disappeared. Their offspring continued to mate with each other, resulting in

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severe inbreeding. In the early 1990s, a third immigrant (from now on referred to as the third founder) entered the peninsula, and eventually formed the Gillhov territory with a daughter to the first founders and had many offspring. This helped the transition from a single inbred family to an actual population (Liberg et al., 2005), and over the following 10 years, the population grew to over 100 individuals (Sand et al., 2022), despite clear signs of inbreeding depression (Liberg et al., 2005). Two new immigrants that both bred in 2008 (in Galven and Kynna) seemed to once more rescue the population, with higher fertility and higher pairing of the their offspring compared to individuals descending only from the three first founders (Åkesson et al., 2016). Another immigrant pair was translocated from northern Sweden to Tiveden in Örebro Län in 2013, where they bred with each other. Later, genetic analysis revealed that this couple was related to each other on the level of full siblings, and their offspring were thus as inbred as many of the Scandinavian wolves (Kardos et al., 2018). After the death of the Tiveden male, the female however formed a new bond with a Scandinavian male, leading to many outcrossed F1 offspring in the subsequent years of which some are now breeding in the population (Svensson et al., 2023). After the Tiveden female, only one more immigrant has had both F1 and F2 offspring (which is needed to be counted as a founder); a male in Norway that entered Scandinavia in the winter of 2019-2020 and was successfully translocated to Setten were he formed a territory. (Svensson et al., 2023).

Wolves are known to disperse over large distances, and immigration events from Finland/Russia are frequently recorded. However, the reason for why so few of the immigrants manage to reach and breed in the population is due to that most of Northern Fennoscandia are reindeer husbandry areas where semi-domesticated reindeers are roaming freely throughout the year. As wolves that enter these lands tend to upset the reindeers to the extent that it becomes a severe problem for the owners, no wolves are allowed to form territories within the reindeer husbandry area, and protective hunting is almost always issued to those applying for it (Swedish Government, 2012). In Norway, there are also over 1.3 million free grazing sheep (Norsk institutt for bioøkonomi, 2022). Because of this, the Norwegian Government has decided that wolves are allowed only in the most south western regions of the country (Innlandet, Viken and Oslo) (Miljødirektoratet, 2023). Despite the low gene-flow, the Scandinavian population has reached numbers over 500 individuals for two consecutive seasons (around 450 in Sweden and the rest in Norway).

For all **Chapters I-V**, I use Scandinavian wolf samples collected between 1977 and 2015. The set includes the female founder, the four reproducing immigrants in Galven, Kynna and Tiveden, seven immigrants that never reproduced, and 91 individuals born in Scandinavia between 1983 and 2014. In **Chapter V**, I also use additional wolves sampled up until 2021.

Wolves in Finland and Russia

Wolves in Finland have been through a similar history of hunting and population decline, with more than 23,000 killed wolves during the last 150 years (Ermala, 2003). In the last century, wolves were extirpated from the western and central parts of the country, and could only be found in the Easternmost part where they were frequently crossing the border from the former USSR (Pulliainen, 1980). It is thought that the population size twice (in the 1920s and 1970s) was as low as a few individuals (Jansson et al., 2014). Since the mid 1990s, the Finnish population has grown thanks to conservation efforts and hunting control, and in the winter of 2022 - 2023, the population was estimated to around 310 individuals (Heikkinen et al., 2023). In recent years the population has expanded from the East towards the Southwest in the regions around Turku and along the Westcoast. With only few packs in central Finland, two subpopulations have emerged, with less genetic diversity in the Western subpopulation (Heikkinen et al., 2023; Valtonen et al., 2021). During the last decades, the connectivity with Russian wolves seems to have decreased and the Finnish wolves have started to become genetically differentiated from Russian wolves (Aspi et al., 2009; Jansson et al., 2012). A recent report from the National Research Institute (Luke) suggests that current immigration from Russia is not enough to maintain a genetically stable population (Valtonen et al., 2021).

The number of wolves in the European parts of Russia has also fluctuated during the last century, but always on considerably higher levels than the Finnish and Scandinavian populations (22,000 to 45,000 individuals; Sastre et al., 2011), with the Karelian subpopulation estimated to 300-350 individuals (Aspi et al., 2009). For **Chapter I-IV** I use 95 Finnish wolves sampled in the years 2000-2017, and 15 Russian wolves sampled between 2015 and 2018.

Wolves and dogs

Dogs are the domesticated form of wolves, and they were the first animals to be tamed by humans. Exactly when and where this happened is still debated. Genomic data from modern samples suggest an origin 20-30 thousand years ago (kya) around the time of the Last Glacial Maximum (Fan et al., 2016) but there are fossil data and genomic data from ancient samples indicating an earlier origin (Botigué et al., 2017; Ovodov et al., 2011; Skoglund et al., 2015; Thalmann et al., 2013). The geographical origin of the domestication event has been pinpointed to East Asia (Pang et al., 2009; Savolainen et al., 2002; Wang et al., 2016), Central Asia (Shannon et al., 2015), Middle East (vonHoldt et al., 2010) and Europe (Thalmann et al., 2013), but also a dual origin has been suggested (Bergström et al., 2022; Frantz et al., 2016). Despite

their striking phenotypic differences, all dogs are still so closely related to wolves that they are genetically the same species, and there are no biological barriers to wolves and dogs reproducing with each other and have fertile off-spring. This is also frequently happening in many places of the world, especially where feral dogs are abundant (Andersone et al., 2002; Fan et al., 2016; Galaverni et al., 2017; Godinho et al., 2011; Pilot et al., 2018). In the modern Scandinavian population, wolf-dog hybridisation has occurred twice; the first time in Norway in 1999 (Vilà et al., 2003) and the second time in Sweden in 2017 (Wabakken et al., 2018). In both cases, all hybrid offspring were shot to prevent backcrossing (breeding between hybrids and pure individuals, mediating gene-flow from the hybrids back into the population). F1 hybrids are easily identified genetically using only a few genetic markers, but smaller levels of dog ancestry (as after two or more generations of backcrossing) are more problematic to verify without whole-genome data. Most researchers and decision makers agree that F1 dog hybrids should be removed before further admixture occurs, to prevent dog introgression into the wild wolves, however it is not clear how to treat individuals with a smaller or uncertain dog ancestry (Donfrancesco et al., 2019). In a current case in Finland, four hybrids have been identified from DNA in scat, but the first decision to cull the entire pack was appealed by the Finnish Nature Conservation Association, and later stopped by the Eastern Finnish Administrative Court (Kauta, 2023). They meant that the time lag between sampling and culling was too long, so there was a substantial risk that the hunt would target the wrong individuals. This can of course also lead to future implications in the Scandinavian wolf management, if those wolf-dog hybrids later emigrate to Scandinavia. I investigate dog and wolf admixture in **Chapter II**.

Wolves, hunters and politics

Though this thesis is focused on evolutionary genetics and conservation genomics rather than political sciences, all wolf research is affected by politics in one way or another. Our findings are noticed and scrutinized by media and different associations (both pro- and against wolves) and are sometimes evaluated by instances like the Environmental Protection Agency and might even indirectly affect decisions concerning management. In the following section I want to give the background to the wolf conflict, and the current political situation in Sweden.

Scandinavia has a strong and long cultural tradition of game hunting using dogs that goes back thousands of years (known from ancient rock carvings; Nationalencyklopedin, 2023). But unleashed hunting dogs in wolf territories are at great risk of getting killed, as the wolves will identify the dog as a conspecific intruder. The conflict between game hunters and wolf protectors goes

back a long time, and is quite infected (TT, 2012). It also divides citizens in urban and rural areas, as well as different political parties. Nature conservationist and biologists argue that wolves are important apex predators, needed above all to control ungulate species. Too many ungulates have negative consequences on the forestry industry, especially moose that preferably feed on pine shoots (Zimmermann et al., 2022). In Sweden with 279,000 square kilometres of predominantly managed forests this is a considerable problem. Many hunters on the other hand think that the ungulate populations are best controlled by human hunting. A general argument from wolf antagonists is that the decision makers and "wolf lovers" who want to keep the wolves mostly live in cities, where they are completely unaffected by these "dangerous" predators in their everyday life. Also in rural areas, there are many people who want to preserve the wolves, but in some parts of the country this is something to keep quiet about, for example out of fear of being excluded from the local hunting club (Ekman, 2010). The hunting association in Sweden has a strong lobby movement, and many members of the parliament are hunters – the parliament even has its own hunting club (Bengtsson, 2020). Over the last decade with increasing numbers of wolves, there has been a clear shift politically. Most political parties now support wolf hunt and a reduction of the population, despite that the wolf is listed as a strictly protected species in the Bern convention (Council of Europe, 2018). The current reference value for wolves in Sweden is a minimum of 300 individuals, given that there is at least one new immigrant reproducing every generation, currently defined as five years (Naturvårdsverket, 2015). As the population has been steady over 300 for several years, licence hunt is permitted annually on a fixed number of individuals decided by the county boards. In 2024, license hunt is planned on 36 individuals (Naturvårdsverket, 2023). In 2022, the Swedish parliament gave the Environmental Protection Agency a commission to evaluate the possibility of decreasing the population size on the Swedish side of the border to 170-270 individuals (Miljödepartementet, 2022). This was immediately criticized by both national and international researchers (Laikre et al., 2022). After the last election that resulted in a right-wing government, an amendment was announced that the investigation should be focused on the conditions required to aim for the lower range of the interval, that is 170 individuals. (Landsbygds- och infrastrukturdepartementet, 2023). Data from **Chapter III** has been provided to the investigation, which is outsourced on two international researchers and will be reported in 2024.

Research aims

The aims of this thesis were two-fold: 1) to pinpoint the geographical and genetic origin of the recently founded Scandinavian wolf population, and 2) to investigate the genetic health of the Scandinavian wolves, by estimating different types of genetic load and how these have fluctuated with inbreeding and immigration following the founding event. Specifically, the thesis is narrowed on the following aims:

Chapter I: The aim of this study was to reconstruct Y chromosome haplotypes from the Scandinavian population and compare them to other wolves and dogs to trace the paternal origin of the population. As no wolf Y chromosome reference sequence was available, a second objective was to characterize and annotate the identified Y sequences to compare genes and copy number differences between wolves and dogs.

Chapter II: In this chapter, the goal was to expand the study in **Chapter I** to investigate if the indicated paternal origin was also supported by whole genome data from both males and females. A more specific goal was to investigate if there were any traces of dog introgression found in any of the Fennoscandian wolf populations, with a focus on recent admixture to see if dogs could have played a role in the founding event of the Scandinavian population.

Chapter III: The purpose of this study was to use all the previously published whole-genome SNP data to investigate genetic load in the Scandinavian wolf population, both in relation to the Finnish and Russian wolves, and over the generations since the founding event.

Chapter IV: This study was a follow-up to **Chapter III**, to continue studying the genetic load, but using structural variants as markers instead of SNPs. A specific focus was how the genetic load was affected after immigration of new wolves to the Scandinavian population. A second objective was to assess the amount and types of structural variation in the wolf genome, which had not previously been done, as well as how they relate to repetitive sequences and genes.

Chapter V: In this chapter, the objective was to find the genetic basis of cryptorchidism, a condition with undescended testis that has negative effects on the fertility of male wolves. Cryptorchidism has been found to increase in the Scandinavian population over time, and could be a sign of inbreeding depression. By comparing genetic data from affected and healthy individuals, this study aimed to pinpoint the genomic loci responsible for the trait.

Methods

This section is not a comprehensive description of all methods used in the chapters, but rather an overview of some of the key methods, especially those used in more than one chapter. All the work I have performed during my PhD has been purely bioinformatic, but as none of these projects could have been accomplished without actual data sampled in the wild, I start with a section on the samples.

Sample acquisition and sequencing

The Scandinavian wolf population is thoroughly monitored on both sides of the Swedish-Norwegian border. Every year, from October 1st to March 31st, the population is surveyed by snow tracking and DNA-analysis of collected scat, urine and hair samples, which are coordinated by the County Administrative Boards (in Sweden) and Norwegian Nature Inspectorate (SNO) and Inland Norway University of Applied Sciences (in Norway) and commissioned by the Swedish Environmental Protection Agency and the Norwegian Institute for Nature Research (Svensson et al., 2023). Known territories with family groups or scent marking pairs are specifically targeted, but also samples collected and sent in by the public are analysed. In addition, all dead wolves – whether found dead or killed in traffic accidents, in protective hunting or licence hunting – are fully examined in a post mortem, performed at the National Veterinary Institute (SVA, in Sweden) or at the Norwegian Veterinary Institute (in Norway). In Sweden, all dead wolves are thereafter sent to the Natural History Museum (NRM) where tissue samples and sometimes pelt, bones and organs are saved in their biobank. Metadata on all dead wolves are saved in the common database Rovbase (Naturvårdsverket & Rovbase, 2014).

All Scandinavian samples used in this study come either from dead wolves (the vast majority from protective or licence hunts, but also some traffic killed, illegally killed and a few individuals found dead by sickness and/or age) or alive wolves sampled during capture as part of the monitoring program (for translocation or GPS collaring purposes), and were provided by either NRM

or SKANDULV (the Scandinavian Wolf Research Project) at the Swedish University of Agricultural Sciences, Grimsö.
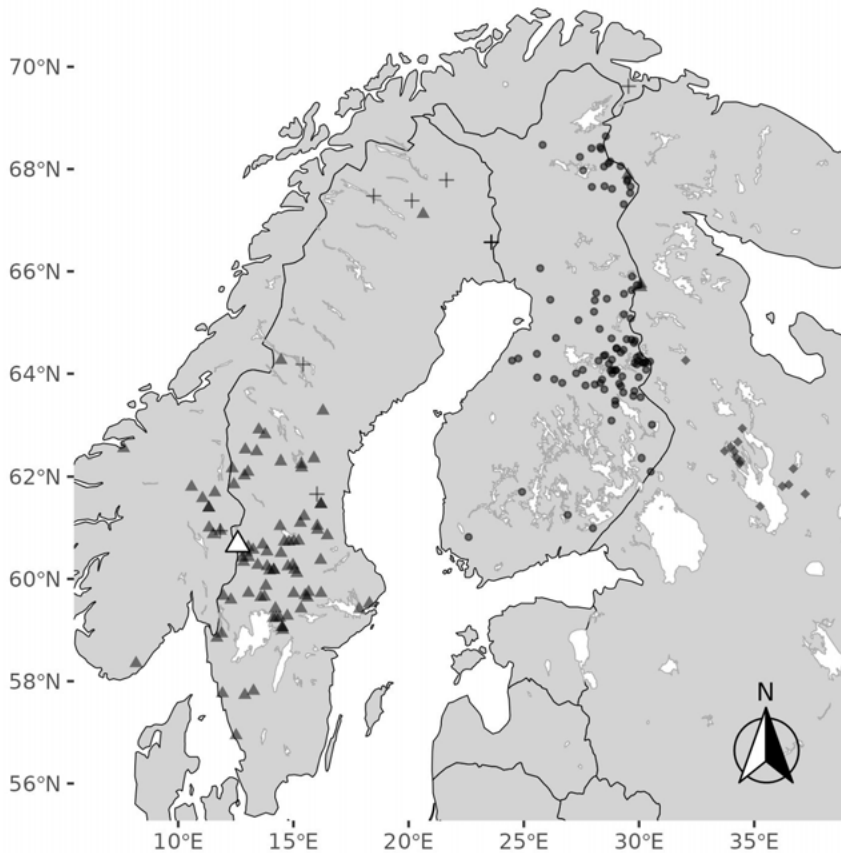

Figure 1. Sample locations for whole-genome sequenced individuals. The large white triangle denotes the female founder of the modern Scandinavian population. Small triangles are individuals born in Scandinavia (three were sampled in Finland); immigrants to Scandinavia from Finland/Russia are denoted with crosses, Finnish samples with circles and Russian samples with diamonds.

Due to the long-term monitoring, there is rigorous information on the relationships between Scandinavian wolves, and a well-established pedigree. The overall number of packs and minimum number of litter size in the first two decades were known from monitoring (Wabakken et al., 2001), but in 2005, the first pedigree from genetic data was constructed (Liberg et al., 2005), and since then the pedigree has been continuously updated with new individuals detected in the yearly monitoring (Åkesson et al., 2023). Information from the pedigree was to some extent used in all chapters, but particularly important for **Chapter III-V**.

Finnish wolves were sampled within the Finnish monitoring program and provided by Ilpo Kojola at the Natural Resources Institute in Finland (Luke). Some of the Finnish individuals were also known to be related (Ilpo Kojola, personal communication). Information on Finnish family trios were used in **Chapter IV**. Russian wolf samples were provided by Konstantin Tirronen at the Institute of Biology, Karelian Research Centre of the Russian Academy of Science, and delivered to Sweden by Jouni Aspi, University of Oulu.

Whole-genome sequencing (WGS) of samples used in **Chapter I-IV** were performed with an Illumina HiSeq X instrument at the SNP&SEQ Technology Platform at Uppsala University. All samples were sequenced with pair-end technology to a read length of 150 bp, with an insert size of 350 bp and an aimed coverage of 30X. The sample locations of all whole genome samples are shown in **Figure 1**. SNP-chip sequencing of samples for **Chapter V** was performed using an Axiom Canine HD Array at the Eurofins Genomics Europe Genotyping in Denmark.

Assessing different types of genetic variation

The basis of all analyses in this thesis is genetic variation. To find differences in DNA between individuals or populations from whole-genome sequences, the sequenced material is typically compared to a reference sequence. The dog genome (Lindblad-Toh et al., 2005) was used as reference in **Chapters II-V**.

SNPs and SVs

Each part of the genome is preferably sequenced multiple times to obtain an excess of reads covering every base-pair (and from both chromosome copies, in the case of diploid organisms). Reads are aligned ("mapped") to the reference, and deviations where some or all of the reads differ from the reference at certain positions can be detected. The simplest form of variation to detect are SNPs. These are the basis for the analysis in **Chapters I-III** and **V**. Larger types of variation as structural variants (SVs) are more complex to detect – especially those that are longer than the read-length and hence cannot be fully spanned within the read sequences. To detect SVs, one can instead use discordant read pair information from paired-end sequencing, or coverage information (Mahmoud et al., 2019). For deletions, no reads should map in the deleted region, and read pairs mapping on each side of the deleted sequence will appear to have a longer insert size than expected. For inversions, reads mapping in the inverted region will map backwards compared to the expected read direction in relation to its mate, if the mate maps outside of the inversion. For duplications, higher read coverage in the variant compared to the flanking regions can be used for detection. Also split read information can be used,

were reads coming from the junction between the two copies map to both ends of the duplication. Structural variants were the basis for all analysis in **Chapter IV**.

When all deviations from the reference have been compiled, the variants are genotyped in each individual, so that the final data format is a table with all variants as rows and the individuals with their genotypes in columns. This type of data (in so called variant call format, VCF) is used in all **Chapters I-V**. Both SNP and SV data contain errors. Throughout the course of my PhD, I have spent a substantial amount of time on variant filtering to optimize the data and to find the sweet spot between filtering away false positives and keeping as much data as possible. For the structural variants which are much more error prone than SNPs, also manual visual inspection was used as an additional filter to remove false positives.

Another method to assess genetic variation is to use already known variants, called markers, and genotype them on an array which target the flanking regions around the desired variants. This sequencing technique was used in **Chapter V**.

Inferring haplotypes

As described earlier, the non-recombining, sex-specific chromosome forms its own haplotype. For **Chapter I**, after mapping male sequences to Y chromosome scaffolds, the haplotypes were constructed directly based on the variable sites. The unsampled male founders were inferred from the haplotypes of their sampled sons. Any missing data (if the sequence reads did not cover all sites for some individuals) were imputed based on the three genetically most similar samples.

For **Chapter II** and **V**, also autosomal chromosomes were used and hence the variants for each individual needed to be separated into one maternal and one paternal haplotype. As a first step, statistical phasing which groups variants depending on their co-occurrence in the different genomes was used on all samples. In **Chapter V**, a combination of SNP-chip data and WGS data was used to maximize the number of individuals. For **Chapter V**, I also developed custom scripts to reconstruct the founder haplotypes based on the approach in Viluma et al. (2022). As a starting point, the haplotypes of the female founder (who is related to all the other samples) were taken directly from the statistical phasing. The subsequent founders were reconstructed in the order they appeared in the population, in segments of 1 million base-pairs (1 Mb windows). If a founder was unsampled (as the first two male founders) the haplotypes of their direct offspring were extracted and compared to the previous founder(s). The direct offspring should carry one haplotype from previous founder(s) and

one from the new founder, and by having access to multiple offspring hopefully both new haplotypes can be recovered. If only one new haplotype was found, it can either mean that one haplotype was not inherited to any offspring, or that the founder was homozygous (carrying two similar haplotypes) for this region. The sampled founders were directly compared to previously reconstructed founder haplotypes and new founder haplotypes were assigned if they have not been seen previously. When all founder haplotypes had been reconstructed, the individuals of interest were compared with the recorded founder haplotypes and assigned to one or two of them for the same 1 Mb windows.

Population genetic analyses

Principal component analysis

Principal component analysis (PCA) is a method to reduce the number of dimensions in a large dataset, and explain the variation seen with less variables (Pearson, 1901). Commonly, only the two most informative dimensions ("PC1" and "PC2") are used to represent the data in a 2D-figure, where each point is a different sample, and the structure should basically reflect the genetic relationship so that samples from the same population cluster together. PCA is used in **Chapter II** and **Chapter IV-V**. A known problem can occur when the samples are related, and the obtained structure actually reflects family relationships rather than population structure, something that was investigated in **Chapter II**.

Admixture

Just as in a PCA, an admixture analysis is searching for structure in the data, but will assign the samples to a predefined number of ancestries using maximum likelihood, so that the fractions of the different ancestries are obtained for each individual (Alexander et al., 2009). Typically, several runs with different number of predefined ancestries are executed, after which the most likely number of ancestries can be obtained using cross-validation. This was used in **Chapter II**. An admixture analysis gives the fraction of ancestries for the whole genome, but does not say anything about the composition over the chromosomes. Therefore, I also used local ancestry analysis, which matches an individual against pure reference populations across genomic regions (Brisbin et al., 2012). As I did not have known pure reference populations, I explored different combinations of available samples, and also evaluated the difference in outcome depending on the number of markers used.

Load estimation

I divided the genetic load into the two components masked load and realized load (Bertorelle et al., 2022), and approximated them using the proportion of heterozygous genotypes of all protein-coding deleterious polymorphic sites (for masked load) and the proportion of homozygous derived sites (for realized load), after all variants had been polarized using two outgroups. This was done based on SNPs in **Chapter III** and SVs in **Chapter IV**. As a whole genome estimate for **Chapter III**, I used Genomic Evolutionary Rate Profiling (GERP) conservation scores (Davydov et al., 2010) based on a multiple whole genome alignments of 100 vertebrate species. For sites that were considered deleterious (GERP>4), the GERP scores were summed up for all derived alleles in each individual, and normalized by the number of called sites to account for differences in genotyping rates.

Genome-wide association

To connect a known phenotype with a genomic region, one can perform a genome-wide association study (GWAS), where a large set of genomic markers from individuals with recorded phenotypes are tested for association to the phenotype, one by one. An ideal GWAS includes thousands of unrelated samples, and a continuous trait with values roughly following a normal distribution (Uffelmann et al., 2021). In **Chapter V**, I sought to find the genetic basis for a binary trait ('sick' or 'healthy') using only 75 (mostly related) samples, and applied a linear mixed model method that took the relatedness of samples into account. I treated the trait as continuous, but only sampled from the extreme ends of the normal distribution as suggested by Zhou and Stephens (2012).

Summary of chapters

Chapter I – The evolutionary history of grey wolf Y chromosomes

The non-recombining part of the male-specific Y chromosome is inherited as a block from father to son, and can therefore be used to trace paternal linages back in time. In this chapter the aim was to investigate the paternal origin of the recently founded Scandinavian wolf population, by comparing the male founders' Y chromosome haplotypes to those seen in male wolves from Finland – the likely geographical origin of the founders – as well as to public data from other wolves and dogs world-wide. First, we identified Y-linked scaffolds in a published, highly fragmented male wolf assembly (Gopalakrishnan et al., 2017) by comparing coverage from mapped male and female data (as the Y chromosome is male specific, female sequence data should not map to scaffolds from this chromosome). We identified 4.7Mb of Y specific sequence, fragmented into many small scaffolds presumably representing most of the nonampliconic sequence from the euchromatic part of the wolf Y chromosome, and annotated 22 protein-coding genes, out of which 18 had been previously identified as Y-linked in dog (Li et al., 2013). Y chromosome haplotypes were constructed from ~1,500 polymorphic sites identified in mapped short-read data from 176 samples, including 145 wolves.

We found 53 different Y haplotypes divided into four major haplogroups. All four groups were represented in the Finnish sample set, indicating that the linages were not partitioned on a larger geographical scale. However, individual haplotypes were only shared between individuals from the same region. The Scandinavian founders and immigrants shared haplotypes with Finnish wolves, but not with wolves from other regions nor with dogs, consistent with an origin from the Finnish-Russian population. A phylogenetic tree showed a deep divergence among haplotypes within the wolve clade, corresponding to a split up to 125,000 years. We noted that dogs were found in several locations in the tree, spread over two different haplogroup clusters, indicating multiple domestication events of dog or possibly later paternal introgression from wolves. The deepest divergence between dog and wolf haplotypes was estimated to 29,000 years, which is consistent with a domestication event predating the Last Glacial Maximum as have been suggested in other studies.

Chapter II – Whole-genome analyses provide no evidence for dog introgression in Fennoscandian wolf populations

We followed-up **Chapter I** in another study aimed to expand the analysis to also include females and using data from the whole genome. Specifically, we investigate whether there were any signs of dog introgression to exclude the possibility that the Scandinavian wolf population could have been partly founded by dogs.

We assessed population structure using a large data comprised of 100 Scandinavian wolves and 100 Finnish and Russian wolves, as well as three known wolf-dog hybrids and over 100 publicly available dogs using principal component analysis. We found that wolves and dogs separated along the first principal component (PC1), with the wolf-dog hybrids placed in the middle. Unexpectedly, the Scandinavian and Finnish-Russian wolves separated on PC2. This seemed to be caused by the strong relatedness between the Scandinavian wolves, which all basically belong to a single family. Repeating the analysis with only unrelated samples (and hence only keeping the female founder of the Scandinavian population), she clustered with the Finnish-Russian wolves, as did offspring from the two unsampled male founders. An admixture analysis showed that Finnish-Russian and Scandinavian wolves shared the same ancestry component that was not found in dogs.

To investigate ancestry on a finer scale, local ancestry analysis was performed after all genomes had been statistically phased into haplotypes. Each individual was analysed separately, and its haplotypes (divided into smaller windows) were compared to two reference ancestry populations. These were approximated using unrelated wolf samples as one group, and all dog samples as the other. The hybrids could serve as validation for both the ancestry approach and the phasing; as expected, all three hybrids had one wolf and one dog haplotype for the vast majority of windows, but they were often shuffled between the parental haplotypes indicating switch errors in the phasing. For assessing potential dog ancestry in wolves, we summed up the windows indicative of mixed ancestry regardless of which of the parental haplotype that showed dog ancestry, so that switch errors should not affect the analysis. For all wolves, the mixed ancestry was small, on average less than one percent per genome. Reversing the analysis to search for wolf ancestry in the dog genomes resulted in very similar numbers. Since most dogs have known pedigrees extending back many generations without wolf ancestry, we conclude that the low levels of mixed ancestry seen in both wolves and dogs are – if real – traces of very old admixture (predating the founder event of the Scandinavian population). However, wolves and dogs share most of the genetic variation, and in

windows with few or no private SNPs, assignment might be less accurate. Finally, we tested how the ancestry assignment was affected by the number of individuals used in the reference groups, and by the number of markers used. Fewer reference individuals resulted in higher levels of mixed ancestries, but the levels flattened when more than 20 individuals were used in each reference group. The number of markers correlated positively with the mixed ancestry, which is likely due to that a dense set of markers can identify older and hence shorter regions of introgression.

Chapter III – From high masked to high realized genetic load in inbred Scandinavian wolves

Large populations can harbour a large number of recessive deleterious mutations that in heterozygotes are essentially invisible to selection. If such population experience a bottleneck event, and inbreeding occurs, the deleterious mutations can become homozygous and exposed to natural selection. Theoretically the deleterious mutations should get purged from a small population, but if the bottleneck is strong so that only very few individuals reproduce, deleterious mutations can instead increase in frequency by genetic drift.

In this chapter, we sought to investigate genetic load – the reduction of fitness in a population due to the accumulation of deleterious alleles – in the recently founded Scandinavian wolf population, and compare it with the estimated load in Finnish and Russian wolves. Also, we investigated how the load is affected by inbreeding and immigration. Deleteriousness of individual mutations in wolves is not known, but it can be predicted in different ways. In protein-coding genes, a common approach is to assume that synonymous mutations (that do not change the amino acid sequence of the protein) have a low impact on fitness, while non-synonymous mutations (that change the protein sequence), and nonsense mutations (that disrupt the protein sequence) have a larger impact. Moreover, the degree of conservation to orthologous proteins in other species can be used to infer if particular non-synonymous changes are generally tolerated or deleterious. As a neutral reference, we used mutations at synonymous sites. A site frequency spectrum showed a clear shift towards rare alleles for deleterious variants, compared to neutral variants, indicative of negative selection.

Genetic load was divided into masked load, defined as the proportion of deleterious mutations in heterozygous state, and realised load, the proportion of deleterious mutations in homozygous state. Scandinavian wolves were partitioned into number of generations since the founding event, which roughly corresponds to their level of inbreeding. Both estimates of genetic load

showed a clear correlation with the number of generations since founding, but in opposite directions. While the masked load decreased for every generation, the realized load increased with inbreeding. New immigrants breeding in the population reverted this pattern, but only temporarily. We also assessed the load in the whole genome by using conservation scores from a multiple-species alignment, and assuming that more conserved sites have some function and that mutations at such sites are likely deleterious. This resulted in the same correlations as for the protein-coding data, with decreased masked load and increased realized load. We conclude that more continuous immigration is needed, even if this will also bring in new deleterious variants, to reduce the inbreeding levels and prevent the realized load to further increase.

Chapter IV – Structural genomic variation in the inbred Scandinavian wolf population contributes to the realized genetic load but is positively affected by immigration

Structural variation comprises rearrangements within the genome, sometimes defined as changes involving more than 50 bp. Due to technical difficulties in identifying and calling SVs, they have been markedly understudied compared to SNPs, especially in wild populations and species of conservation concern.

In this chapter, we aimed to expand the study from **Chapter III** on genetic load to also include structural variants (SVs). We identified deletions, duplications and inversions in relation to the dog reference genome based on discordant read-pair information and coverage differences in 212 wolf genomes. In total almost 80,000 variants were detected. Previous studies have shown that up to 80% of the SV calls could be false positives, and to address this issue we applied both stringent quality filtering and manual curation. For the latter, individuals that were either homozygous for the reference, heterozygous, or homozygous for the variant, respectively, were randomly extracted and assessed by visual inspection. We requested that two individuals of each genotype were available for inspection to have high confidence in judging the correctness of the SVs. This also served as a genotype frequency filter, similarly to a commonly used minor allele frequency filter. All filters together removed two-thirds of the original calls, keeping 26,552 high-confidence variants for further analyses. The vast majority of SVs were deletions, with only a small fraction of duplications and inversions remaining. Structural variant lengths distributions were strongly skewed towards the left. Both deletions and duplications had visible length peaks with an excess of variants just below 200bp in length, which were shown to correspond to certain classes of transposable elements (TEs); SINE/tRNA-Lys for deletions and LINE/L1 for duplications.

To assess the genetic load, we used SVs in genes and considered all variants overlapping with protein-coding regions to be deleterious. SVs in introns were used as a putatively neutral comparison. When comparing the allele frequency spectra of the different categories of SVs, deleterious variants were clearly shifted to the left, consistent with a higher negative selective pressure. We further partitioned the individuals into generations as in **Chapter III**, and estimated the masked and realized load separately. The realized load from SVs increased with inbreeding just as that from SNPs, while the masked load decreased. We also specifically investigated load before and after a genetic rescue event, and found that immigration counteracted this pattern in that the realized load was significantly reduced after immigration. Our results demonstrate the importance to study also structural variation in endangered populations, and reinforce the conclusion from **Chapter III** that more continuous gene-flow is needed into the Scandinavian wolf population.

Chapter V – A scan for the genetic basis of cryptorchidism in inbred Scandinavian wolves using GWAS and haplotype analysis

All populations carry deleterious mutations to some extent, but in natural populations we can typically only *estimate* their deleteriousness, and have even less knowledge about their actual function in the genome. However, for some particular traits, like diseases, it might be possible to pinpoint the underlying deleterious variants. As a continuation of **Chapter III** and **IV**, we wanted to study the genetic basis cryptorchidism, a deleterious trait occurring in the Scandinavian wolf population that appears to be increasing in frequency and that could potentially be related to inbreeding depression. Cryptorchidism is a urogenital condition where one or both testicles fail to descend properly into the scrotum, and is connected to lower fertility and different forms of testis cancer.

We genotyped 75 males that had been subject to post-mortem (by which 22 cryptorchid and 53 healthy individuals were defined), for over 500,000 genetic markers, and sought to associate those with their cryptorchid status using a linear mixed model. We found a weak association to a region on chromosome X, that only became statistically significant after correcting the Bonferroni threshold for linkage between sites. A gene-set analysis associating full genes instead of SNPs resulted in the same peak on chromosome X, however not statistically significant. None of the genes in this region had previously been associated to cryptorchidism in other species. An estimation of the trait variance explained by different factors suggested that 38% of the phenotypic

variance could be explained by genetics, but no single marker explained more than 0.5%, indicating that the trait is highly polygenic.

Due to the small number of founders in the Scandinavian population, and the large amount of available genotype data, we also reconstructed the founder haplotypes using statistical phasing and custom developed scripts taking advantage of the known pedigree. Each individual was subsequently assigned to founder haplotypes for each 1 Mb window of the genome. Similar to above, however, we found no clear association between haplotype and cryptorchidism. We conclude that to disentangle the genetic basis of this likely highly polygenic trait, a much larger sample set will be needed than what is currently available. As this type of data is very hard to retrieve from natural populations, there is a general problem in pinpointing the genetic basis of inbreeding depression in endangered species.

Conclusions and future perspectives

When I started the work with this thesis, I was not sure where it would end up. My interests were purely bioinformatical and data driven rather than aiming at answering specific biological questions. However, over the years I grew into the field of conservation genomics, and through my desire for trying out new methods on the same dataset ("genomic recycling"), the outline was suddenly very clear.

In this thesis, the origin of the Scandinavian population that has been so debated over the years was confirmed to be the Finnish/Russian populations, first using only male-specific markers in **Chapter I**, and then supported by whole genome data in **Chapter II**. We could also conclude that there has not been any recent introgression from dogs into the Scandinavian population, and definitely put a stop to the conspiracy theory claiming that the population were partly founded by dogs. I also explored the large effect that relatedness of samples can have on many standard population genetic analyses – something that might not be an issue when working with large populations, but is of great relevance in many species of conservation concern.

Small inbred populations can accumulate deleterious mutations and hence have a higher genetic load than outbred populations. At the same time, some levels of inbreeding can lead to purging of highly deleterious alleles which will result in a lower genetic load (Dussex et al., 2023). In **Chapter III** and **IV**, we could conclude that the genetic load changes fast with the level of inbreeding, likely mostly due to drift. We also showed that it makes sense to separate the load into realized and masked load, since those two components correlate with inbreeding in opposite directions. Gene-flow through immigration alleviates the masked load, but only temporary. I suggest that even more immigration would be beneficial to the Scandinavian population, even if immigrants also bring in new deleterious variants. The gene-flow needs to be continuous such that the deleterious variants not end up contributing to the realized load. Further work is needed to investigate the dominance and distribution of fitness effects in this population, and possibly also investigate the most deleterious category of variation ('nonsense' mutations), which was omitted due to poor annotation.

I have shown that the realized load increases with inbreeding, and from the monitoring we know cryptorchidism is increasing in the Scandinavian population. However, to show that these two are connected turned out to be a difficult task. Having access to 75 samples with a known disease phenotype from a population in the wild is remarkable, but it is not enough to pinpoint the genetic basis of a complex polygenic trait, as attempted in **Chapter V**. Reconstructing the founder haplotypes did not help to improve the association at a first glance, but I think more work on this using more advanced statistical methods could lead to new insights.

To switch from SNP data to structural variants was an interesting experience. Although this field is in an early phase with many challenges to overcome, SVs can clearly be studied in wild populations using only short read data. They roughly show the same patterns of genetic load as SNP data, but the relative contribution of these very different types of mutations remains to be explored, with more thorough comparative studies. Long read sequencing data will be necessary for a full characterization of all types of SVs in wolves. Once this is achieved, they can serve as an extended set of markers for future association studies, as SVs are known to be of great functional importance and could represent some of the missing heritability in GWAS (Gupta, 2021; Ho et al., 2020).

One caveat that has concerned me over the years is that we have used the dog genome as reference (simply because there was no wolf reference genome available at the time I started). As stated above, dogs and wolves are still the same species, but it is likely that the dog through thousands of years of artificial selection has many fixed derived variants that are lacking in wolves, or only segregating at a very low frequency. By using other outgroup species for polarising the alleles, as in **Chapter III** and **IV**, there should not be a bias in ancestry assignment. However, now when the Darwin Tree of Life project has published a wolf reference, future studies should aim at switching to this reference (Sinding et al., 2021) to avoid unnecessary biases.

The current Scandinavian population is now 40 years old, and has been followed since the start. For every year to come, new generations are born and sampled and will contribute to an astonishing dataset, probably one of the best available for a wild mammalian population. I hope future studies will continue with the genomics research, as there is such a great potential. This thesis has explored the genomic perspectives on the founding event and the first few generations of inbreeding and immigration. We see that patterns change fast and that drift has had a huge impact, so I anticipate that exciting results are to come following another 40 years of Scandinavian wolf generations.

Svensk sammanfattning

All biologisk mångfald vi ser på jorden kan spåras tillbaka till ett och samma ursprung. Genom miljarder år av evolution har nya arter utvecklats, samtidigt som andra har dött ut. Källan till all denna variation är mutationer i arvsmassan, vårt DNA. Dessa kan uppstå antingen genom att det blir fel i replikationen – när kromosomerna kopieras inför en celldelning – eller genom skada från kemikalier eller strålning. Om felet uppstår i könscellerna så kan det ärvas vidare till nästa generation och spridas i populationen. De allra flesta mutationer är neutrala, och påverkar inte individen. En del är skadliga på olika sätt, och andra är till och med dödliga för bäraren, men dessa ser vi mycket sällan. Ju skadligare en mutation är, desto snabbare försvinner den ur populationen genom naturligt urval – selektion. Mycket få mutationer som uppstår är fördelaktiga för bäraren, men dessa kan å andra sidan spridas i populationen genom det naturliga urvalet – ju fördelaktigare desto snabbare – tills slutligen alla individer har den muterade varianten som således har fixerats i populationen. Detta öde – förlust eller fixering – drabbar alla mutationer förr eller senare, och hur snabbt det går beror på storleken på populationen. I små populationer går förloppet snabbare, och det naturliga urvalet fungerar sämre, både på så sätt att skadliga mutationer kan fixeras och fördelaktiga mutationer kan gå förlorade bara på grund av slumpen, så kallad genetisk drift. Inavel, som ofta är oundvikligt i väldigt små populationer, är ytterligare en bidragande faktor. Dessa i kombination kan leda till sämre fertilitet och reproduktion och resultera i ännu färre individer i nästa generation, vilket gör att små populationer riskerar att hamna i en så kallad utrotningsvirvel.

I och med människans stora påverkan på jorden med habitatförstörning och klimatförändringar så har antalet hotade arter ökat, och många populationer balanserar på gränsen till utrotning. Bevarandegenomik är ett forskningsområde som ökat markant de senaste åren, i vilket man studerar utrotningshotade populationer med hjälp av helgenomsdata, och framförallt undersöker om och varför hotade populationer har en så kallad högre genetisk belastning än en motsvarande icke-hotad population. Det som bidrar till den genetiska belastningen är just ansamlandet av skadliga mutationer, som resulterar i sämre fitness – ett biologiskt mått på överlevnad och reproduktionsförmåga. I den här avhandlingen har jag använt bevarandegenomik och olika bioinformatiska analysmetoder för att studera den skandinaviska vargstammen – en liten

population som först utrotades helt och sedan kom tillbaka, återbildad av ett fåtal invandrade vargar från Finland/Ryssland för bara 40 år sedan. Genom att använda genetisk variation baserat på helgenomsdata från över hundra skandinaviska och hundra finsk-ryska vargar så ville jag först bekräfta grundarnas ursprung – som under åren har varit föremål för debatt – och därefter undersöka deras genetiska belastning i förhållande till inavel och immigration.

I **Kapitel I** identifierade jag sekvenser från den könsspecifika Y-kromosomen (som bara förekommer i hanar) och jämförde Y-haplotyper från de skandinaviska grundarna med Y-haplotyper sedda i finska vargar, samt med ett stort antal publika vargdata från hela norra halvklotet och även ett antal Y-haplotyper från olika hundraser. De skandinaviska grundarna delade haplotyper med finska vargar, men inte med vargar från andra platser, eller med hundar.

I **Kapitel II** byggde jag vidare på samma frågeställning som i **Kapitel I**, men utökade studien med genetisk variation från hela genomet från både hanar och honor, och jämförde med fler hundar och vargar samt inkluderade data från tre kända varg/hund-hybrider. Med en så kallad admixtureanalys undersöktes olika genetiska ursprung, som visade att de skandinaviska vargarna delade ursprung med de finska och ryska, samt att hybriderna som väntat var precis hälften varg och hälften hund. Jag delade även upp genomet i mindre bitar och undersökte lokalt ursprung, ifall någon varg skulle vara en tillbakakorsning mellan en varg och en hybrid och ha kvar en mindre andel hundursprung, men fann inte mer hund i vargarna än varg i hundarna.

Efter att ursprunget var helt klarlagt gick jag vidare till att undersöka den genetiska belastningen hos de skandinaviska vargarna i **Kapitel III**. Jag utgick från att de flesta skadliga mutationer är recessiva, det vill säga bara uttrycks om de förekommer i två kopior i genomet. Sen delades den genetiska belastningen upp i maskerad belastning och realiserad belastning, där den förstnämnda bestod av proteinkodande skadliga mutationer i heterozygotform (en kopia) och den sistnämnda bestod av proteinkodande skadliga mutationer i homozygotform (två kopior). Den realiserade genetiska belastningen ökade för varje generation i den skandinaviska populationen, medan den maskerade belastningen minskade. Efter att nya immigranter kommit in och förökat sig så hävdes mönstret, men bara mycket tillfälligt. Jag provade även att använda konserverade positioner i genomet – baspar som bevarats trots miljontals år av evolution och således kan antas ha en viktig funktion – och betraktade mutationer i dessa regioner som skadliga, med precis samma resultat.

I **Kapitel III** användes bara SNPar – mutationer som var och en bara berör en enskild nukleotid – men det finns även en annan grupp av varianter i genomet som spänner över betydligt större regioner, och som kallas för strukturella varianter (SV). Dessa undersöktes i **Kapitel IV**, och användes därefter till att

återigen studera den genetiska belastningen. Strukturella varianter är notoriskt svåra att identifiera och genotypa, men hård filtrering och efterföljande manuell kurering resulterade i ett set av mycket troliga deletioner (när en bit av genomet saknas), duplikationer (när en bit av genomet duplicerats) och inversioner (när en region vänt på sig och hamnat i omvänd ordning). Dessa visade sig till största del överlappa med repetitiva sekvenser, oftast olika transponerbara element (TE). Ett fåtal överlappade dock med kodande gener, och dessa användes för att ta fram mått på den maskerade och den realiserade belastningen precis på samma sätt som i **Kapitel III**. Det visade sig att mönstren är precis samma för SV som för SNPar. Ett extra fokus lades på den genetiska räddning som skedde i och med att två nya immigranter reproducerade i populationen 2008, och jag fann både att den realiserade belastningen signifikant minskade, samtidigt som den maskerade belastningen ökade efter händelsen.

Den genetiska belastningen uppskattas utifrån antagande om vilka mutationer som är skadliga och inte, men i de flesta vilda arter har man inte en aning om vad alla dessa mutationer faktiskt gör. I **Kapitel V** undersöktes den genetiska orsaken till kryptorkidism, en åkomma som gör att testiklarna inte vandrar ner i pungen som de ska, vilket kan orsaka lägre fertilitet och testikelcancer. Med genotypdata från över 500,000 varianter i 22 sjuka och 53 friska skandinaviska individer, letade jag efter association mellan varianter och sjukdomstillstånd med en GWAS (Genome-Wide Association Study). Jag fann en svag association till en region på X-kromosomen, vilken även syntes i en gensetanalys som sökte efter association mellan varje gen och sjukdomen, den var dock inte signifikant. En analys av hur mycket fenotypisk varians som kan förklaras genetiskt tydde på att ett stort antal genetiska markörer är inblandade, vilket innebär att kryptorkidism med största sannolikhet beror på en kombination av många olika gener. Jag drog slutsatsen att datasetet trots sin imponerade storlek för att komma från en vild population, inte är tillräckligt för att närmare precisera den genetiska orsaken till kryptorkidism.

Med den här avhandlingen har jag undersökt grundandet och olika genomiska aspekter av inavel och immigration i en liten ny population. Jag har visat hur snabbt den genetiska belastningen kan ändras i en liten population till följd av drift, och hur viktigt det är med ny immigration för att bibehålla den genetiska variationen.

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*Crazy Birding Buddy

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