From high masked to high realized genetic load in inbred Scandinavian wolves

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July 29, 2022

Abstract

Any random genetic change is more likely to impair than improve fitness, a situation that owes to the fact that contemporary genotypes bear a history of having been shaped by natural selection for a very long time. Most mutations are thus deleterious and generate a genetic load that can be difficult to handle in small populations and increase the risk of extinction. We used functional annotation and evolutionary conservation scores to study deleterious variation in 200+ genomes from the highly inbred Scandinavian wolf population, founded by only three wolves and suffering from inbreeding depression, and neighboring populations in northern Europe. The masked load was high in Russia and Finland with deleterious alleles segregating at lower frequency than neutral variation. Genetic drift in the Scandinavian population led to the loss of ancestral alleles and fixation of deleterious variants. The per-individual realized load increased with the extent of inbreeding and reached several hundred homozygous deleterious genotypes in protein-coding genes, and a total of more than 50,000 homozygous deleterious genotypes in the genome. Arrival of immigrants gave a temporary genetic rescue effect with ancestral alleles re-entering the population and moving deleterious alleles into heterozygote genotypes. However, in the absence of permanent connectivity inbreeding has then again led to the exposure of deleterious mutations. These observations provide genome-wide insight into the character of genetic load and genetic rescue at the molecular level, and in relation to population history. They emphasize the importance of securing gene flow in the management of endangered populations.

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Keywords: Genetic load, deleterious mutations, conservation genetics

ABSTRACT

Any random genetic change is more likely to impair than improve fitness, a situation that owes to the fact that contemporary genotypes bear a history of having been shaped by natural selection for a very long time. Most mutations are thus deleterious and generate a genetic load that can be difficult to handle in small populations and increase the risk of extinction. We used functional annotation and evolutionary conservation scores to study deleterious variation in a total of 200+ genomes from the highly inbred Scandinavian wolf population, founded by only three wolves and suffering from inbreeding depression, and neighboring wolf populations in northern Europe. The masked load was high in Russia and Finland with deleterious alleles segregating at lower frequency than neutral variation. Genetic drift in the Scandinavian population led to the loss of ancestral alleles and fixation of deleterious variants. The per-individual realized load increased with the extent of inbreeding and reached several hundred homozygous deleterious genotypes in protein-coding genes, and a total of more than 50,000 homozygous deleterious genotypes in the genome. Arrival of immigrants gave a temporary genetic rescue effect with ancestral alleles re-entering the population and moving deleterious alleles into heterozygote genotypes. However, in the absence of permanent connectivity inbreeding has then again led to the exposure of deleterious mutations. These observations provide genome-wide insight into the character of genetic load and genetic rescue at the molecular level, and in relation to population history. They emphasize the importance of securing gene flow in the management of endangered populations.

INTRODUCTION

At least two processes can place small populations in genetic peril. First, mating between relatives (inbreeding) tends to increase the proportion of homozygous loci (Charlesworth & Willis, 2009; Fisher, 1965; Franklin, 1977). This will expose recessive alleles to selection and in the case of deleterious alleles increase the risk for inbreeding depression (Hedrick & Garcia-Dorado, 2016; Keller & Waller, 2002). Inbreeding can be difficult to avoid in small populations, like following a population bottleneck. Second, the magnitude of genetic drift is inversely proportional to the effective population size (N_e). This means that the efficacy of selection, as given by the scaled selection coefficient $\gamma = 2N_e s$¸ is lowered in small populations. As a consequence, deleterious mutations can increase in frequency and eventually get fixed (Charlesworth, 2009).

Empirical studies aimed at addressing the genetic vulnerability of populations have traditionally used genetic markers to assess the degree and character of genetic diversity (Allendorf, 2017; Avise, 1994). These markers often represent neutral loci that are not targets for selection and thus only provide indirect estimates of levels of genomic diversity, let alone can pinpoint the character of functional diversity. With large-scale genomic re-sequencing data from population samples it became possible to obtain better estimates of genetic diversity, including in genome-wide coding sequences (Davey et al., 2011; Luikart et al., 2003). Yet, it is not straightforward to directly translate such data into information about deleterious variation. In particular, the distribution of fitness effects of new mutations in natural populations is often unknown. This has been overcome, at least to some extent, by prediction of functional consequences of new mutations and/or assuming that derived variation at evolutionary conserved sites represent candidates for deleterious variation (Zoonomia Consortium, 2020; Lindblad-Toh et al., 2011; Marguliesset al., 2003; Miller et al., 2007). For example, the Genomic Evolutionary Rate Profiling (GERP) score uses comparative genomic data from multi-species alignments to quantify the reduction in the number of substitutions across a phylogeny compared to neutral expectations (Cooper et al., 2005; Davydov et al., 2010). When the reduction is significant, sites are interpreted to evolve under the influence of purifying selection and derived variants at such sites thus potentially deleterious.

The genetic load is the occurrence of deleterious alleles in the population, and can be divided into realized load (expressed load) and masked load (potential load, inbreeding load) (Bertorelle et al., 2022). The realized load is formed by all sites where a deleterious allele is expressed, mainly sites that are homozygous for recessive deleterious alleles. The masked load consists of hidden deleterious alleles, sites that are heterozygous where a recessive deleterious allele does not contribute to loss of fitness. As long as a population remains large, leaving limited room for genetic drift, most recessive deleterious alleles will segregate at low frequency and rarely be exposed to selection. As a consequence, the masked load can be high without immediate costs. If the population experiences a significant decline leading to inbreeding, several scenarios for the resolution of the masked load are possible. Exposure of recessive deleterious mutations in homozygous state can purge the gene pool from unfavorable variants and the rate of loss of such alleles may be further accelerated by genetic drift. However, it comes with the cost of inbreeding depression. Drift can also lead to the opposite, that is, fixation of recessive deleterious mutations (mutational meltdown) and thereby an increase in the drift load of the population, and decline in fitness (Lynch, Conery, & Burger, 1995a,b). There is currently little empirical data at the molecular level available to illustrate how the genetic load responds to sharp changes in demography.

The grey wolf is a keystone apex predator in large parts of the world and at the same time a flagship mammalian species in the context of biodiversity conservation (Chapron et al., 2014; Hindrikson et al., 2017). The decline of wolf populations is a concrete example of human-induced alteration in the abundance of a once-common species, since the main reason for its disappearance from many areas is human persecution (Mech, 1995). Many studies have addressed the genetic consequences of decreased size of wolf populations, including in North America (Adams et al., 2011; Hedrick et al., 2014; Hedrick et al., 2019; Hervey et al., 2021; Leonard et al., 2005; Muñoz-Fuentes et al., 2010; Robinson et al., 2019; Sinding et al., 2018; vonHoldt et al., 2016), Asia (Fan et al., 2016; Zhang et al., 2014) and Europe (Aspi et al., 2006; Gómez-Sánchez et al., 2018; Pilot et al., 2010). Several studies have provided evidence for inbreeding depression in wolf populations (Liberg et al., 2005; Räikkönen et al., 2006; Räikkönen et al., 2009).

After functional extinction, the Scandinavian wolf population was re-established in the 1980s by the arrival of three immigrants (Wabakken et al., 2001). The population is highly inbred with a mean inbreeding coefficient of 0.25–0.30 among reproducing pairs (Bensch et al., 2006; Flagstad et al., 2003; Vilà et al., 2003; Wabakken et al., 2001; Åkesson et al., 2016). Genome-wide analysis has shown that the genome of most individuals contain very large tracts of runs of homozygosity, reflecting chromosomal regions identical by descent from a recent common ancestor (Kardos et al., 2018). Limited immigration has counteracted depletion of genetic diversity and provided genetic rescue effects (Vilà et al., 2003; Åkesson et al., 2016). There are strong opposing views on how the population should be managed (Immonen & Husby, 2016; Laikre et al., 2022). Here we seek to assess the genomic incidence of deleterious alleles and the character of the genetic load in relation to inbreeding and gene flow in this population.

MATERIALS AND METHODS

Variant detection

We used published high-coverage, whole genome sequencing data of 209 wolves from three previous studies (Kardos et al., 2018; Smeds et al., 2021; Smeds et al., 2019), with a known pedigree for the Scandinavian population (Åkesson & Svensson, 2016). Reads had already been mapped with BWA-MEM version 0.7.17 (Li & Durbin, 2009) to the dog reference genome (CanFam 3.1 (Lindblad-Toh et al., 2005)), and sorted, deduplicated, base-recalibrated and individually variant called using samtools version 1.9 (Li et al., 2009), PICARD version 2.10.3 (http://broadinstitute.github.io/picard/) and GATK v 3.8 (McKenna et al., 2010). For the present study, variant calls from the 209 individuals (gvcf format) were jointly genotyped using GATK's GenotypeGVCFs (v 3.8). Only biallelic single nucleotide polymorphisms were used, and these variants were further "hard filtered" using GATK's VariantFiltration (settings from Alternative protocol 2 in GATK Best Practices (Van der Auwera et al., 2013)). To ensure high quality of calls and reduce the risk of including duplicated regions, we only kept sites (*a*) with an overall coverage between 10X and twice the genome-wide coverage, (*b*) that had a genotype quality of at least 30 and (*c*) less than 10% missing data. Moreover, for all analyses except the calculation of site frequency spectrums, we only included sites that had a minor allele count of at least 2 in the whole data set. The X chromosome was analyzed separately and only females were included for these sites to avoid SNP calling issues for the haploid males.

Polarization of alleles

We used publicly available short read data from two outgroups, *Canis lupaster* (African wolf, SRA accession: SRR8049196) and *Lupulella mesomelas* (black-backed jackal, SRA accession: ERR3210523), and mapped the sequences to the dog reference genome using the same procedure as described above, keeping only sites covered with at least five reads per outgroup. To avoid ascertainment bias towards the dog reference allele, we did not use called genotypes for the outgroups but instead pseudo-haploidized the genomes by randomly drawing one allele for each species using the read coverage as weight. This was done for each filtered variant site from above using custom python scripts.

The ancestral state of polymorphisms segregating in wolves was inferred for all sites where the outgroups agreed on one of the two alleles present in the wolf data set. Sites where the outgroups did not agree or agreed on a third allele not present among the wolves were removed from the analysis. The use of five agreeing outgroups (also including *Canis simensis*, *Canis adustus* and *Cuon alpinus*) did not impact the results of the polarization per se, but decreased the proportion of sites that could be polarized due a lower average coverage of these three genome samples. The ancestral alleles were added to the vcf file using a custom perl script. For all sites considered to contain deleterious variation, the derived allele was assumed to be the deleterious allele. All analyses were based on polarized mutations only.

Inferring genotypes of founder males

Assigned haplotypes for 73 Scandinavian wolves, and the two male founders of the population, for 1Mb windows were taken from (Viluma et al., 2022). Since the male founders had not been sequenced, Viluma et al. inferred their haplotypes based on observed haplotype combinations in their offspring. We translated founder male haplotypes to genotypes by for each variant site matching haplotypes to genotypes in all sequenced individuals. For example, if all individuals with haplotype A|A had the genotype 0/0, and all individuals with haplotype A|B had the genotype 0/1, we could infer that allele 0 was associated with haplotype A and allele 1 was associated with haplotype B. When all haplotypes had been associated with an allele at each variant site, the two male founders were added to the vcf file with genotypes entirely based on their inferred haplotypes. As a validation of this approach, we also inferred the genotypes of the sequenced female founder and compared these to the calls from the genotyping, which were identical at 58,806 out of 59,323 sites (99.1%).

Deleteriousness in coding regions

The command line version of Ensembl's Variant Effect Predictor (VEP; (McLaren et al., 2016)) release 99 was run using the settings --species "canis_familiaris" and --sift b to get predictions of deleteriousness based on SIFT (Sorting Tolerant From Intolerant) scores. SIFT uses both sequence homology and physical properties to predict if an amino acid substitution has an impact on the protein function (Kumar et al., 2009). In overlapping genes or transcripts, a single site can have more than one prediction, for example a site can be synonymous in one gene but non-synonymous in another gene. For such cases, we only kept the most severe effect for each site.

We focused the analysis of deleterious alleles representing missense rather than nonsense mutations. The latter involve start, stop or splice codon(s), and mutations at such sites might intuitively be considered candidates to affect gene function and be deleterious. However, the distribution of conservation scores for this rather small category of mutations (1,023 in our total data set) was similar to the genome-wide pattern (Figure S4) indicating technical issues with gene annotation (like the presence of alternative splice variants or incorrectly placed start or stop positions for translation), not uncommon in non-model species.

The Miyata score (Miyata et al., 1979) and Sneath's Index (Sneath, 1966) were assigned for each amino acid change reported in the VEP output using a custom python script inspired by simpred (https://github.com/NBISweden/simpred.git). These models calculate the distance between replaced amino acids; the Sneath's index uses 134 categories of activity and structure, and the Miyata's distance is based on volume and polarity. A site was assigned deleterious if the Miyata score was higher than 1.85 or if the Sneath Index was higher than 20, respectively (thresholds taken from (Williamson Scott et al., 2005)).

Deleteriousness based on GERP scores

A multiple alignment with 100 vertebrate species ("100way alignment") including the CanFam3.1 reference genome was downloaded from the UCSC genome browser (http://hgdownload.soe.ucsc.edu/goldenPath/hg38/multiz100way/maf/). To avoid biases towards the focal genome, the dog genome was removed from the alignment using MafFilter version 1.1.2 (Dutheil et al., 2014) before GERP++ (version 20110522; (Davydov et al., 2010)) was run using the tree file provided by UCSC (http://hgdownload.soe.ucsc.edu/goldenPath/hg38/multiz100way/hg38.100way.nh) and hg38 as reference. The GERP scores were subsequently transferred to dog reference coordinates using LiftOver between hg38 and canFam3.1 (Kuhn et al., 2013).

The range of GERP scores obtained from a particular alignment depends on the width of the corresponding phylogenetic tree. Suitable thresholds for judging whether mutations shall be considered deleterious or not will depend on the phylogenetic relationships among the species included in the multiple alignment. As a guide for setting a threshold we compared the distributions of GERP scores for sites assigned either synonymous or deleterious with VEP, and found a GERP score of 4 to represent a compromise between excluding as many as possible of tentatively neutral sites while including as many as possible of potentially deleterious sites.

Calculations of genotype proportions

The vcfR package was used to read the vcf files into R (version 4.1.1; (R Team, 2021)), and all subsequent calculations were performed in R using the package tidyverse (Wickham et al., 2019). When calculating the proportions of heterozygous genotypes and homozygous derived genotypes in an individual, we divided the number of sites of each genotype with the total number of called genotypes for that individual (including sites homozygous for the ancestral allele, genotyped because they were polymorphic in other individuals in our dataset).

RESULTS

We used whole-genome SNP data from 100 individuals of the highly inbred Scandinavian wolf population and from an additional 109 wolves from Finland and Russia Karelia. The Scandinavian wolves consisted of 73 animals sampled 1984–2015 that descend from the three wolves that founded the population ("the original population"), 11 immigrants of which four became integrated with the population 2008–2013 and bred in Scandinavia, and 16 offspring from matings between immigrants and individuals of the original population sampled 2010–2015 ("immigrant descendants").

We identified 10,622,231 autosomal variant sites of which 8,313,538 could be polarized using two outgroups. We began by focusing on protein-coding regions in the genome and identified 59,323 SNPs in 14,261 different genes. These SNPs were classified as synonymous (33,895), missense (24,405) and nonsense (1,023) mutations. Further, 17,790 of the missense variants could confidently be divided into deleterious (4,809) and tolerated (12,981) mutations based on SIFT scores. We will mainly focus on synonymous and deleterious missense variants to contrast a category of potentially neutral mutations with mutations that are likely to contribute to the genetic load.

The effect of genetic drift

Most variants in a population typically segregate at low frequency (Figure 1A). An unfolded site frequency spectrum for alleles in the three founders (Figure 1B) was shifted further to the left for deleterious mutations compared to synonymous mutations, just as in a large population (Figure 1A), consistent with purifying selection. About half of the deleterious mutations that entered the Scandinavian population were represented by only one copy in the founders. On the opposite side of the spectrum, all three founders were homozygous for 47 deleterious mutations segregating in neighboring populations; these mutations were thus directly fixed in the Scandinavian population. The number of variants, both synonymous and deleterious, decreased over time in the original Scandinavian population (Table S1). For example, there were 1,369 deleterious alleles segregating in the three founders, but only 1,006 remaining after five generations of inbreeding; about one-quarter of alleles had thus become lost by genetic drift.

To further examine the effect of drift we compared allele frequencies in the three founders with that in the population after five generations of inbreeding, represented by 11 wolves sampled 2007–2015 (Figure 2). For 30 deleterious and 618 synonymous mutations that were polymorphic in the founders, all 11 inbred individuals were homozygous for the derived genotype, indicating that these sites had become fixed in the population. The significant variation in allele frequencies among inbred wolves for each of the six possible starting frequencies (given three founders; Figure 2) indicates that the power of genetic drift in this small and bottlenecked population was strong. We note that of the tentatively fixed sites, which had three copies of each allele in the founders, 61% became fixed for the ancestral allele and 39% for the derived allele (χ^2 test, p=0.028).

The arrival and breeding of four immigrants 2008–2013 meant that many new alleles entered the population, including alleles that had become lost by drift in the original population. Of the 30 sites that tentatively had become fixed for the derived deleterious allele in the original population, 28 had regained the ancestral allele in 16 immigrant descendants sampled 2010-2015. For the 47 sites fixed for the derived allele already among the three original founders, 21 had regained the ancestral allele in immigrant descendants. Immigrants also contributed additional deleterious alleles (1,890 in total). There were 1,993 deleterious variants in immigrant descendants, almost twice as many as in the 11 inbred wolves with only original Scandinavian ancestry sampled approximately during the same time period.

The number of variants was higher in Finland and Russia than in the original Scandinavian population for all functional categories (Table 1). For example, while 4,640 and 3,756 deleterious missense mutations were seen in the Finnish and Russian samples, respectively, only 1,404 were present in the original Scandinavian population. The number of variants in a sample depends on the number of unrelated individuals studied. Although the sample size from the original Scandinavian population was large, the lower number of detected variants can clearly be attributed to its very narrow genetic basis.

The effect of inbreeding

Inbreeding is expected to shift genotype frequencies. To examine if this led to the exposure of deleterious mutations in the Scandinavian population, we followed changes in genotype frequencies over time and compared frequencies between populations. First, it was clear that the three founders of the original population had a lower proportion of heterozygous sites (deleterious: mean = 0.111 ± 0.020; synonymous: mean = 0.174 ± 0.029) than immigrant wolves (0.179 ± 0.017; 0.238 ± 0.016) as well as wolves from Finland (0.190 ± 0.015; 0.243 ± 0.017) and Russia (0.186 ± 0.008; 0.241 ± 0.009) (Figure 3A). The population thus started with less neutral and functional diversity than would have been the case with any three random individuals from the samples of Finnish, Russian and immigrant wolves.

Second, we found a clear and continuous reduction over time in the proportion of heterozygous genotypes in the original population, both for synonymous and deleterious mutations (Figure 3A). Third, the pattern for homozygous derived genotypes was essentially reversed (Figure 3B). Inbreeding resulted in an increased proportion of homozygous genotypes, both for neutral sites and deleterious mutations. The same patterns were found when grouping the individuals according to the fraction of the genome represented by runs of homozygosity (F_{RoH}), a measure of inbreeding (Table S2): the proportion of homozygous derived genotypes increased with F_{RoH}.

Fourth, immigrants contributing to reproduction in Scandinavia 2008–2013 were genetically more variable (had a higher proportion of heterozygous genotypes) than individuals of the inbred population (Figure 3A). Offspring from matings between immigrants and inbred individuals of the original population had a higher proportion of heterozygous genotypes and lower proportion of homozygous derived genotypes than inbred wolves from the same time period. However, just as in the original population, the proportion of heterozygous genotypes again decreased following new generations of inbreeding.

To test if the observations made above were robust to the method used for assessing the deleteriousness of non-synonymous mutations, we also applied two classical models of deleteriousness based on physiochemical properties of amino acids: the Sneath's index (7,185 deleterious mutations identified) and the Miyata's distance (8,668). The relative patterns of diversity differences among groups of wolves were similar for all methods (see Figure S1A-B).

Finally, we considered the absolute number of sites with homozygous deleterious missense mutations in protein-coding genes per individual. The three founders of the Scandinavian population had 175, 205 and 236 such sites, respectively. However, after six generations of inbreeding, the number of homozygous deleterious missense sites had increased to a mean of 278 ± 10.7 per individual, some of which may contribute to inbreeding depression. The number was higher than in the Russian population (mean 218 ± 19.1), among immigrants (mean 221 ± 20.8) and in the Finnish population (mean 240 ± 35.2).

Genes on the X-chromosome

We identified 1,473 synonymous and 191 deleterious variants in 432 X-linked genes segregating in 74 females from the total sample. Of these variants, 275 and 25 were detected in the original Scandinavian population, respectively. With less data we could not perform the same analyses as with autosomal sequences but it was clear that deleterious alleles on the X-chromosome segregated at lower frequency than deleterious alleles on autosomes (Figure S2). As an example, in the Russian population the frequency of singleton deleterious alleles was 2.1 times higher than the frequency of singleton synonymous alleles on the X-chromosome compared to 1.5 higher on autosomes. Since recessive X-linked alleles are exposed to selection in males, purifying selection (and thus purging) should be more effective on the X-chromosome than on autosomes.

Analyses based on GERP

An alternative way to assess the potentially deleterious effects of mutations is to use conservation scores based on alignment of homologous sequences from a large number of species. This allows studying any alignable region of the genome, i.e. also including non-coding sequences, and provides a quantitative estimate of deleteriousness. Figure 4A shows the distribution of GERP scores for synonymous and deleterious missense mutations in protein-coding genes, and Figure 4B the distribution of scores for 4,995,746 polymorphic sites across the whole wolf genome. The density plot for deleterious mutations in protein-coding genes is heavily skewed towards high GERP scores, as expected, although we note that some missense mutations considered deleterious by VEP/SIFT do not appear particularly conserved. Technical (for instance, incorrect polarization of segregating alleles) or biological reasons (like turnover of conserved sequences; Huber et al., 2020) could potentially explain this seemingly unexpected observation.

Based on the distributions of synonymous and deleterious missense mutations we set a GERP score threshold of 4 for defining a mutation as potentially deleterious (see Methods). With this threshold, 7.5% (376,835) of all mutations present in alignable regions of the 200+ wolf genomes analyzed in this study were deemed potentially deleterious. In Finland, Russia and among immigrants to Scandinavia the mean number of deleterious sites per individual genome was about 90,000 (Table S3). It was lower among the three founders of the original Scandinavian population and further decreased to 35,000–40,000 after six generations of inbreeding in the population.

Using polymorphism data from the whole genome we estimated the individual masked load as the sum of the GERP scores of all deleterious derived alleles in heterozygous genotypes, divided by the number of called genotypes per individual to account for differences in callability between individuals. The load was highest in wolves from Finland and Russia, and in immigrants to Scandinavia (Figure 5). The three founders of the original Scandinavian populations had somewhat lower masked load, and the load further decreased during subsequent generations of inbreeding. Like for VEP/SIFT data on heterozygous genotypes, the arrival and breeding of new immigrants to the Scandinavian population increased the masked load but this was followed by a decrease during subsequent generations of inbreeding.

The realized load, the sum of GERP scores of deleterious derived alleles in homozygous genotypes divided by all called sites, showed the opposite pattern (Figure 5B), again similar to VEP/SIFT data on homozygous genotypes in protein-coding genes. In this case the load was generally lowest in the larger populations and in immigrants (about 40,000 derived homozygous sites per individual). In Scandinavia, the realized load increased with inbreeding (up to over 52,000 sites), was balanced by the integration of new immigrants (≈43,000 sites) and then again increased with subsequent inbreeding (≈47,000 sites after three generations).

Finally, we considered a set of mutations in protein-coding genes that are candidates for being truly deleterious, namely the intersect of deleterious missense mutations and mutations with a GERP score >4. This set shows a site frequency spectrum that is further shifted to the left compared to synonymous mutations and deleterious missense mutations with a GERP score ≤4. (Figure S3). In the 11 inbred wolves sampled 2007–2015, there were on average 75.5 ± 10.5 homozygous "highly deleterious" genotypes per individual, compared to 47.9 ± 5.0 in the first-generation offspring to the founders.

DISCUSSION

Although the concept of genetic load was formulated more than 70 years ago (Muller, 1950), it is not until very recently it has become possible to estimate the load with other than quantitative genetic approaches. While such approaches have provided important insights into the relationship between inbreeding and fitness (Morton et al., 1956), they cannot address the molecular basis of the genetic load or be used in natural populations of non-model species without information on inbreeding coefficients and access to phenotypic data. Those obstacles can be overcome by whole-genome sequencing of population samples followed by analyses of the functional character of segregating variation in the data, a direction of research with considerable current interest (Barbosa et al., 2021; Benazzo et al., 2017; Dussex et al., 2021; Freedman et al., 2014; Grossen et al., 2020; Han et al., 2019; Khan et al., 2021; Kleinman-Ruiz et al., 2022; Pérez-Pereira et al., 2022; van Oosterhout, 2020; von Seth et al., 2021).

Our results demonstrate that the masked genetic load in wolf populations in northern Europe is high. For example, in the Russian reference sample of 14 individuals we found more than 20,000 missense mutations in protein-coding genes, of which 3,756 were confidently assigned as deleterious with an average of more than 1,000 mutations per individual. Most deleterious variants were rare and segregated at lower frequency than neutral alleles, consistent with the action of purifying selection. Considering the whole genome, Russian wolves showed on average some 90,000 mutations with a GERP score above 4, again indicating that mutations with potentially negative effects on fitness are common in wolf genomes. Since this analysis was only possible for regions of the genome alignable across a very large number of species, the actual number of deleterious mutations in wolf genomes is likely to be higher. In large populations such mutations will rarely drift to high frequencies and become exposed to selection in homozygote form. In other words, they are not purged.

The cost for high levels of masked load in neighboring populations is paid by the wolf population in Scandinavia. After functional extinction in the 1960s (preceded by a rapid population decline; wolves were common over the Scandinavian peninsula until the 19^{th} century), re-establishment by immigration of three founders from Finland or Russia in the 1980s meant that the population became highly inbred and likely affected by strong genetic drift. We could see genetic signatures of both these processes. Although some deleterious alleles became lost after a number of generations of inbreeding, the proportion of homozygous genotypes of derived deleterious alleles increased. This was seen both for deleterious missense mutations and potentially deleterious mutations with high GERP scores across the whole genome. Moreover, some deleterious alleles more or less directly became fixed in the Scandinavian population, either because all three founders were homozygous or because deleterious alleles reached fixation after a few generations only (Figure 2). The most inbred individuals showed nearly 300 sites homozygous for deleterious alleles in protein-coding genes, and more than 50,000 such sites in the rest of the genome, which gives a quantitative estimate of the magnitude of the realized load.

Inbreeding depression has been documented in the Scandinavian wolf population, involving morphological (Räikkönen et al., 2006; Räikkönen et al., 2013) as well as fitness-related traits (Bensch et al., 2006; Liberg et al., 2005). Inbreeding depression has also been recorded among wolves on Isle Royal (Robinson et al., 2019), in red wolves (Brzeski et al., 2014) and in Mexican wolves (Fredrickson et al., 2007). Wolves were once abundant and widespread over the northern Hemisphere. Analyses of ancient wolf genomes indicate that connectivity between wolf populations across continents was high, resembling panmixia, throughout Late Pleistocene (Bergström, 2022); indeed, the dispersal capacity of wolves is significant (Mech, 2020). Contemporary populations in Eurasia share a common ancestry that can be traced back to unidirectional gene flow from Siberia during the Last Glacial Maximum, although the survival of deep local ancestries argues against local extinctions during this process (Bergström, 2022; Loog et al., 2020; Ramos-Madrigal et al., 2021). There are thus reasons to believe that the high masked load we detected in Finland and Russia, and in immigrants to Scandinavia, was characteristic to many wolf populations before human persecution in the last centuries led to rapid and significant population declines and fragmented distributions (e.g. Hindrikson et al., 2017). With this demographic history, and without pronounced periods of purging as seen in some other species (Grossen et al., 2020; Khan et al., 2021; Kleinman-Ruiz et al., 2022; Robinson et al., 2018), contemporary wolf populations may be particularly sensitive to inbreeding depression by carrying a high masked load. We suggest that this could be the case as well for other vertebrate predators that suffered from human persecution during Anthropocene, increasing the risk for extinction (Kyriazis et al., 2021).

The arrival and breeding of new immigrants to the Scandinavian wolf population had positive effects on genetic diversity. New alleles arrived, ancestral alleles that had become lost in the original population re-entered the population and the proportion of homozygous genotypes of derived deleterious alleles decreased. These observations are concrete manifestations of genetic rescue at the molecular level and are consistent with concurrent population expansion and increased breeding success in the Scandinavian population (Vilà et al., 2003; Åkesson et al., 2016). Empirical evidence (from data on demography or fitness-related traits) for genetic rescue have been reported in several species (Frankham, 2015), including in other wolf populations (Adams et al., 2011; Fredrickson et al., 2007), but have rarely included genomic data demonstrating how deleterious alleles get masked.

Management of endangered and isolated populations emphasizes the importance of gene flow to counteract inbreeding and loss of genetic diversity (Whiteley et al., 2015). Our results demonstrate such effects in the Scandinavian wolf population and they also show that continuous immigration is necessary to make rescue effects other than just temporary. Genomic signatures of inbreeding were soon again apparent after the arrival of new immigrants 2008–2013, with decreased proportions of heterozygous genotypes and increased proportions of homozygous derived alleles. Maintaining connectivity to the larger populations in Finland and Russia should thus be of prime importance to wolf conservation in Scandinavia (Laikre et al., 2016). This recommendation is independent of whether maintaining neutral or functional diversity is considered the most important conservation goal (DeWoody et al., 2021; Kardos et al., 2021; Kyriazis et al., 2021; Ralls et al., 2020; Teixeira & Huber, 2021).

ACKNOWLEDGEMENTS

We acknowledge funding from the Swedish Research Council and the Knut and Alice Wallenberg Foundation and bioinformatic advice from Marcin Kierczak at the National Bioinformatics Infrastructure Sweden at SciLifeLab.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

HE conceived of the study, LS performed all analyses, LS and HE interpreted the data and wrote the paper.

DATA AVAILABILITY STATEMENT

Raw data in this study are publicly available with the following accession numbers: PRJEB20635, PRJEB28342 and PRJEB38198. The final vcf files (both coding and genome wide) will be made available on Dryad. All custom scripts and all commands for running the software used in the study are available on github (https://github.com/linneas/wolf-deleterious).

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Table 1. Number of single nucleotide variants per functional category of protein-coding genes for the different wolf populations. Number of individuals per sample in parenthesis.

	Original Scandinavia (n=73)		**Finland (n=95)**		**Russia (n=14)**	
	No	**Mean per ind**	**No**	**Mean per ind**	**No**	**Mean per ind**
Total	25,992		57,722		50,128	
Synonymous	15,588	8,194 ± 951	33,024	11,794 ± 344	29,015	11,675 ± 185
Missense	9,951	5,080 ± 619	23,702	7,681 ± 230	20,225	7,563 ± 127
Tolerated*	5,616	2,898 ± 357	12,618	4,266 ± 124	10,858	4,202 ± 71
Deleterious*	1,404	603 ± 85	4,640	1,144 ± 50	3,756	1,109 ± 33
Nonsense	453	236 ± 30	996	349 ± 14	888	343 ± 11

*Confidently assigned by SIFT.

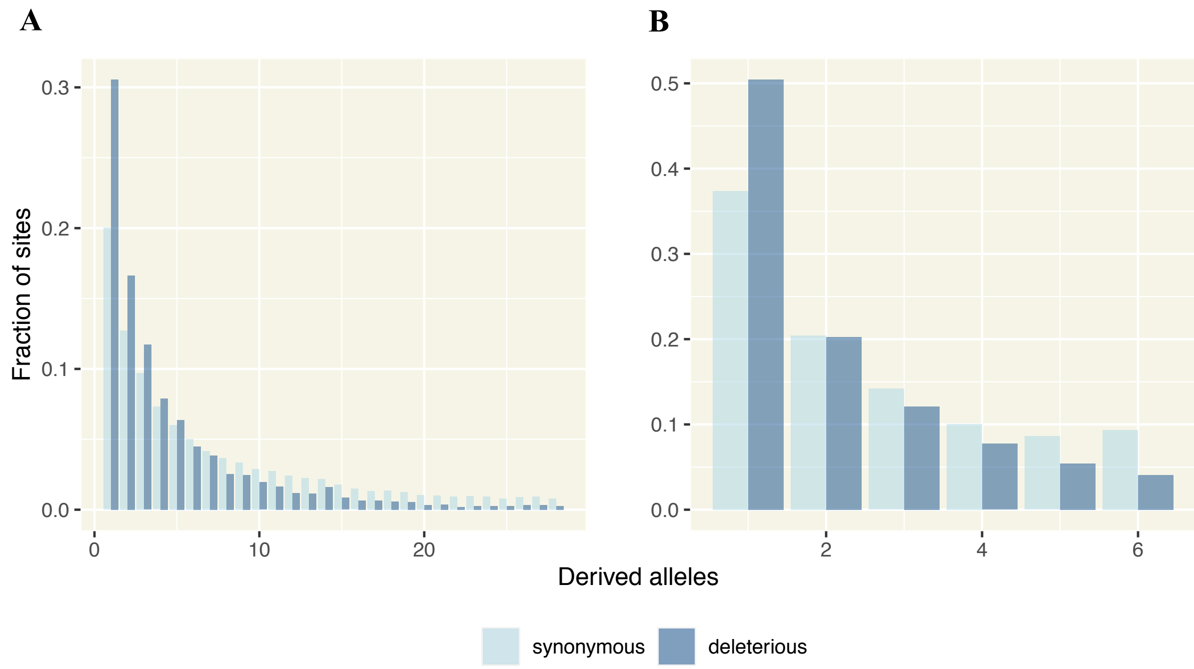


Figure 1. Site frequency spectrum for synonymous (light blue) and deleterious missense (dark blue) mutations in **A)** 14 Russian wolves and **B)** the three Scandinavian founders.

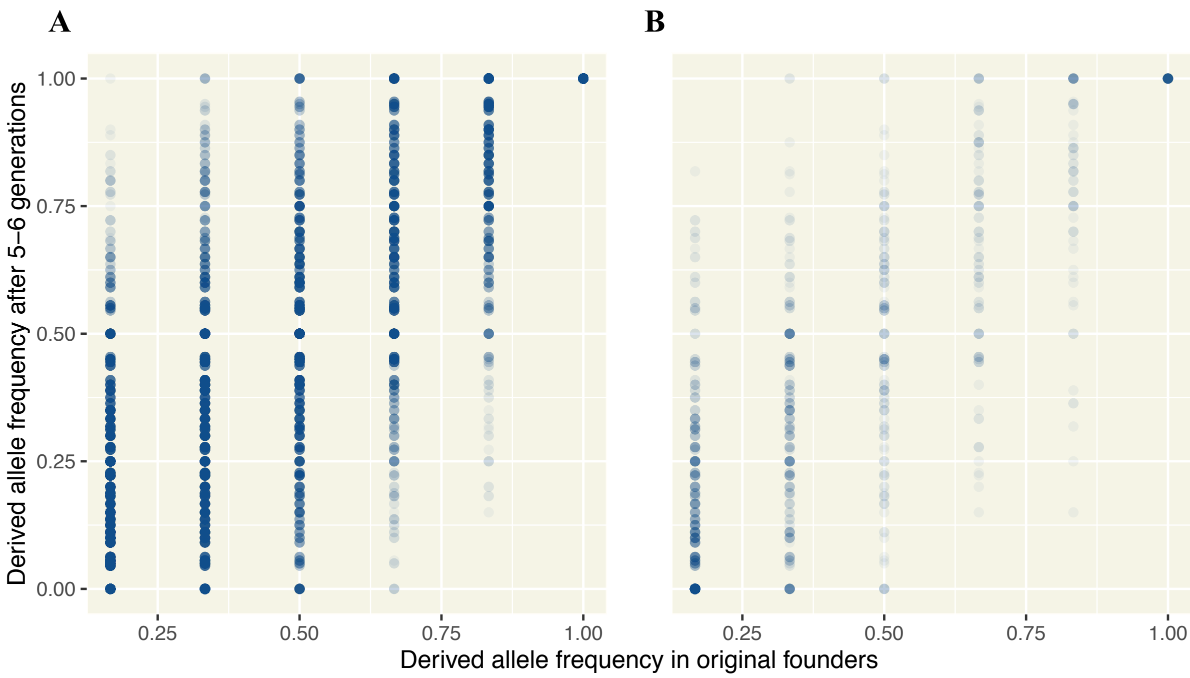


Figure 2. Allele frequency changes in Scandinavian wolves after five generations of inbreeding at **A)** synonymous and **B)** deleterious missense sites. Only sites with data for all three founders and at least eight individuals after five generations are included.

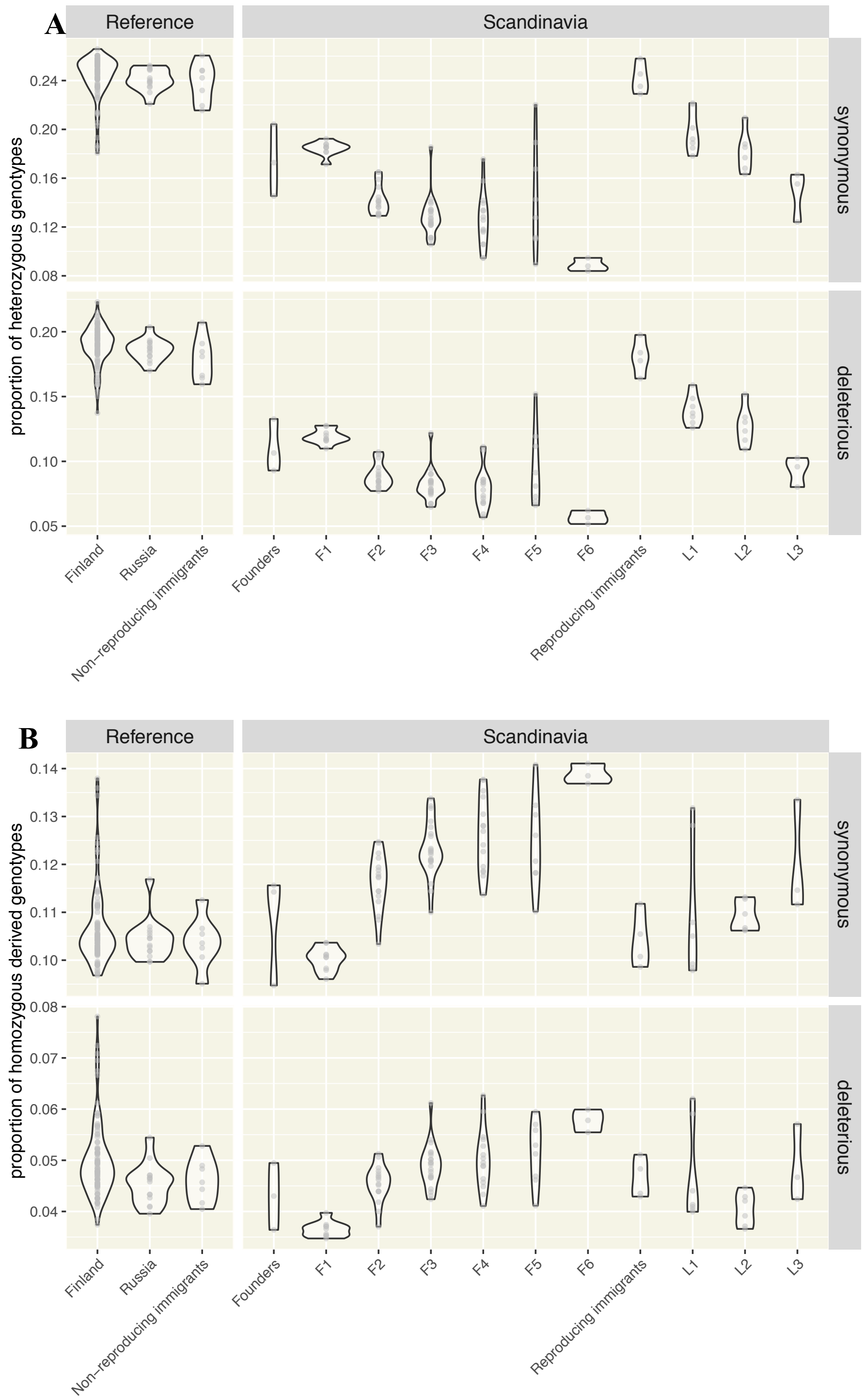


Figure 3. Proportion of **A)** heterozygous genotypes and **B)** homozygous derived genotypes for synonymous sites (top) and deleterious missense sites (bottom) in different wolf samples. Scandinavian-born wolves are separated by number of generations to closest founder. Descendants to the first three founders are denoted F1-F6, while descendants to later reproducing immigrants are denoted L1-L3.

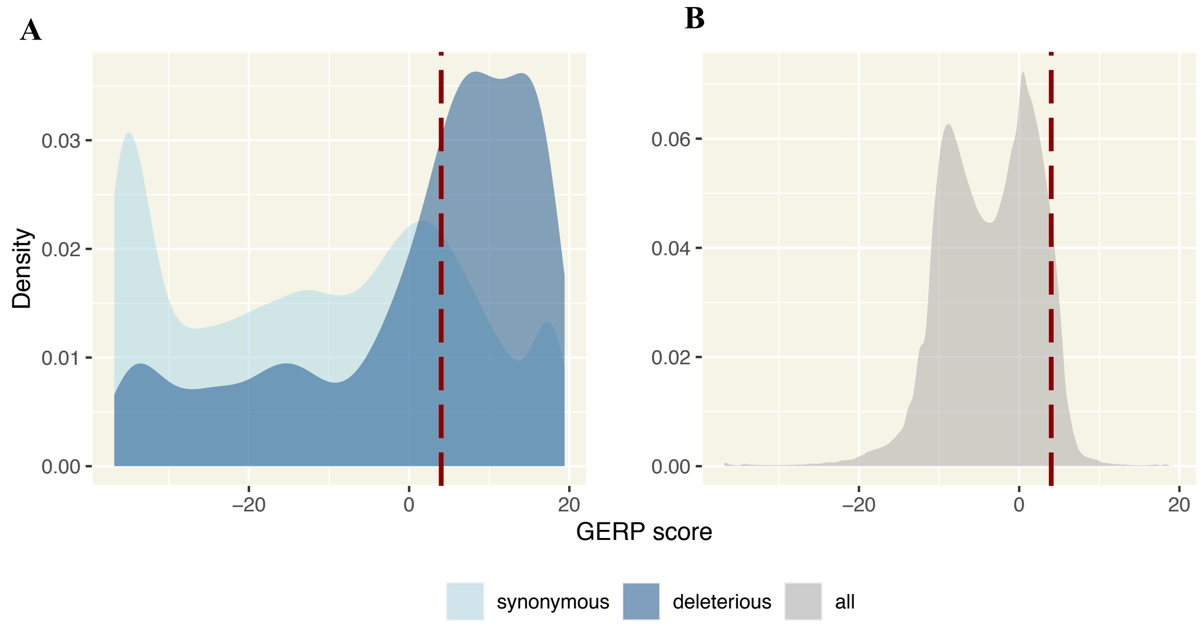


Figure 4. GERP score distributions for **A)** protein-coding regions categorized with VEP/SIFT as synonymous or deleterious, **B**) the whole genome (all alignable sites). The red line at GERP=4 marks the threshold used for assigning deleteriousness on the genome-wide scale.

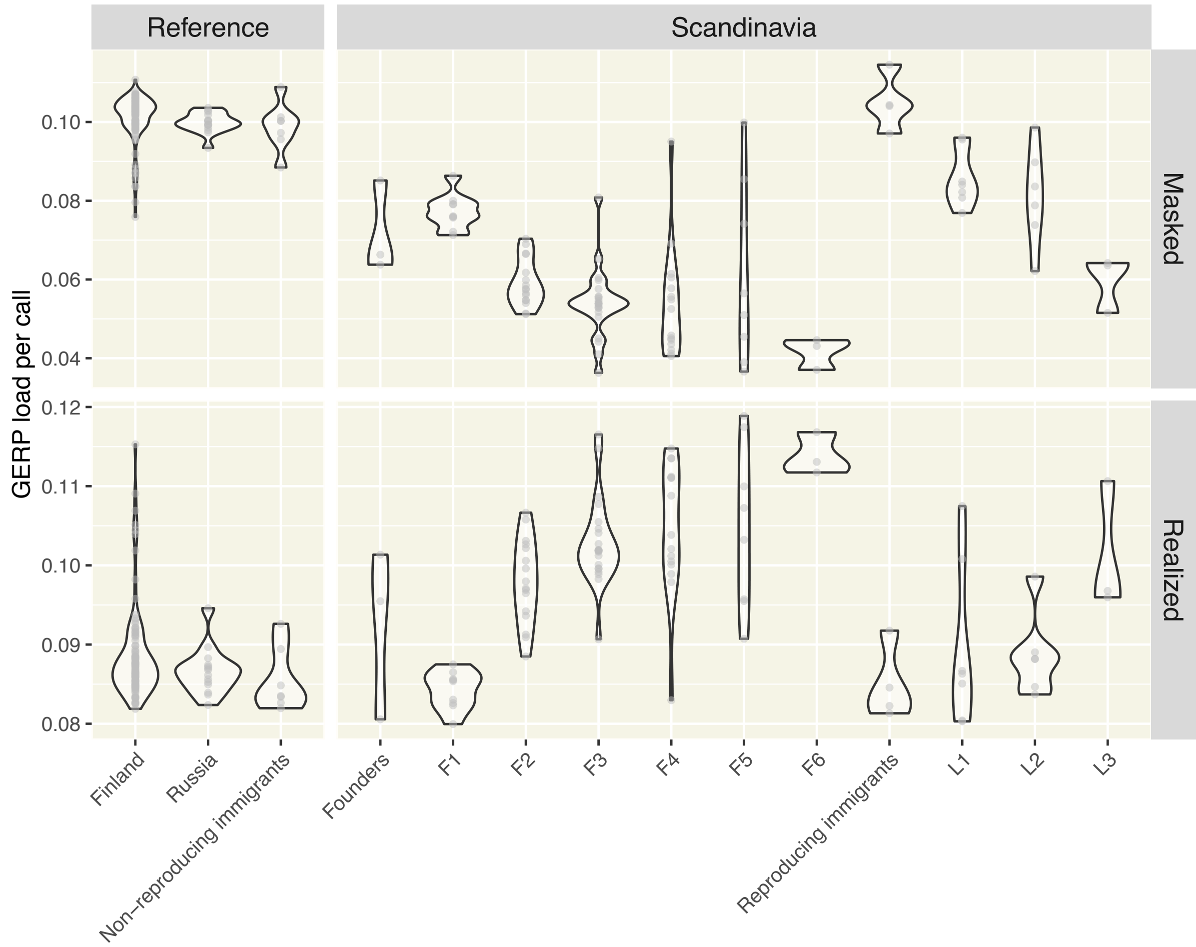


Figure 5. Genetic load divided into the components masked load (top) and realized load (bottom) estimated as the sum of GERP scores over all deleterious derived alleles in heterozygous and homozygous genotypes respectively, divided by the total number of calls in each individual.

SUPPLEMENTARY INFORMATION

From high masked to high realized genetic load in inbred Scandinavian wolves

Linnéa Smeds & Hans Ellegren

Supplementary Tables

Table S1. Number of derived mutations in protein-coding genes per generation in the Scandinavian population. The number of sampled individuals for each generation is denoted in parenthesis.

	Generations to original founders					
	1 (n=9)	**2 (n=16)**	**3 (n=21)**	**4 (n=15)**	**5 (n=8)**	**6 (n=3)**
Synonymous	15,232	14,563	14,156	13,555	12,508	8,854
Deleterious	1,347	1,270	1,211	1,118	1,006	634

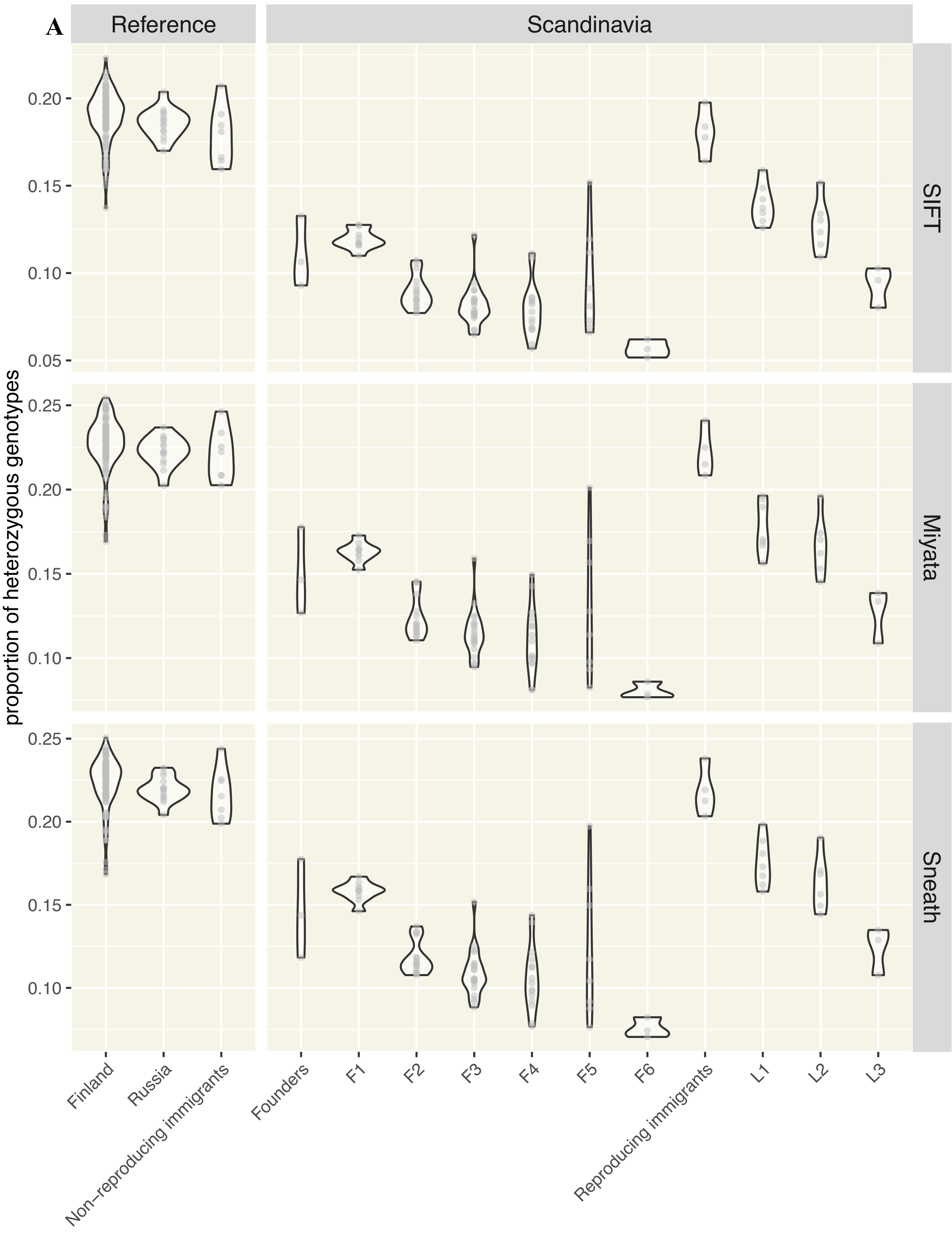
Table S2. Number of variants seen and proportion of homozygous derived genotypes in Scandinavian-born wolves grouped according to inbreeding measured as proportion of the genome in runs of homozygosity (F_{RoH}). The number of sampled individuals for each group is denoted in parenthesis.

	F_{RoH} in descendants to original founders			
	0-0.2 (n=13)	**0.2-0.3 (n=25)**	**0.3-0.4 (n=19)**	**0.4< (n=15)**
Synonymous				
variants seen	15,373	14,216	14,231	13,502
prop. homozygous der.	0.103 ± 0.005	0.118 ± 0.004	0.123 ± 0.005	0.134 ± 0.005
Deleterious				
variants seen	1,369	1,228	1,224	1,125
prop. homozygous der.	0.038 ± 0.003	0.046 ± 0.003	0.049 ± 0.003	0.056 ± 0.005

Table S3. Average number of sites contributing to load in the different groups (number of individuals in parenthesis). Numbers are normalized by the fraction of total calls per individual to account for differences in missing data.

	Average number of sites contributing to load	
Group (n individuals)	**Masked**	**Realized**
Finland (95)	92,161 ± 5,747	41,061 ± 2,756
Russia (14)	91,270 ± 2,374	40,119 ± 1,393
Non-reproducing immigrants (7)	90,403 ± 5,633	39,609 ± 1,838
Founders (3)	65,882 ± 10,734	42,918 ± 4,945
F1 (9)	70,979 ± 4,245	39,164 ± 1,141
F2 (16)	54,383 ± 5,564	45,446 ± 2,489
F3 (21)	49,647 ± 8,335	47,763 ± 2,606
F4 (15)	42,911 ± 5,706	48,556 ± 3,958
F5 (8)	56,028 ± 21,236	48,528 ± 4,908
F6 (3)	38,158 ± 3,623	52,739 ± 1,235
Reproducing immigrants (4)	96,112 ± 6,574	39,357 ± 2,131
L1 (7)	78,692 ± 6,687	41,503 ± 4,783
L2 (6)	74,569 ± 11,770	41,075 ± 2,488
L3 (3)	54,793 ± 6,606	46,946 ± 3,782

Supplementary Figures



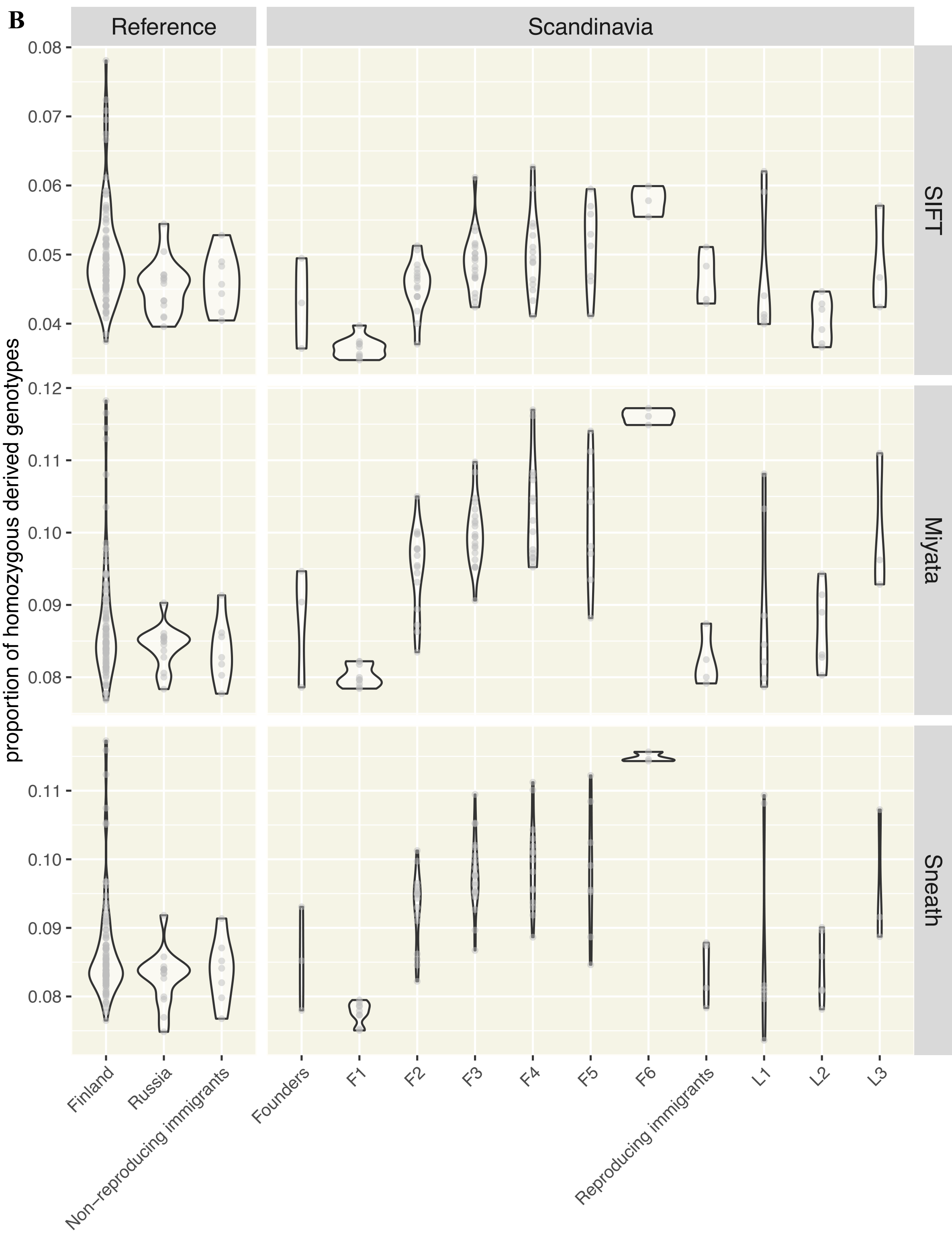


Figure S1. Proportion of **A)** heterozygous genotypes and **B)** homozygous derived genotypes for deleterious sites inferred from SIFT scores (top), Miyata distances (middle) and Sneath's Index (bottom). Descendants to the first three founders are denoted F1-F6, while descendants to the reproducing immigrants are denoted L1-L3.

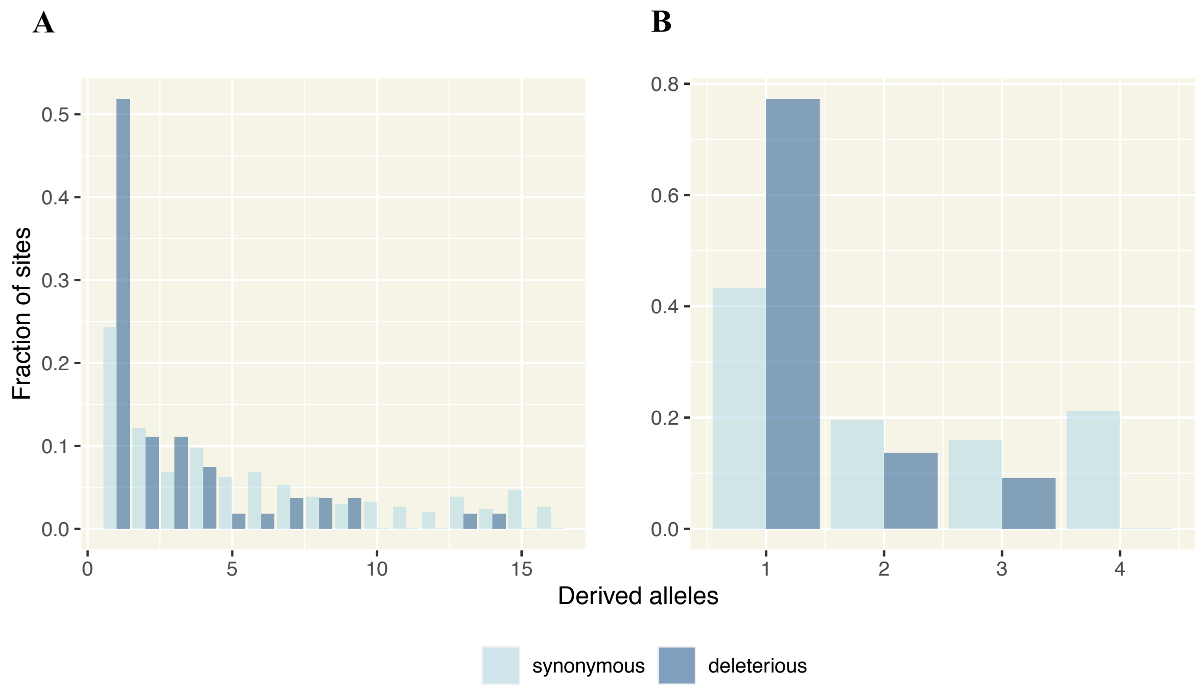


Figure S2. Site frequency spectrum for synonymous and deleterious missense of protein-coding genes on the X-chromosome (outside the pseudo-autosomal region) sites in **A**) eight Russian females and **B**) the three Scandinavian founders.

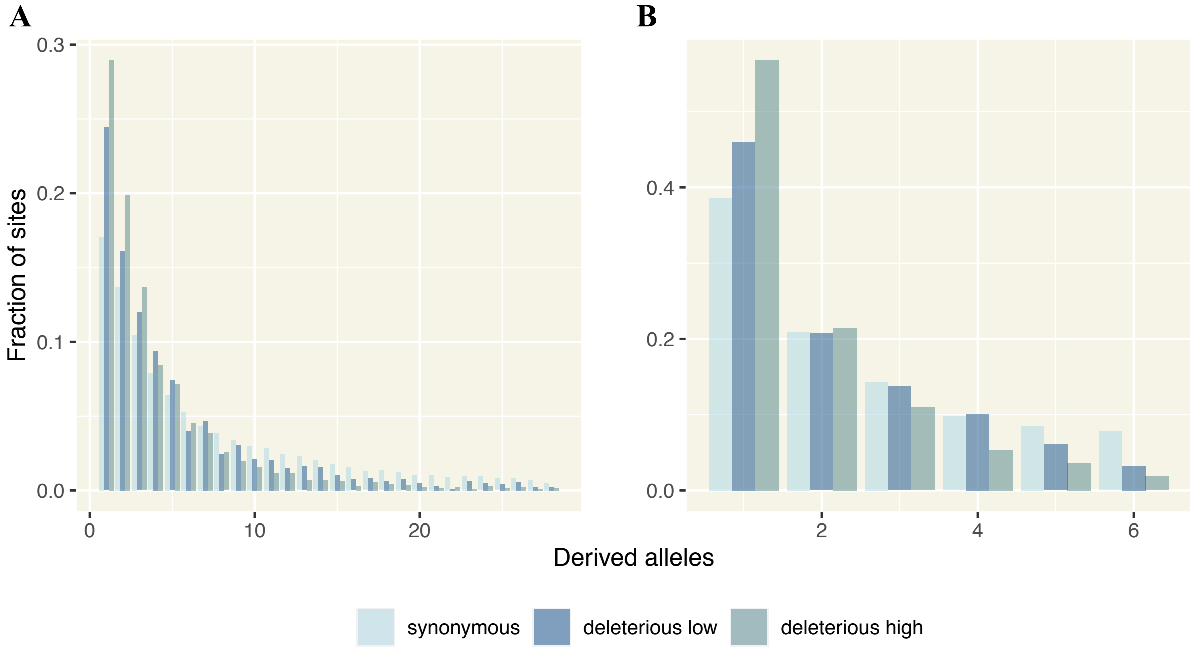


Figure S3. Site frequency spectrum for synonymous (light blue) and deleterious missense sites in protein-coding genes divided into those with GERP score <4 (dark blue) and ≥4 (green) in **A**) 14 Russian wolves and **B**) the three Scandinavian founders.

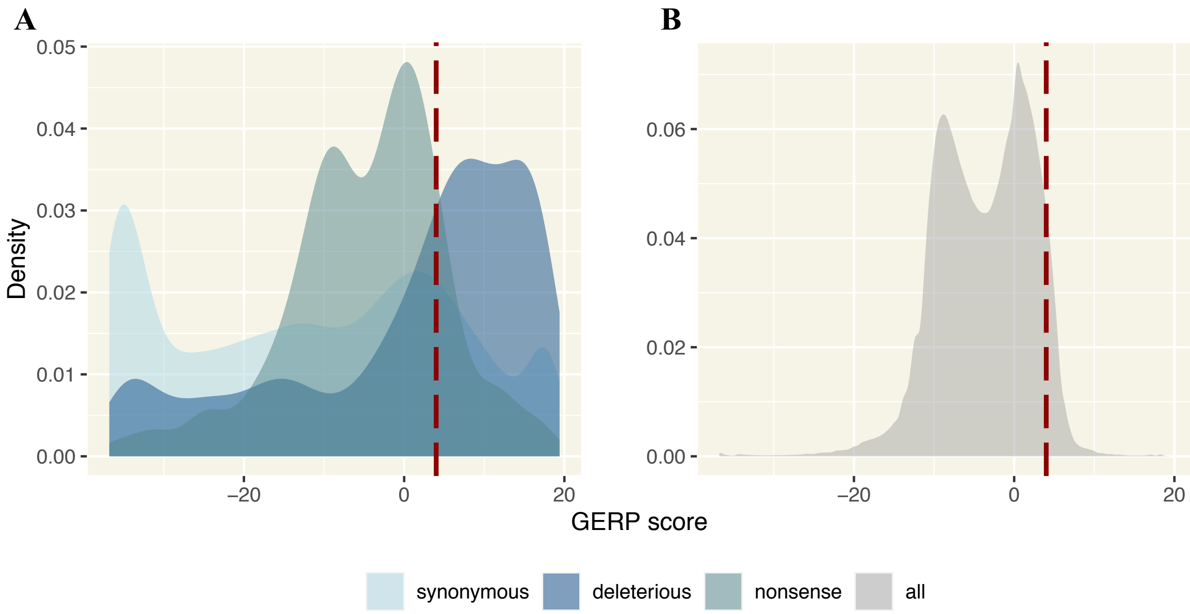


Figure S4. GERP score distributions for **A)** synonymous (light blue), deleterious missense (dark blue) and nonsense (green) mutations, and **B)** the whole genome (grey).