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Publication Date

2017

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UNIVERSITY OF CALIFORNIA

Los Angeles

The effects of small population size and inbreeding
on patterns of deleterious variation in
Channel Island foxes and Isle Royale gray wolves

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biology

by

Jacqueline Anita Robinson

2017

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ABSTRACT OF THE DISSERTATION

The effects of small population size and inbreeding
on patterns of deleterious variation in
Channel Island foxes and Isle Royale gray wolves

by

Jacqueline Anita Robinson

Doctor of Philosophy in Biology

University of California, Los Angeles, 2017

Professor Robert Wayne, Chair

In small isolated populations the fate of mutations is governed largely by chance rather than natural selection. Most mutations are deleterious, thus the inability of selection to effectively eradicate them incurs a cost to the genetic health of individuals in small populations. These detrimental consequences have been observed in numerous captive and wild populations, such as the gray wolves (*Canis lupus*) of Isle Royale, which suffer from severe inbreeding depression. However, other small populations appear healthy, and even persist for thousands of generations, such as the foxes (*Urocyon littoralis*) of the Channel Islands near the coast of Southern California. In this dissertation, we explore the genome-wide effects of small population size and isolation in these two extreme examples. First, we show that long-term small population size and isolation has led to a striking lack of diversity in island foxes, particularly in San Nicolas foxes,

which are virtually monomorphic across the entire genome. Additionally, island foxes have an elevated burden of deleterious mutations relative to mainland gray foxes (*U. cinereoargenteus*), but show no signs of inbreeding depression. Second, we explore the possibility that strongly deleterious recessive alleles have been purged from island fox populations, facilitating their persistence, and allowing them to recover rapidly from extreme bottlenecks. We found that recent, short-term bottlenecks have had little impact on island fox genomes, and confirm through simulations that island populations are expected to have a reduced burden of strongly deleterious recessive alleles relative to mainland foxes. In contrast, Isle Royale wolves suffer from severe inbreeding depression, and are on the verge of extinction less than a century after the population was founded. Third, we show through whole genome sequencing of Isle Royale wolves that intense inbreeding has eliminated variation in large genomic segments, causing the increased homozygosity of deleterious mutations. Our results suggest that persistence is possible in small populations large enough for selection to purge strongly deleterious recessive alleles without causing extinction, although the long-term effects of the elevated burden of weakly deleterious alleles and the loss of diversity in these small populations are difficult to predict.

The dissertation of Jacqueline Anita Robinson is approved.

Kirk Edward Lohmueller

Howard Bradley Shaffer

Bridgett vonHoldt

Robert Wayne, Committee Chair

University of California, Los Angeles

2017

This dissertation is dedicated
with utmost gratitude to
Jean and Richard Robinson.

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ACKNOWLEDGMENTS

To adapt a phrase, it takes a village to complete a dissertation. I therefore begin by acknowledging a few of the people who were truly instrumental in the process of bringing forth this one, while recognizing that there are many others who played a part that I shall fail to mention in these few pages. First, I thank my committee members, Dr. Bridgett vonHoldt, Dr. H. Bradley Shaffer, Dr. Kirk Lohmueller, and Dr. Robert Wayne for the invaluable role that they played in the completion of this work. Their questions, advice, and encouragement were particularly vital during the final stages and greatly contributed to improving this manuscript.

The following chapters resulted from the combined efforts of many. The first chapter is a reformatted version of “Genomic Flatlining in the Endangered Island Fox” by Jacqueline A. Robinson, Diego Ortega-Del Vecchyo, Zhenxin Fan, Bernard Kim, Bridgett vonHoldt, Clare Marsden, Kirk Lohmueller, and Robert Wayne, published in *Current Biology* in 2016. JAR primarily carried out the genomic analyses, with assistance from DODV, ZF, BK, BvH, CM, and KL. Demographic inference and simulations were performed by DODV. The manuscript was written by JAR, DODV, KL, and RW, with input from all authors. The second chapter is the work of Jacqueline A. Robinson, Caitlin Brown, Bernard Kim, Kirk Lohmueller, and Robert Wayne. CB conducted the morphological analysis, JAR conducted the genomic analysis, and JAR and BK conducted the simulations. The manuscript was written by JAR, with contributions from CB, KL, and RW. The third chapter is the work of Jacqueline A. Robinson, Jannikke Räikkönen, Kirk Lohmueller, and Robert Wayne. JR performed the morphological analysis, and the remaining analyses were carried out by JAR. The manuscript was written by JAR, with contributions from KL and RW. The authors thank Rolf Peterson, Leah Vucetich, and Philip Hedrick, for samples, financial support, and advice in designing the experiment.

I now address these acknowledgments to the individuals whom I would most like to thank. To my advisor, Dr. Robert Wayne: Years ago, when I was a total stranger to you and came to you with nothing but the goal of one day becoming a scientist, you gave me a chance, and it changed the course of my life. I will never be able to sufficiently express my gratitude to you for that, nor for the subsequent years in which you challenged me, encouraged me, and provided me with the means to achieve my goal. I feel incredibly fortunate to have been your student. To my other advisor, Dr. Kirk Lohmueller: Before you came along, I did not even know what I did not know. Now, I think maybe I do know one or two things, and I learned them from you. As a teacher, as a scientist, and as a mentor, you inspire me. To Dr. John Pollinger: During my first few years conducting research, you did me the great favor of passing on crucial knowledge that you had accumulated over your years as a pillar of the lab. In your absence, I did my best to repay your selflessness by sharing what you bestowed on me.

To the members of the Wayne and Lohmueller labs, past and present: I have thoroughly enjoyed working with and learning from all of you over the years. I credit you for the wonderfully supportive and fun environment I enjoyed throughout this process. Specifically, thank you to Rachel Johnston, Annabel Beichman, Rena Schweizer, Devaughn Fraser, and Tiffany Armenta: You are my role models. And thank you to Annabel and Rachel for all the great times outside of the lab.

To Bernard Kim, Grace John, and Jonathan Chang: You are the joy in my life! Graduate school has its ups and downs – mostly downs – but taking this journey with you made all the difference. My favorite memories since we started on this arduous path are the times we spent together. To Bernard: I rely on you in too many ways to describe, so thank you from the bottom of my heart for all the ways in which you enrich my life.

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Schweizer, R.M., **Robinson, J.**, Harrigan, R., Silva, P., Galverni, M., Musiani, M., Green, R.E., Novembre, J. And Wayne, R.K., 2016. Targeted capture and resequencing of 1040 genes reveal environmentally driven functional variation in grey wolves. *Molecular Ecology* 25, 357-379.

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February 2016

Plant & Animal Genome Conference; San Diego, CA, January 2016

Chapter 1: Genomic flatlining in the endangered island fox

Summary

Genetic studies of rare and endangered species often focus on defining and preserving genetically distinct populations, especially those having unique adaptations (Crandall et al. 2000; Funk et al. 2012). Much less attention is directed at understanding the landscape of deleterious variation, an insidious consequence of geographic isolation and the inefficiency of natural selection to eliminate harmful variants in small populations (Lande 1994; Lynch et al. 1995; Kirkpatrick and Jarne 2000). With population sizes of many vertebrates decreasing, and isolation increasing through habitat fragmentation and loss, understanding the extent and nature of deleterious variation in small populations is essential for predicting and enhancing population persistence. The Channel Island fox (*Urocyon littoralis*) is a dwarfed species that inhabits six of California's Channel Islands, and is derived from the mainland gray fox (*U. cinereoargenteus*). These isolated island populations have persisted for thousands of years at extremely small population sizes (Wayne et al. 1991; Aguilar et al. 2004) and, consequently, are a model for testing ideas about the accumulation of deleterious variation in small populations under natural conditions. Analysis of complete genome sequence data from island foxes shows a dramatic decrease in genome-wide variation and a sharp increase in the homozygosity of deleterious variants. The San Nicolas Island population has a near absence of variation, demonstrating a unique genetic flatlining that is punctuated by heterozygosity hotspots, enriched for olfactory receptor genes and other genes with high levels of ancestral variation. These findings question the generality of the small population paradigm that maintains substantial genetic variation is necessary for short and long-term persistence.

Results and Discussion

To determine the extent of genetic variation in coding and non-coding regions in the island fox genome, and the role of demography and natural selection in shaping patterns of variation, we sequenced genomes of seven island foxes representing each of the island populations and a mainland gray fox from Southern California (Figures 1-1A, 1-1B). We included two genomes of the San Nicolas Island fox to better assess genome-wide patterns of variation because past research suggested a dramatic loss of variation in hypervariable loci (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999). Each of these island populations represents a morphologically and genetically distinct subspecies, four of which (San Miguel, Santa Rosa, Santa Cruz, Santa Catalina) have been listed as endangered under the US Endangered Species Act following catastrophic declines due to predation by non-resident golden eagles (*Aquila chrysaetos*) and introduced canine distemper virus (Roemer et al. 2001; Timm et al. 2009; USFWS 2004). The individuals sequenced in this study were sampled in 1988, prior to the recent declines of these four populations. Each genome was sequenced with an Illumina HiSeq 2000 and aligned to the domestic dog reference genome, canFam3.1, followed by joint genotyping, filtering, and annotation of variants, yielding ~13-23X coverage (Table 1-1).

Genome-wide autosomal heterozygosity is high for the mainland gray fox ($12.0 \times 10^{-4}/\text{bp}$), whereas heterozygosity in island foxes is reduced by 3- to 84-fold ($4.08 - 0.142 \times 10^{-4}/\text{bp}$, Figures 1-1A, 1-2). The most extreme reduction of heterozygosity is found in the San Nicolas population ($0.142 - 0.190 \times 10^{-4}/\text{bp}$), which has a genome that is almost entirely monomorphic (Figure 1-3A). Both San Nicolas individuals are nearly identical, differing at fewer than two sites per 100 kb. This remarkable absence of genomic variation in San Nicolas foxes is unprecedented in an outbreeding species (Figure 1-1C), though it is conceivable that our

heterozygosity estimates could be biased downward by conservative data quality filters designed to mitigate the inclusion of sequencing errors. However, previous studies of hypervariable loci have consistently shown San Nicolas to be among the most monomorphic outbreeding animal populations (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999). After San Nicolas, the second most monomorphic fox genome is found on San Miguel, the smallest of the fox-inhabited islands at 37 km². However, the San Miguel fox genome still has approximately seven times as many heterozygous sites as the San Nicolas foxes, although the populations have similar census sizes on the order of a few hundred individuals (Figure 1-1A) (Wayne et al. 1991). This finding suggests that, although a critical variable, long-term small population size does not completely account for the striking lack of diversity in San Nicolas foxes. Indeed, an extreme population bottleneck likely occurred in the San Nicolas population in the early 1970s (Aguilar et al. 2004; Laughrin 1980). Nonetheless, the San Nicolas population subsequently rebounded without human assistance, despite the drastic reduction in genetic variation.

We used an approximate Bayesian computation (ABC) approach to estimate the parameters of demographic models that might account for the observed low heterozygosity in island populations. We utilized the distribution of heterozygous genotypes in a set of 13,647 1 kb putatively neutral regions distributed across the genome to infer demographic parameter values (see Appendix 1-I). Based on previous genetic analyses (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Aguilar et al. 2004; Hofman et al. 2015) and our inferred phylogenetic tree (Figure 1-1B), we assumed a bottleneck associated with the initial founding of the island fox, followed by island-specific colonization bottlenecks with no gene flow between islands or between the islands and the mainland (Figures 1-4, 1-5). We focused on modeling the demographic history of three populations: 1) the mainland gray fox, representing a population

with no history of founder events or bottlenecks; 2) San Miguel, the smallest island population that nonetheless has greater diversity than the San Nicolas foxes, representing a population with small long-term effective size following island colonization; and 3) San Nicolas, the island population with the lowest observed heterozygosity, which has a history similar to that of San Miguel, but with a recent extreme bottleneck. These three models therefore exemplify the three major types of demographic history of individuals in our dataset (mainland, island, island+bottleneck). We estimated a large effective population size equal to 5,185 during the last 500 generations in the gray fox, consistent with greater levels of variation in the large outbreeding mainland population. In contrast, we found that the San Nicolas and San Miguel populations had effective population sizes of 64 and 133 individuals, respectively, during more than 93% of the last 500 generations (Figure 1-5). These results confirm that long-term small population size is a key element explaining the extremely low variation observed in the island populations, and that the exceptional lack of diversity in San Nicolas is the result of a recent severe bottleneck.

Given that small population size and population bottlenecks, such as those inferred in the island fox populations, are predicted to affect the efficacy of selection (Lande 1994; Lynch et al. 1995; Kirkpatrick and Jarne 2000; Sousa et al. 2014), we assessed the distribution of heterozygosity found in island fox genomes according to sequence context. We used the annotation of the dog genome to extract sequences corresponding to putatively neutral regions, exons of protein-coding genes, and conserved non-coding regions (Figure 1-6A). The island genomes are characterized by decreased heterozygosity overall, however, with the dramatic exception of San Nicolas, levels of variation match general expectations of reduced heterozygosity in putatively functional (exonic and conserved non-coding) regions relative to

neutral regions. To more directly assess the efficacy of selection, we examined the ratio of heterozygosity of zero- to four-fold degenerate sites (zero-fold degenerate sites: all mutations are non-synonymous, four-fold degenerate sites: all mutations are synonymous). This ratio is predicted to be elevated in small populations, since deleterious alleles can increase in frequency under strong drift and weakened selection (Lohmueller et al. 2008; Sousa et al. 2014). Consistent with this prediction, we found a negative relationship between the ratio of zero-fold to four-fold heterozygosity and neutral heterozygosity, with smaller populations having lower neutral heterozygosity but higher zero-fold heterozygosity (Figure 1-6B). San Nicolas is the most extreme in this regard with highly elevated levels of putatively deleterious zero-fold heterozygosity. Using forward in time population genetic simulations, we found that our demographic models, combined with a distribution of fitness effects inferred from human polymorphism data (Boyko et al. 2008), are sufficient to explain the observed ratios (Figure 1-6B). Therefore demographic history can account for the observed increase of deleterious heterozygosity in the island genomes, demonstrating the long-term effects of small population size on reducing the efficacy of selection in small, isolated populations.

To further investigate the consequences of weaker selection in the island populations, we annotated variants within coding regions with respect to their effect on the amino acid sequence, and polarized alleles as ancestral or derived using the dog as an outgroup (Figure 1-6C). We utilized the prediction algorithm *Sorting Intolerant From Tolerant (SIFT)* (Kumar et al. 2009), which estimates if missense mutations are likely to be damaging by assessing evolutionary constraint in homologous protein alignments, with the assumption that mutations observed at positions highly conserved across taxa are likely to disrupt function (DEL: *SIFT* score < 0.05), whereas others are more likely to be tolerated (TOL: *SIFT* score \geq 0.05). We also catalogued loss-

of-function mutations, as these mutations have a high probability of impacting fitness by reducing or eliminating gene functionality (MacArthur et al. 2012; Sulem et al. 2015). In the mainland gray fox, genotypes containing putatively derived alleles (heterozygous and homozygous derived) are lowest for deleterious variants (loss-of-function and deleterious) relative to benign variants (tolerated and synonymous), demonstrating that purifying selection in the large mainland population effectively removes deleterious variation (Figure 1-6C). In contrast, all island populations have reduced heterozygosity and an elevation of homozygosity for derived alleles, suggesting the conversion of deleterious alleles from the heterozygous to the homozygous state through strong genetic drift reducing the efficacy of purifying selection. The number of missense derived deleterious alleles per individual is 6.2% higher in the island populations than in the gray fox ($P < 0.007$, Table 1-2) suggesting a greater additive genetic load in the island populations. Additionally, the island foxes have more than twice the number of homozygous loss-of-function genotypes compared to the mainland fox ($P < 1.75 \times 10^{-5}$, Figure 1-6C, Table 1-2), implying a substantial genetic load, particularly if loss-of-function variants are recessive (Kimura et al. 1963; Peischl and Excoffier 2015). The general increase in loss-of-function alleles in all the island populations indicates that the accumulation and fixation of deleterious variants is a feature associated with long-term small population size and isolation, rather than a recent extreme bottleneck as observed in the San Nicolas population. In combination, these results argue that small population sizes have not resulted in more efficient purging of deleterious alleles, and have instead significantly increased the genetic load of the island populations.

A previous genotyping study of five major histocompatibility complex (MHC) loci in the San Nicolas population found evidence for balancing selection (Aguilar et al. 2004). However,

coverage limitations and the frequent presence of pseudogenes in our sequence data complicate assessments of heterozygosity at the MHC region. Consequently, we instead searched for regions of elevated heterozygosity throughout the genome at sites passing our series of quality filters. Within each genome, peaks greater than two standard deviations above the mean heterozygosity in a sliding window analysis across the autosomes were considered to be elevated (Figures 1-3A, 1-2). We found 66 and 48 discrete peaks in the San Nicolas genomes (526 - 1,092 peaks in other island fox genomes, 763 peaks in the gray fox genome). We found that peak regions were not enriched for genic content ($P \geq 0.595$), but were significantly enriched for a variety of gene ontology (GO) terms (Table 1-3). In the two San Nicolas fox sequences and the mainland gray fox, we found enrichment of olfactory receptor (OR) genes (*KEGG:04740*) in heterozygosity peaks (San Nicolas 1: $P = 3.20 \times 10^{-20}$, San Nicolas 2: $P = 5.68 \times 10^{-4}$, gray fox: $P = 1.16 \times 10^{-15}$). A result this extreme rarely occurs by chance (San Nicolas 1: $P = 10^{-3}$, San Nicolas 2: $P = 0.033$, gray fox: $P < 10^{-3}$). However, OR genes commonly occur in clusters, a spatial factor that is not formally considered in the GO enrichment test that could inflate apparent enrichment. Additionally, OR genes have high ancestral levels of variation, as suggested by their presence in peaks in the gray fox and high rates of OR gene polymorphism observed in mammals (Gilad et al. 2003; Alonso et al. 2008; Chen et al. 2012).

To assess whether these peaks of heterozygosity remaining in the San Nicolas genome could be the result of neutral demographic processes, we conducted simulations of 1,000 full genomes under our San Nicolas demographic model. We find that the simulated genomes contain as many or more peaks of similar magnitude, suggesting that peaks of heterozygosity are expected even after severe bottlenecks, demonstrating that balancing selection is not necessary to account for the pattern of heterozygosity observed in the San Nicolas genomes (Figures 1-3B, 1-

3C). Therefore, the remaining heterozygosity peaks in the San Nicolas genomes appear to be the remnants of high heterozygosity in the ancestral population that has yet to be eliminated through drift, rather than being actively maintained through balancing selection. This effect is more likely to occur in regions of the genome that have elevated polymorphism in the ancestral population, such as olfactory receptor genes. As we have shown in the San Nicolas fox, consideration of demographic models is essential to determine if the number of high heterozygosity peaks could be due to demographic history and drift alone.

We show that island fox populations have experienced a dramatic reapportionment of deleterious variation and have two to three times more variants in the homozygous state for loss-of-function and deleterious missense variants. In the San Nicolas Island fox, heterozygosity has flatlined genome-wide such that the population has a near absence of genetic variation, with remaining variation evident in only a few genes with high initial levels of variation, such as the OR gene family. This lack of variation can be explained by an extremely small effective size of about 64 individuals over 500 generations ago, followed by a severe bottleneck ~30 generations ago that reduced the population to fewer than a dozen individuals. The dominant effect of demography across all island populations is a reduction in the efficacy of selection and a consequent increase in the load of deleterious variation. Nonetheless, the island populations appear healthy, can recover from disease epidemics, and the four recently bottlenecked populations recovered rapidly under human management after introduced non-native disease or predation threats were removed (Coonan et al. 2014; King et al. 2014). The unaided persistence of the San Nicolas population, and the successful recovery of the four endangered populations, contrasts with other cases in which loss of genetic diversity following population declines has resulted in apparent inbreeding depression, hampering recovery (Westemeier et al. 1998;

Johnson et al. 2010). The long-term persistence of island foxes despite their small population sizes and increased genetic load presents a challenge for the small population conservation paradigm (Caughley 1994), which emphasizes population size and its effects on genetic variation as crucial factors in long-term persistence or endangerment. The absence of obvious negative effects on population persistence from genetic deterioration may in part reflect a more benign island environment, given the lack of competitors and predators that exist on the mainland. If island environments are more benign in general, then island populations may often tolerate higher levels of genetic load than mainland counterparts. Notably, our results contradict the notion that long-term small effective population size and inbreeding on the islands have enhanced purging and decreased their genetic load (Xue et al. 2015). Conceivably, phenotypic plasticity mediated by regulatory and epigenetic mechanisms may help compensate for the lack of genomic variation, a possibility that can now be explored in small populations using new and developing molecular techniques (Kristensen et al. 2010; Vergeer et al. 2012).

Materials and Methods

Samples and sequencing

DNA samples representing each of the Channel Island fox populations and one mainland gray fox from southern California were used for whole genome sequencing on an Illumina HiSeq 2000. Island fox DNA samples were originally obtained for a population genetic study in 1988, prior to subsequent population declines due to predation and disease in four of the island populations (Gilbert et al. 1990). Samples for sequencing were selected to maximize DNA quality and quantity, evaluated by gel electrophoresis, NanoDrop spectrophotometer, and Qubit

fluorometer. Each individual was sequenced with at least one lane of paired-end 100 bp reads. Sample and sequence information is summarized in Table 1-1.

Alignment and annotation

Reads were trimmed and filtered for quality before being aligned to the domestic dog genome (canFam3.1). Over 90% of reads successfully aligned to the dog reference genome yielding ~13-23X coverage per individual (Table 1-1). We applied conservative ad hoc filters to minimize the inclusion of erroneous genotypes. We used the genomic coordinates identified in Freedman et al. (2014) to extract coding, conserved non-coding, and putatively neutral sequences. Briefly, coordinates of 196,668 exons were obtained from a filtered set of transcripts originating from NCBI, Ensembl, and UCSC. 319,958 mammalian conserved non-coding sequences were identified from a multi-genome alignment of 11 Euarchontoglires as regions longer than 50 bp with a phastCons score > 0.7 (Siepel et al. 2005). The putatively neutral regions comprise a set of 13,647 1 kb loci located at least 30 kb apart, at least 10 kb from coding sequence, and 100 bp from conserved non-coding sequence while avoiding regions of the genome with repeats, poor assembly, or low mappability. Coordinates of zero-fold and four-fold degenerate sites within coding transcripts were those identified as positions where mutations would always or never change the encoded amino acid, respectively (Marsden et al. 2016). Variant annotation was performed with *VEP* (McLaren et al. 2010) running *SIFT* (Kumar et al. 2009), using Ensembl's *C. familiaris* annotation database (release 78), to identify loss-of-function, deleterious (*SIFT* score < 0.05), tolerated (*SIFT* score ≥ 0.05), and synonymous mutations. Loss-of-function mutations were defined as those that encoded a premature stop codon. Further details are provided in Appendix 1-I.

Heterozygosity peak analysis

Heterozygosity was calculated as the fraction of heterozygous genotypes of all genotypes passing filters in 100 kb windows with a 10 kb step size. Heterozygosity “peaks” were identified as windows with heterozygosity in excess of two standard deviations above the mean, calculated per genome (Fig. 1-2). Heterozygosity peak regions were tested for enrichment of genic content by tallying the number of bases within them that overlapped with genes, and testing whether this proportion was significantly higher than expected by chance. Gene ontology (GO) enrichment analysis was performed on peak coordinates using *gProfileR* with the Ensembl *C. familiaris* annotation (release 79) (Reimand et al. 2007).

Demographic inference and simulations

We used approximate Bayesian computation (ABC) to infer the demographic history of the San Nicolas, San Miguel, and mainland gray fox populations (Figures 1-5B, 1-5C). Demographic models and prior distributions of parameters were based on estimates from our own analyses (Figures 1-1B, 1-4, 1-5A) and the literature (Aguilar et al. 2005; Hofman et al. 2015). We conducted forward in time simulations with a modified version of the forward simulator used by Lohmueller (2014) to jointly assess the effects of demography and purifying selection on levels of deleterious variation. We conducted simulations with *MaCS* (Chen et al. 2009) to determine whether the observed number of high heterozygosity peaks observed in the San Nicolas genome is expected under neutrality using our inferred demographic model. Full descriptions of these methods are provided in Appendix 1-I.

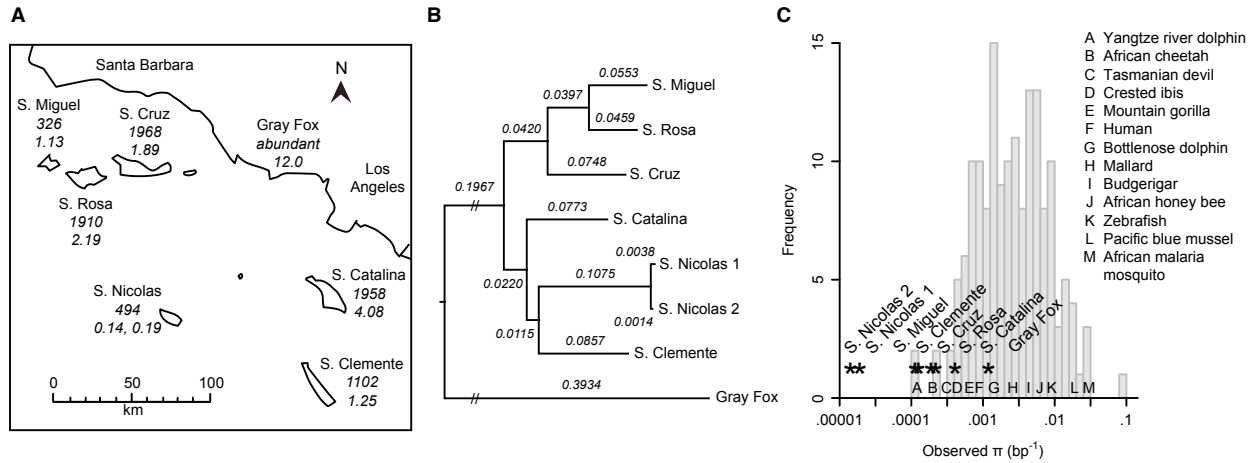


Figure 1-1. Island foxes show exceptionally low heterozygosity. (A) Map showing island geography, estimated census size at time of sampling (upper number) (Wayne et al. 1991), and genome-wide heterozygosity per 10 kb of autosomal sequence (lower number). (B) Neighbor-joining tree constructed from a genome-wide pairwise distance matrix, displaying reciprocal monophyly of northern and southern island populations, and the southern California gray fox as the outgroup. Genetic distance is indicated on the branches. All nodes have 100% bootstrap support. (C) Histogram showing the distribution of published genome-wide estimates of π from 159 outbreeding species (137 animal, 11 plant, 8 fungus, and 3 protist taxa; sources denoted with † in the Bibliography of this chapter), with the position of island and gray fox heterozygosity values indicated by asterisks.

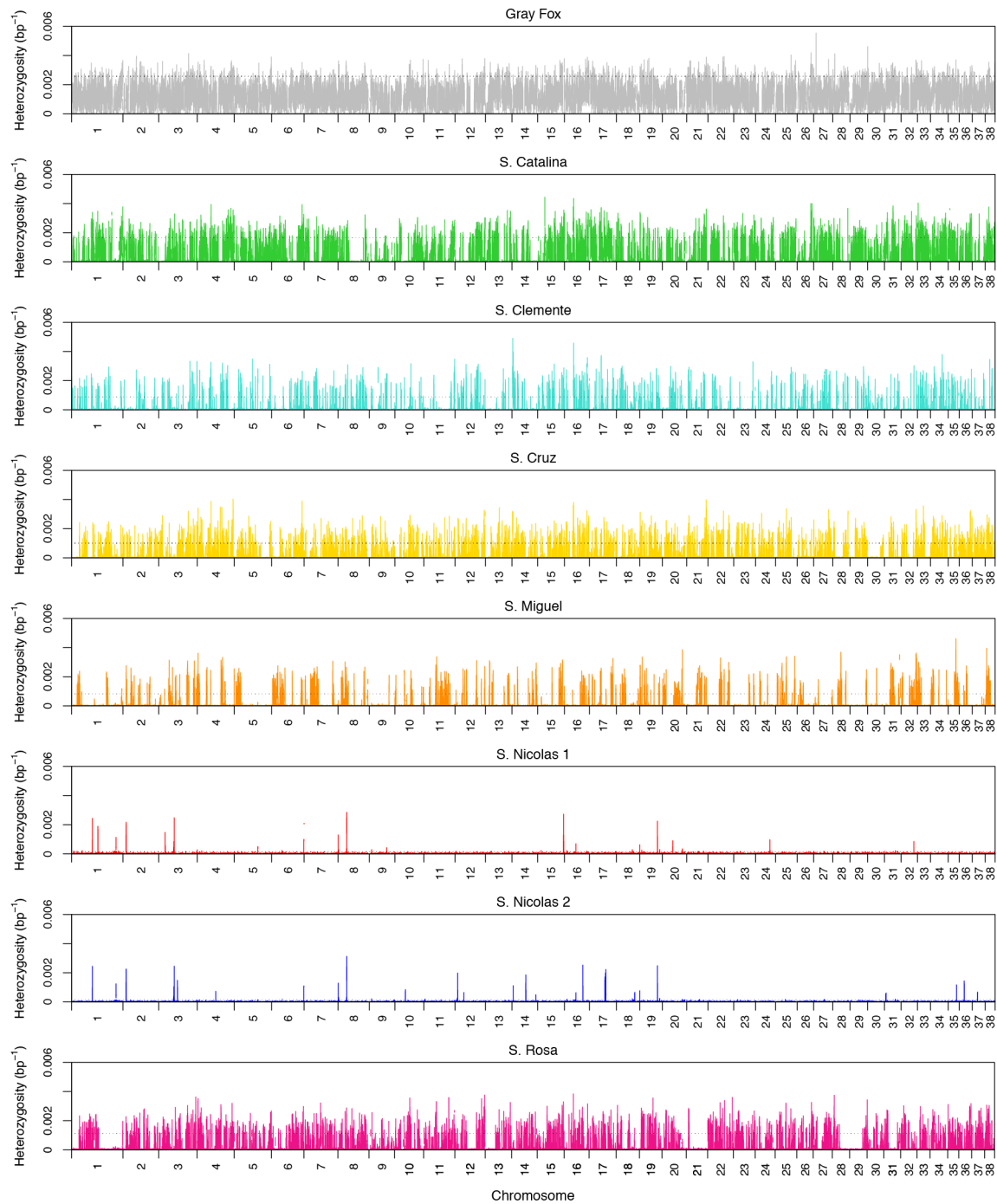


Figure 1-2. Per-site heterozygosity across the autosomes per individual in sliding windows of 100 kb with a 10 kb step size. For inclusion, windows were required to have at least 10 kb of sequence, and no more than 10% of sites within the window could be failing filters. Dotted lines represent the threshold for calling peaks of heterozygosity (heterozygosity mean plus two standard deviations).

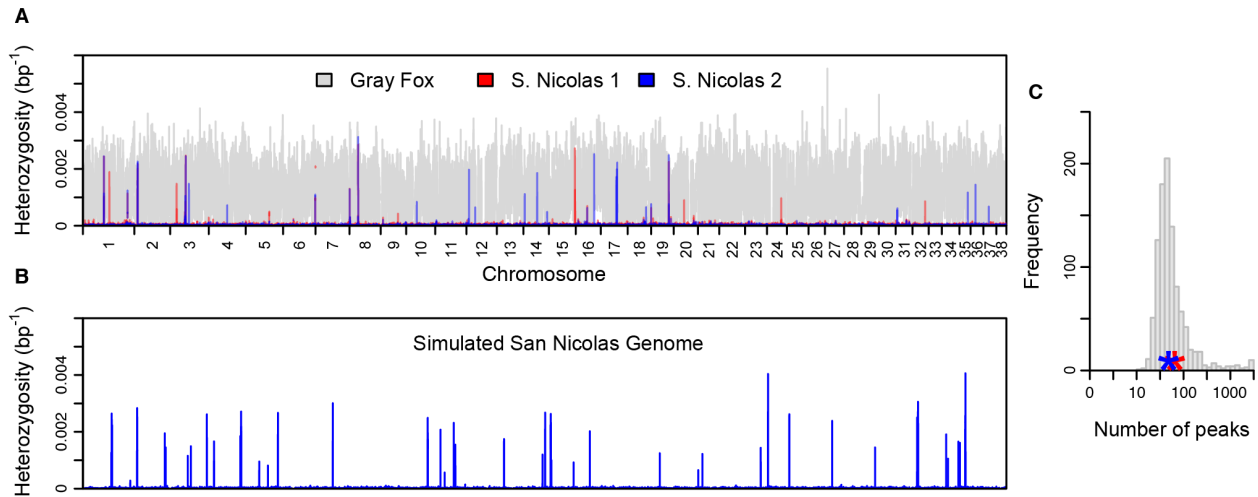


Figure 1-3. Distribution of peaks of heterozygosity across the island and mainland fox genomes. (A) Empirical heterozygosity per 100 kb window with a 10 kb step size across the genome in two San Nicolas foxes (red and blue lines, joint purple lines) and the mainland gray fox (gray lines). The mainland fox exhibits heterozygosity across the entire genome, whereas the San Nicolas foxes have virtually no heterozygosity except at a few distinct peaks. (B) An example of a simulated genome using the San Nicolas population demographic model, showing a similar lack of heterozygosity genome-wide except at a few distinct peaks. According to these simulations, peaks of equivalent magnitude and number can be generated by genetic drift alone. (C) Histogram showing that the observed number of peaks in the San Nicolas genomes (colored asterisks) falls near the mode of the distribution of the number of peaks generated in genomes simulated under the San Nicolas demographic model ($P = 0.257-0.451$).

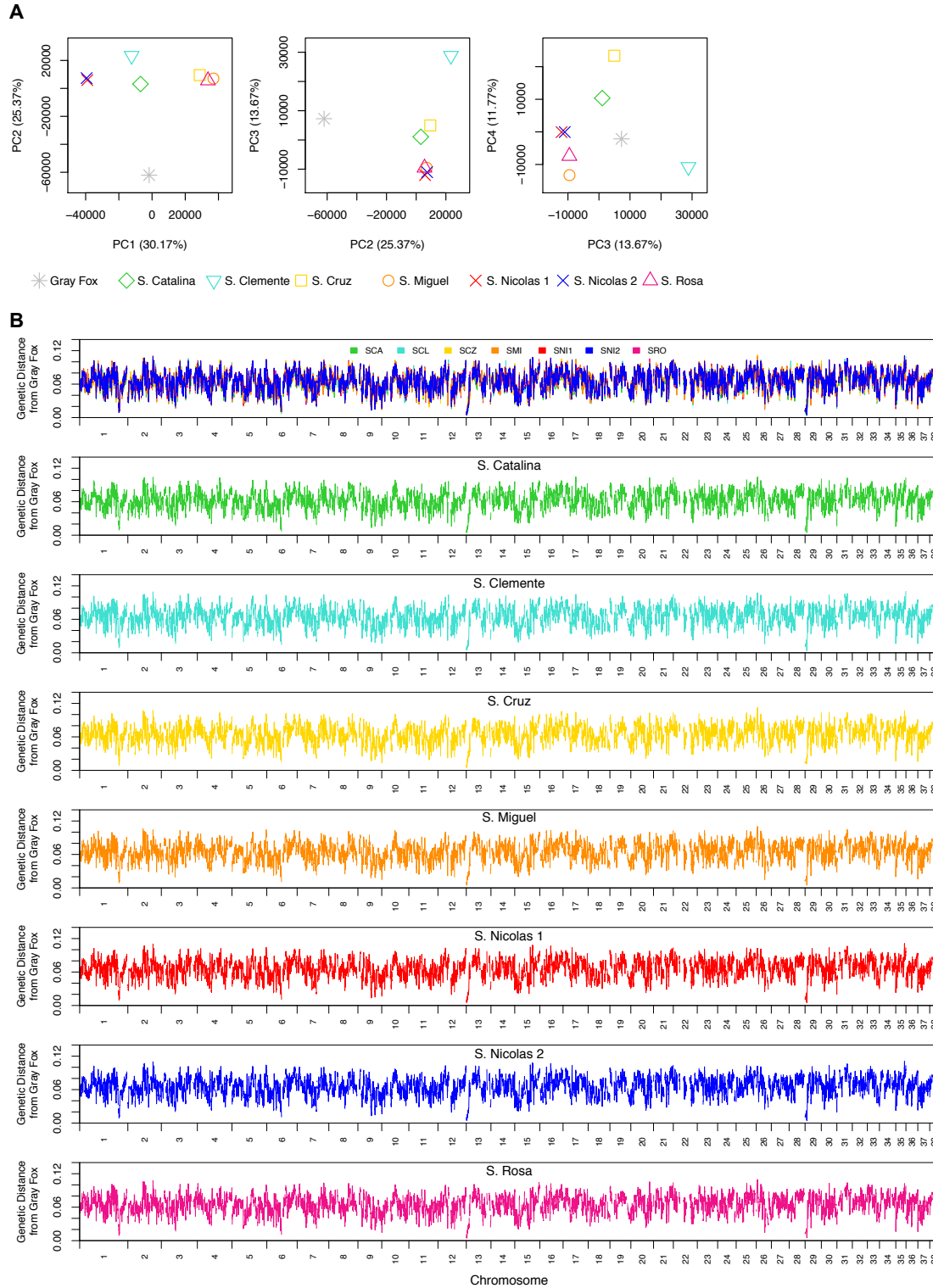


Figure 1-4. (A) Genetic covariance of SNPs pruned for linkage disequilibrium (86,994) is largely dictated by geography (PC1: 30.17%, PC2: 25.37%). No island fox appears to cluster with the gray fox, and island foxes cluster with one another according to expectation. (B) Genetic distance from the gray fox was calculated for each island fox in 1 Mb windows with 100 kb step size. The overall similarity of distances across islands suggests the absence of recent gene flow from the mainland.

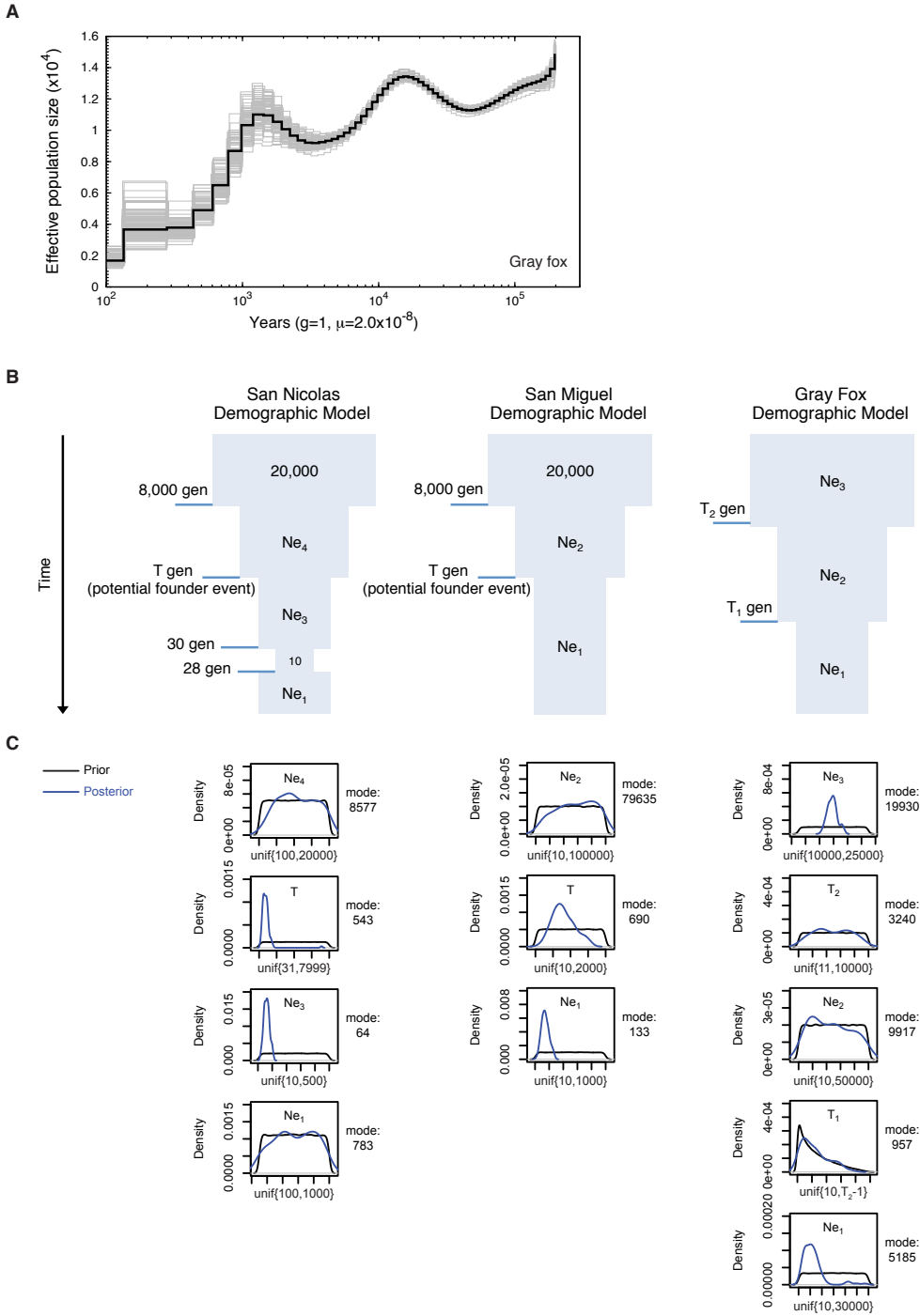


Figure 1-5. (A) *PSMC* (Li and Durbin 2011) analysis suggests a population decline in the mainland gray fox. The dark line represents the inferred effective population size of the gray fox over the past 2×10^5 generations, and light lines represent bootstrap replicates. Time and population size were scaled under the assumption of a one-year generation time and a mutation rate of 2.0×10^{-8} /site/generation. (B) Models used for inference of demographic parameters were based on estimates from literature (Aguilar et al. 2004; Hofman et al. 2015) and *PSMC* results. (C) Plots of prior (black line) and posterior (blue line) distributions of parameter values in demographic inference, shown below corresponding model. See Appendix 1-I for full details.

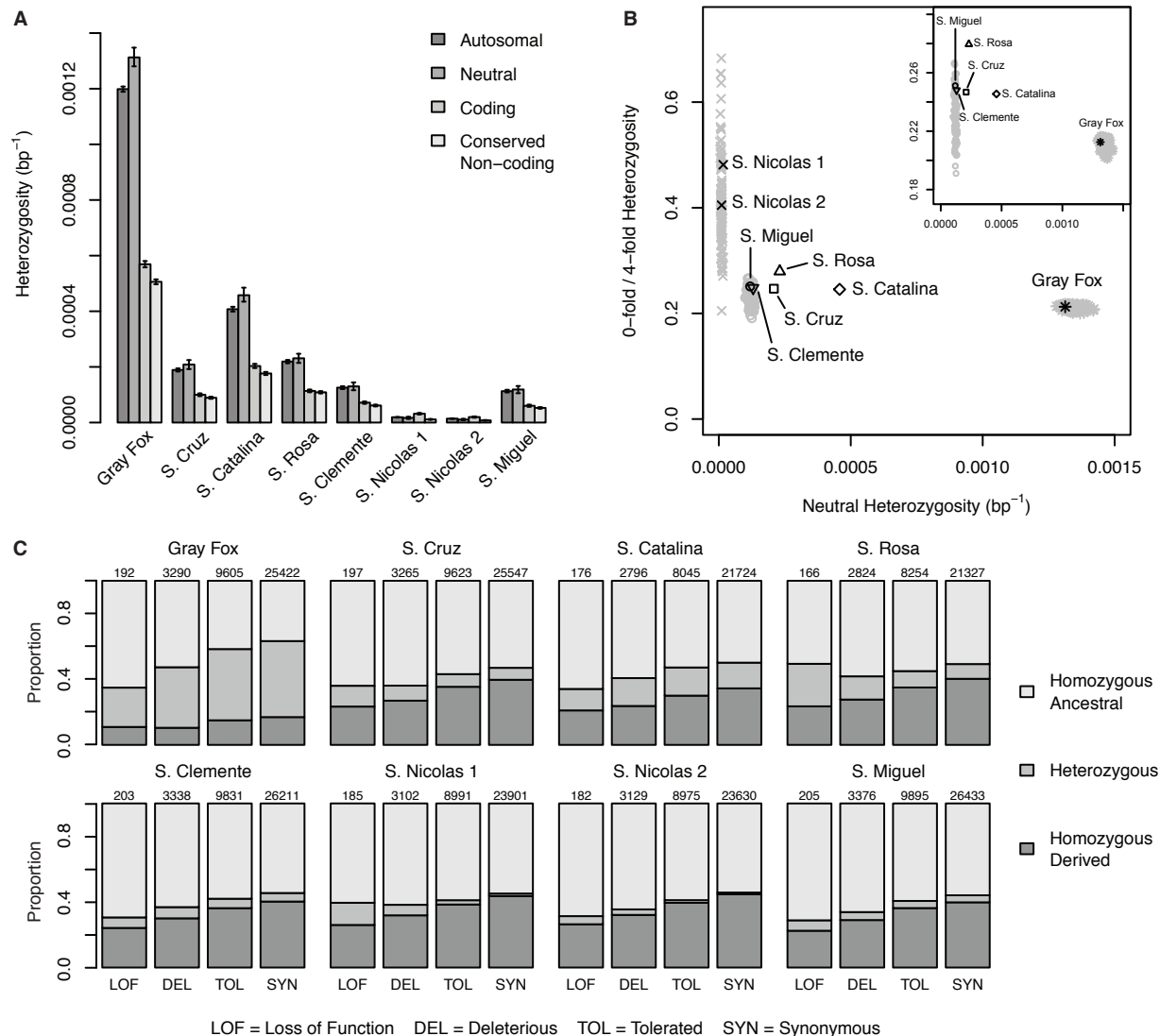


Figure 1-6. Extreme drift in island foxes reapportions deleterious genetic variation. (A) In all foxes, except San Nicolas, autosomal and neutral diversity is higher than in regions putatively under purifying selection (coding and conserved non-coding sequence). Error bars represent 95% confidence intervals from bootstrap resampling. Individuals are ordered according to census sizes (Figure 1-1A). (B) The negative relationship between neutral diversity (influenced by effective population size), and the ratio of heterozygosity at zero-fold relative to four-fold sites (influenced by the efficacy of purifying selection). Light gray symbols represent values obtained from simulations using the distribution of selective effects inferred in humans (Boyko et al. 2008) and our demographic models (Figure 1-5). A negative relationship between the zero-fold/four-fold heterozygosity ratio and neutral diversity also persists after excluding the San Nicolas foxes (inset). At extremely low effective population size, as in San Nicolas, there is high variance in the ratio of zero-fold/four-fold heterozygosity in simulations due to low counts of heterozygous genotypes. (C) Proportion of genotypes in each individual that are homozygous ancestral, heterozygous, or homozygous derived (with respect to the dog reference genome) at segregating sites within coding regions. Total genotype counts are indicated at the tops of bars for each category. Loss-of-function mutations are those that encode a premature stop codon, whereas “deleterious” and “tolerated” missense mutations are categorized by *SIFT* (Kumar et al. 2009).

Table 1-1. Sample, sequence, alignment, and filtering statistics.

Species	Location	ID	Sample #	Sex	Year Sampled	Sequencing Center	Base Pairs (Gbp)	Total # of reads aligned* (x 10 ⁶)	# of reads aligned, post-filtering (x 10 ⁶)	Mean depth of coverage (X)	95%-ile of coverage (X)	% of reference covered by ≥ 6 reads	# of genotypes passing all filters (x 10 ⁶)
ULI	Santa Catalina	SCA	SCA16/ RKW4644	F	1988	UC Berkeley	82.5	384	315	12.90	36	83.53	970
ULI	San Clemente	SCL	SCLV4/ RKW4045	F	1988	UC Berkeley	73.2	525	453	18.72	31	92.59	1168
ULI	Santa Cruz	SCZ	SCZ05/ RKW12331	M	1988	UC Berkeley	77.9	411	352	14.54	26	91.17	1153
ULI	San Miguel	SMI	SMI15/ RKW12354	F	1988	UC Berkeley	80.5	628	562	23.27	38	92.83	1165
ULI	San Nicolas	SNI1	SNI05/ RKW4038	F	1988	UC Los Angeles	66.1	403	342	13.88	28	88.18	1082
ULI	San Nicolas	SNI2	SNI41/ RKW12349	F	1988	UC Berkeley	84.3	390	333	13.70	24	91.54	1150
ULI	Santa Rosa	SRO	SRO40/ RKW12359	F	1988	UC Berkeley	82.8	365	313	12.86	24	90.38	1119
UCI	SMMNRA**	UCI	GFO46/ RKW14819	F	2012	UC Berkeley	67.5	477	412	17.06	28	92.46	1164

* Following duplicate removal

** Santa Monica Mountains National Recreation Area

Table 1-2. Comparison of the proportions of homozygous derived genotypes or derived alleles per individual between gray fox and island foxes.

Proportion of Homozygous Derived Genotypes					
Sequence category	Null model ^a		Alternative model ^b		Likelihood ratio test ^c
	MLE	Log-likelihood	MLE	Log-likelihood	
Synonymous	$p_i=p_g=0.375$	-128330.2	$p_i=0.404,$ $p_g=0.169$	-125440.6	$\Lambda=5779.31, P<<10^{-16}$
Tolerated	$p_i=p_g=0.332$	-46521.10	$p_i=0.358,$ $p_g=0.150$	-4.5592.58	$\Lambda=1857.04, P<<10^{-16}$
Deleterious	$p_i=p_g=0.264$	-14501.63	$p_i=0.287,$ $p_g=0.104$	-14209.25	$\Lambda=584.76, P<<10^{-16}$
Loss of function	$p_i=p_g=0.229$	-837.61	$p_i=0.245,$ $p_g=0.116$	-828.39	$\Lambda=18.45, P<1.75 \times 10^{-5}$
Proportion of Derived Alleles					
Sequence category	Null model ^d		Alternative model ^e		Likelihood ratio test ^f
	MLE	Log-likelihood	MLE	Log-likelihood	
Synonymous	$p_i=p_g=0.431$	-265431.5	$p_i=0.435,$ $p_g=0.401$	-265327.8	$\Lambda=207.37, P<<10^{-16}$
Tolerated	$p_i=p_g=0.390$	-97909.18	$p_i=0.393,$ $p_g=0.367$	-97884.76	$\Lambda=48.85, P<2.77 \times 10^{-12}$
Deleterious	$p_i=p_g=0.326$	-31698.67	$p_i=0.331,$ $p_g=0.289$	-31674.95	$\Lambda=47.45, P<5.65 \times 10^{-12}$
Loss of function	$p_i=p_g=0.294$	-1883.56	$p_i=0.302,$ $p_g=0.237$	-1880.15	$\Lambda=18.45, P<1.75 \times 10^{-5}$
Tolerated ^g	$p_i=1.085p_g,$ $p_g=0.363$	-97885.54	$p_i=0.393,$ $p_g=0.367$	-97884.76	$\Lambda=1.56, P=0.22$
Deleterious ^g	$p_i=1.085p_g,$ $p_g=0.303$	-31678.59	$p_i=0.331,$ $p_g=0.289$	-31674.95	$\Lambda=7.28, P<0.007$
Loss of function ^g	$p_i=1.085p_g,$ $p_g=0.272$	-1881.53	$p_i=0.302,$ $p_g=0.237$	-1880.15	$\Lambda=2.74, P=0.098$

a. Here the null model constrains the proportion of homozygous derived genotypes per individual (p) to be the same in the island and mainland (i.e. $p_g=p_i$). MLEs of p and the corresponding log-likelihoods are shown.

b. Here the alternative model allows the proportion of homozygous derived genotypes per individual to differ between the island and the mainland (i.e. $p_g \neq p_i$). MLEs of p_g and p_i as well as the corresponding log-likelihoods are shown.

c. Likelihood ratio test for differences in the number of homozygous derived genotypes per individual between the island and mainland foxes. $\Lambda = -2(\log\text{-likelihood}_{\text{null}} - \log\text{-likelihood}_{\text{alternative}})$. Asymptotically, Λ is χ^2 distributed with one degree of freedom. This distribution was used to calculate P -values.

d. Here the null model constrains the proportion of derived alleles per individual (p) to be the same in the island and mainland (i.e. $p_g=p_i$). Maximum Likelihood Estimates (MLEs) of p and the corresponding log-likelihoods are shown.

e. Here the alternative model allows the proportion of derived alleles per individual to differ between the island and the mainland (i.e. $p_g \neq p_i$). MLEs of p_g and p_i as well as the corresponding log-likelihoods are shown.

f. Likelihood ratio test for differences in the proportion of derived alleles per individual between the island and mainland foxes. $\Lambda = -2(\log\text{-likelihood}_{\text{null}} - \log\text{-likelihood}_{\text{alternative}})$. Asymptotically, Λ is χ^2 distributed with one degree of freedom. This distribution was used to calculate P -values.

g. Here the null model assumes the ratio of p_i to p_g found at synonymous SNPs (i.e. $p_i=1.085355p_g$). The alternative model is the same as before. The purpose of this comparison is to test whether putatively deleterious SNPs show a greater difference in the proportion of derived alleles between island and mainland than that seen at synonymous SNPs.

Table 1-3. Gene ontology (GO) enrichment results of heterozygosity peaks. Abbreviations used in the table for island names are as in Table 1-1.

Population	P value ^a	Term size ^b	Query size ^c	Overlap size ^d	Precision	Recall	Term ID	Term name
SCA	0.00499	855	1434	84	0.059	0.098	GO:0098772	molecular function regulator
	0.00619	278	1434	37	0.026	0.133	GO:0070838	divalent metal ion transport
	0.0181	13970	1434	894	0.623	0.064	GO:0008150	biological_process
SCL	0.000547	252	1949	45	0.023	0.179	GO:0043235	receptor complex
SCZ	0.00435	37	1555	12	0.008	0.324	GO:0002548	monocyte chemotaxis
	0.0179	21	1555	7	0.005	0.333	KEGG:04977	Vitamin digestion and absorption
	0.023	13	1555	6	0.004	0.462	REAC:5950285	Organic cation/anion/zwitterion transport
SMI	8.25E-05	4163	1924	414	0.215	0.099	GO:0051179	localization
	0.0102	1403	1924	156	0.081	0.111	GO:0007399	nervous system development
	0.0118	440	1924	59	0.031	0.134	REAC:5949615	GPCR downstream signaling
SNI1	3.01E-26	545	216	44	0.204	0.081	GO:0004984	olfactory receptor activity
	3.25E-26	546	216	44	0.204	0.081	GO:0050911	detection of chemical stimulus involved in sensory perception of smell
	3.20E-20	446	216	34	0.157	0.076	KEGG:04740	Olfactory transduction
	4.64E-17	1163	216	49	0.227	0.042	GO:0007186	G-protein coupled receptor signaling pathway
	1.17E-08	3157	216	66	0.306	0.021	GO:0031224	intrinsic component of membrane
	4.54E-05	131	216	11	0.051	0.084	GO:0005549	odorant binding
	0.000761	3081	216	54	0.25	0.018	GO:0005886	plasma membrane
	2.54E-12	12	163	8	0.049	0.667	GO:0005149	interleukin-1 receptor binding
SNI2	1.06E-07	35	163	8	0.049	0.229	GO:0002437	inflammatory response to antigenic stimulus
	7.02E-06	545	163	19	0.117	0.035	GO:0004984	olfactory receptor activity
	7.23E-06	546	163	19	0.117	0.035	GO:0050911	detection of chemical stimulus involved in sensory perception of smell
	0.000568	446	163	13	0.08	0.029	KEGG:04740	Olfactory transduction
	0.00093	145	163	9	0.055	0.062	GO:0005125	cytokine activity
	0.00455	131	163	8	0.049	0.061	GO:0005549	odorant binding
	0.0116	250	163	10	0.061	0.04	GO:0019221	cytokine-mediated signaling pathway
	0.0155	1163	163	22	0.135	0.019	GO:0007186	G-protein coupled receptor signaling pathway
SRO	0.0261	8	163	3	0.018	0.375	GO:0001660	fever generation
	0.024	524	2025	69	0.034	0.132	REAC:5949307	Transmembrane transport of small molecules
	0.0284	921	2025	113	0.056	0.123	GO:0005887	integral component of plasma membrane
	0.0474	1063	2025	126	0.062	0.119	GO:0005215	transporter activity

	4.67E-18	545	989	76	0.077	0.139	GO:0004984	olfactory receptor activity
	5.22E-18	546	989	76	0.077	0.139	GO:0050911	detection of chemical stimulus involved in sensory perception of smell
	7.19E-16	3100	989	224	0.226	0.072	GO:0016021	integral component of membrane
	1.16E-15	446	989	62	0.063	0.139	KEGG:04740	Olfactory transduction
	7.29E-13	1163	989	108	0.109	0.093	GO:0007186	G-protein coupled receptor signaling pathway
UCI	0.0028	131	989	19	0.019	0.145	GO:0005549	odorant binding
	0.00536	78	989	14	0.014	0.179	GO:0000786	nucleosome
	0.0159	18	989	6	0.006	0.333	REAC:5949377	RNA Polymerase I Promoter Opening
	0.0202	3	989	3	0.003	1	REAC:5950344	GABA A (rho) receptor activation
	0.031	20	989	6	0.006	0.3	REAC:5950622	Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3
	0.031	20	989	6	0.006	0.3	REAC:5950590	DNA methylation

a. Significance is based on the *g:SCS* method within *gProfileR* [S4], which takes non-independence of GO terms into account

b. Number of genes within a GO term.

c. Number of genes found overlapping or within peak coordinates.

d. Intersection between genes within GO term and genes overlapping or within peak coordinates.

Appendix 1-I: Supplemental Methods

Alignment and genotyping

Summary statistics pertaining to sequencing, alignment, genotyping, and filtering are provided in Table 1. Reads were trimmed and filtered for quality before being aligned to the domestic dog genome (canFam3.1) with *bwa MEM 0.7.7* (Li 2013). Alignments were processed and filtered to remove duplicate reads with *picard tools 1.80* (<http://picard.sourceforge.net>). Indel realignment and base quality score recalibration were performed with the *Genome Analysis Toolkit 3.1-1 (GATK)* (McKenna et al. 2010). Base quality scores were recalibrated with the *GATK BaseRecalibrator* tool. To obtain a set of “known variants” for use in recalibration, raw variant genotypes were called with three different algorithms using default parameters and a minimum base quality Phred score of 20: the *GATK UnifiedGenotyper*, *samtools mpileup 1.1-26* (Li et al. 2009), and *FreeBayes 0.9.18-17* (Garrison and Marth 2012). Concordant genotypes found in two or more call sets were extracted with *bcftools, 1.1-108* (Li et al. 2009) and used as the set of “known variants” for *BaseRecalibrator*. This process was repeated three times to reach convergence between reported and empirical quality scores. Recalibrated alignments of reads that were properly paired, and mapped uniquely and high quality Phred scores (≥ 30) were used as input for joint genotype calling with *FreeBayes*. Only bases with a minimum Phred score of 20 were considered, and genotypes at all positions were called using the following parameters: --no-population-priors, --report-monomorphic, --genotype-qualities.

We applied conservative ad hoc filters to minimize the inclusion of erroneous genotypes. CpG islands and repeats were removed, following Marsden et al. (2016). Sites with combined read depth across individuals less than 32 or greater than 176 were filtered out (5th and 95th percentiles for total depth, respectively). Variant sites were further filtered on the basis of

genotype quality ($GQ \geq 50$), and required at least 2 alternate allele observations on each of the forward and reverse strands. Multi-nucleotide polymorphisms, indels, complex variants, and sites with more than one alternate allele were excluded, as were sites with more than two missing genotypes. Finally, sites with excess heterozygosity, defined as sites with six or more heterozygotes called out of the eight individuals, were excluded.

Additionally, filters were applied to individual genotypes. All genotypes required a minimum of six supporting reads. Maximum depth thresholds were based on the 95th percentiles of depth for each individual (Table 1-1). Genotypes with quality scores less than 20 were removed. Finally, heterozygous genotypes required an allele balance (ratio of reads containing the reference allele out of total reads in individuals called as heterozygous) between 0.23 and 0.76. Sequence and variant annotation was performed as described in the Results section above. Ancestral and derived alleles were polarized with respect to the dog genome. While we recognize that evolution certainly occurred on the dog lineage since the common ancestor with foxes, and that this evolution, may affect the accuracy of polarization, it should not bias relative comparisons between the island and mainland foxes. Further, as evidence that this polarization approach works well, we found that loss-of-function sites contained fewer homozygous derived genotypes than deleterious or synonymous variants. This is the predicted result if derived deleterious mutations are removed from the population by selection.

We were unable to reliably analyze diversity within MHC genes to replicate the results of Aguilar et al. (2004) due to problems with alignment and filtering in this region. Moreover, much of the MHC region was excluded by our CpG and repeat filtering. Finally, the genes sequenced by Aguilar et al. (DRB and DQB) had approximately double the expected coverage in our sequence data, implying possible gene duplication.

Test for elevated homozygosity of derived genotypes in island foxes

We tested whether the number of homozygous derived genotypes per individual differed between the gray fox and the seven island foxes (Table 1-2). For a given set of genotypes \mathbf{g} , the number of SNPs that an individual will carry in the homozygous derived state will be binomially distributed with parameters p , and g . Here p is the proportion of SNPs over which an individual is homozygous. In the null model, $p_i = p_g$, where p_i refers to the proportion of homozygous derived genotypes per individual for the island foxes and p_g refers to the proportion of homozygous derived genotypes in the gray fox. We computed the log-likelihood for the null model as:

$$l(p | \mathbf{x}, \mathbf{g}) = \sum_{j=1}^8 x_j \log(p) + (g_j - x_j) \log(1 - p)$$

where x_j denotes the number of homozygous derived genotypes for individual j and g_j denotes the total number of called genotypes in individual j . Note that the sum is taken over all eight foxes. This is a binomial log-likelihood function, without the unnecessary – due to the invariance principal of Maximum Likelihood Estimates (MLEs) – binomial coefficients.

Under the alternative model, the island foxes can have a different number of homozygous derived genotypes compared to the gray fox. Here $p_i \neq p_g$. We computed the log-likelihood of the full model as:

$$l(p_i, p_g | \mathbf{x}, \mathbf{g}) = x_g \log(p_g) + (g_g - x_g) \log(1 - p_g) + \sum_{j=1}^7 x_j \log(p_i) + (g_j - x_j) \log(1 - p_i)$$

where x_g denotes the number of homozygous derived genotypes for the gray fox, g_g the total number of called genotypes for the gray fox, x_j the number of homozygous derived genotypes for the j th island fox, and g_j the total number of called genotypes for the j^{th} island fox. Note that the summation over j includes the seven island foxes.

We then tested whether the alternative model fit the data significantly better than the null model using a likelihood ratio test (LRT). Specifically, $\Lambda = -2(l(p | \mathbf{x}, \mathbf{g}) - l(p_i, p_g | \mathbf{x}, \mathbf{g}))$. Asymptotically, Λ is χ^2 distributed with one degree of freedom, allowing calculation of P -values. We applied this LRT approach for all different functional categories of variants (Table 1-2).

Test for elevated proportion of derived alleles in island foxes

We tested whether the proportion of derived alleles per individual differed between the gray fox and the island foxes using a LRT similar to that described above. However, here p denotes the proportion of derived alleles per individual at the g alleles, and x denotes the number of derived alleles.

We found that $p_i > p_g$ for all functional comparisons ($P < 2 \times 10^{-5}$, Table 1-2). For the putatively neutral SNPs, this result is unexpected, as the number of neutral derived alleles per individual is expected to be the same between populations. However, we reasoned that this difference could be due to under calling heterozygous genotypes. Because more of the derived alleles in the gray fox are carried in the heterozygous state than alleles in the island fox are carried in the heterozygous state, under calling heterozygous sites will lead to $p_i > p_g$, even if the rate of under calling is the same in both populations. A similar effect was noted recently for comparisons of dogs and wolves (Marsden et al. 2016).

To account for this effect, we considered a second null model where instead of constraining $p_i = p_g$, we constrained $p_i = R p_g$, where R is the ratio of p_i to p_g inferred from synonymous sites. Specifically, the log-likelihood function for our second null model is

$$l(p_i | \mathbf{x}, \mathbf{g}) = x_g \log(p_g) + (g_g - x_g) \log(1 - p_g) + \sum_{j=1}^7 x_j \log(R p_g) + (g_j - x_j) \log(1 - R p_g)$$

where x_g is the number of derived alleles in the gray fox, g_g the total number of alleles in the gray fox, x_j is the number of derived alleles in island fox j , and g_j is the total number of alleles in island fox j .

We then compared this second null model to the alternative model using a LRT, for the loss-of-function, deleterious, and tolerated variants. This test assesses whether the increase in p_i over p_g is significantly larger for the other category of SNPs than predicted based upon the synonymous SNPs. This approach conservatively accounts for under calling heterozygous genotypes and other possible biases in the data.

Heterozygosity peak analysis

For each individual, heterozygosity was calculated as the fraction of heterozygous genotypes of all those passing filters in 100 kb windows with a 10 kb step size. Heterozygosity “peaks” were identified as windows with heterozygosity in excess of two standard deviations above the mean, calculated per genome (Figure 1-2). The coordinates of these windows were extracted, and overlapping windows were merged using *bedtools 2.21.0* (Quinlan and Hall 2010). We observed that the ends of chromosomes typically had high heterozygosity, which likely reflects highly repetitive regions common to telomeric regions making short read alignment challenging. We therefore removed peaks that overlapped the first or last 5% of chromosomes. The finding that the gray fox does not possess the greatest number of peaks, despite having the highest heterozygosity, is somewhat counterintuitive. However, the threshold for windows constituting a peak in the gray fox (heterozygosity mean + 2 standard deviations, within a given individual) is much higher than for any island fox population (see peak threshold dotted lines in Figure 1-2). Furthermore, peaks vary in width, as they are often made up of

multiple overlapping 100 kb windows that exceed the threshold. A greater number of peaks does not, therefore, indicate a higher overall level of heterozygosity. The number, magnitude, and width of the peaks is not directly evaluated as evidence for or against balancing selection, with the exception of San Nicolas, for which we show through simulations that the observed number of peaks is expected under neutrality with our demographic model.

Heterozygosity peak regions were tested for enrichment of genic content by tallying the number of bases within them that overlapped with genes, and testing whether this proportion was significantly higher than expected by chance. Specifically, we resampled random regions from the genome equal in size and magnitude to the observed peaks 1,000 times using *bedtools*, excluding the ends of chromosomes, and calculating the proportion of genic content in these random sets. The *P*-value was calculated as the rank of the observed proportion of genic content within this null distribution.

Gene ontology (GO) analysis was performed on the coordinates of peaks using *gProfileR* (Reimand et al. 2007) with the Ensembl *C. familiaris* annotation (release 79). Gene lists were tested for significant enrichment of GO terms (FDR <0.05) using the *g:SCS* correction method, which simultaneously corrects for multiple-testing and takes non-independence of GO terms into account (Table 1-3). To assess whether the enrichment of olfactory receptors within peaks could occur by chance due to their overrepresentation within the genome, we permuted the coordinates of the peaks 1,000 times as described above, and performed GO analysis with these random sets. We focused on the “olfactory transduction” term (*KEGG:04740*), and found that the term appears 1, 33, and 0 times with a smaller *P*-value than observed in San Nicolas fox 1, San Nicolas fox 2, and gray fox peaks, respectively, yielding *P*-values of 10^{-3} , 0.033, and $<10^{-3}$.

Genetic distance and phylogeny

Previous studies using limited markers have found that genetic relationships between island fox populations appears to be largely driven by geography (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Hofman et al. 2015). We built a distance-based phylogeny and conducted principal components analysis (PCA) to examine the relationships between our sampled individuals. Calculation of pairwise divergence with the metric in Gronau et al. (Gronau et al. 2011) was used to construct a distance matrix from 1,357,951 single nucleotide polymorphisms (SNPs) with no missing genotypes. Bootstrap replicates were obtained by resampling 500 kb regions of the genome with replacement. Distance matrices from the replicates were used to construct a consensus neighbor-joining tree (Figure 1-1B) with the *neighbor* program of *PHYLIP* (Felsenstein 1989). Genetic distance between each island fox and the gray fox was also calculated in this manner (Figure 1-4). PCA was used to quantify and visualize the genetic covariance amongst individuals (Figure 1-4). SNPs passing all filters were pruned for linkage disequilibrium in *PLINK* (Purcell et al. 2007) using `--indep-pairwise 50 5 0.2`. PCA was conducted on the 86,994 remaining SNPs with *flashPCA* (Abraham and Inouye 2014). Both methods show the expected relationships amongst the islands, with no evidence that any particular island possesses closer ties with the mainland gray fox used in this study. Furthermore, the southern islands appear approximately equally diverged from the northern islands and from one another, implying that any possible history of gene flow between the northern and southern islands has not greatly impacted diversity within island fox genomes.

Demographic inference

We used approximate Bayesian computation (ABC) (Tavaré et al. 1997; Pritchard et al. 1999) to infer the demographic history of the San Nicolas, San Miguel, and mainland gray fox populations. Demographic models and prior distributions of parameters for the island fox histories were based on the literature (see *Demographic Models* below). Very little is known about the history of the mainland population, so we used the *Pairwise Sequentially Markovian Coalescent (PSMC)* (Li and Durbin 2011) to gain insight for constructing a plausible model and prior distributions for the gray fox (Figure 1-5). *PSMC* input was generated and bootstrapping performed following the developer’s recommendations. We found that 25 iterations of the program with the parameters `-t10 -r5 -p “64*1”` yielded reasonable results, as there were more than 10 recombination events inferred in each of the 64 time intervals. To match our simulations, we assumed a mutation rate of 2.0×10^{-8} /site/generation and a generation time of one year for scaling the effective population size and time axes.

We created demographic models for the San Nicolas, San Miguel, and mainland gray fox populations, drawing on previous estimates regarding the timing and severity of bottlenecks (see *Demographic models* below) and our *PSMC* results. To perform the inference of the demographic parameters, we used data from a set of 13,646 1 kb putatively neutral regions (Freedman et al. 2014). Neutral sequences from San Nicolas 2 were used for inference. The ABC approach takes the following steps to infer demographic history from neutral sequence heterozygosity:

- 1) Out of the 13,646 1 kb neutral regions in the data, we use the subset of X neutral regions containing >200 bp of called sites passing all filters. We keep a vector L with the number of called sites in each of the X neutral regions retained.

- 2) We count the number of heterozygous sites in each of the X neutral regions and create a vector E , where each element E_i of the vector contains the number of regions with i heterozygous sites. The vector E ranges from $i = 0$ to $i = 15$ in the San Nicolas fox, and from $i = 0$ to $i = 30$ in the San Miguel and mainland fox. We let the upper bound of i take a higher value in these latter two populations because their neutral regions contain a higher number of heterozygous sites.
- 3) We draw one parameter value from each of the prior distributions of demographic parameters (Figure 1-5). Parameter values were rounded to the nearest integer.
- 4) We use *ms* (Hudson 2002) to simulate X independent neutral regions with lengths L based on the demographic model and the parameter values sampled from the prior distributions. A mutation rate of 2×10^{-8} /bp is used. A different recombination rate for each independent neutral region is drawn from the distribution of the mean recombination rate per base pair in non-overlapping windows of 1 Mb, developed from the dog genome recombination map (Wong et al. 2010). The distribution of the recombination rates per base pair is $\text{gamma}(0.1, 0.0000001)$, with a mean of 1×10^{-8} and a standard deviation of 3.16×10^{-8} .
- 5) We count the number of heterozygous sites in each of the X simulated regions and create a vector S , where each element S_i has the number of simulated regions with i heterozygous sites.
- 6) We assessed the goodness of fit between the empirical data (E) and the simulated data (S) using the following distance metric:

$$\alpha = \sum_{i=0}^M |E_i - S_i|$$

The M value is set to be higher than the maximum number of heterozygous sites found in any neutral region in each analyzed genome to allow for values of S_i with a higher number of heterozygous sites than what is observed empirically. The San Nicolas, San Miguel and mainland gray fox had a maximum of 4, 12, and 13 heterozygous sites in a given window, respectively. Consequently, M was set to be 15, 30 and 30 for the three populations.

- 7) We repeat steps 2-6 until 100,000 simulations have been generated.
- 8) Finally, we keep the 100 simulations with the smallest values of α . The values of the demographic parameters in those 100 simulations with the best fit define the posterior distributions (Figure 1-5).

Demographic models

Pictorial depictions of the demographic models and their respective prior and posterior distributions are given in Figures 1-5B and 1-5C. The two island fox demographic models start with a change in effective population size consistent with Aguilar et al. (2004), who assumed, based on archaeological records, that the island foxes potentially first colonized the northern Channel Islands about 8,000 generations ago, in agreement with recent analysis of complete mitochondrial sequence data that suggests island foxes diverged from gray foxes ~7,100-9,200 years ago (Hofman et al. 2015). The size of the ancestral mainland population was set to 20,000, which is a rough estimate of the mainland effective population size (see *Inferred demographic histories* below).

Definitive colonization histories for the island fox populations have not been well established. Many details are not known with certainty, such as population founding times, or

whether the islands were colonized independently, or through serial founder events. We incorporated these uncertainties by including two epochs, demarcated at time T , with wide ranges for all three parameters (N_e after 8,000 generations ago, T , and N_e after time T). Thus, the model allows for multiple changes in population size, to reflect the potential dynamic history of the islands, permitting one or more bottlenecks. Finally, the San Nicolas model incorporates an additional recent bottleneck occurring between 30-28 generations ago, consistent with reports of a population crash during the early 1970s (Laughrin 1980).

We did not incorporate migration between populations into the model for several reasons. Our phylogenetic tree shows deep splits between all island populations, as well as reciprocal monophyly between northern and southern islands. PCA indicates the expected relationships between individuals based on geographic distance. Furthermore, there is no indication of recent ancestry from the mainland, and population genetic studies have not shown strong signals of migration (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Hofman et al. 2015). Finally, migration would lead to higher levels of diversity, which would not be consistent with our observations of extremely low heterozygosity in the San Nicolas and San Miguel populations.

The mainland population is under-studied, and therefore little information is available regarding its history. As described above, we used *PSMC* to gain a general idea of the demographic history of the gray fox (Figure S1-5A). The mainland fox model includes three epochs with varying population sizes. We kept broad prior distributions on the two most recent epochs to allow the data to inform us whether the population has undergone expansion or contraction.

Inferred demographic histories

Characteristics of the inferred posterior distributions are given in Figure 1-5C. The prior and posterior distribution plots were made by creating gaussian kernel density estimates with the density function in *R* (R Core Team 2014). We utilize the mode of the posterior distributions created with the Gaussian kernel density estimates as a point estimate of each demographic parameter.

In the inferences made for the San Nicolas demographic model, the similarity between the prior and the posterior distribution of the parameters Ne_1 and Ne_4 indicates that the data are not informative of these parameters. However, the posterior distribution of the effective population size following the putative colonization bottleneck, Ne_3 , is narrow, with a mode of 64 individuals. We infer that this took place 543 generations ago. The low effective population size inferred during most of the last 543 generations is consistent with the low heterozygosity values found in the San Nicolas genome.

In the San Miguel demographic model, the data are informative for the parameters Ne_1 and T . We estimate that the recent population size in San Miguel, given by the parameter Ne_1 , is equal to 133. The population of San Miguel reached this effective population size after a contraction event that took place 690 generations ago. Observed heterozygosity in San Miguel is higher than in San Nicolas, consistent with the recent higher effective population size inferred in the model.

The inferences of population size changes in the mainland gray fox were consistent with a historic decrease in effective population size. We estimated that the mainland fox had an ancient population size Ne_3 equal to 19,930 individuals. The inferred effective population size in the present day, Ne_1 , is 5,185. However, 5 out of 100 values from the posterior distribution of

N_{e1} were larger than 15,000 individuals. In these five replicates, the population size in the preceding epoch (N_{e2}), was less than 9,000. This result indicates that these cases are also consistent with a history of declining effective population size, although that decrease takes place in a different point in the demographic model. The higher heterozygosity values in the mainland fox relative to the San Miguel and the San Nicolas populations is also reflected in the higher average effective population sizes inferred in the mainland fox. Although we used the *PSMC* results to inform our model construction and ABC inference for the mainland fox, we allowed the data to inform these parameters by using prior distributions that permitted either population expansion or contraction. Using the ABC method, we infer a population contraction that is broadly consistent with the *PSMC* results.

Forward simulations using the inferred demographic models

We used forward-in-time simulations under the Poisson Random Field (Sawyer and Hartl 1992) model to determine values of expected heterozygosity at neutral, zero-fold and four-fold degenerate sites with the inferred demographic models described previously. We employed a modified version of the forward simulator from Lohmueller (2014). Under this model, the number of mutations that take place each generation i follows a Poisson distribution with a mean equal to $2N_i\mu L$, where N_i is the effective population size in generation i , μ is the mutation rate, and L is the number of independent sites simulated. Each mutation changes its frequency each generation according to the Wright-Fisher model with selection, where the allele frequency follows a binomial distribution $Bin(N_{i+1}, p')$, such that

$$p' = \frac{(1-s)p^2 + (1-hs)pq}{q^2 + (1-hs)2pq + (1-s)p^2}$$

where p is equal to the derived allele frequency in generation N_i , q is the frequency of the ancestral allele, s is the selective coefficient of the allele, and h is the dominance coefficient.

To compute the values of heterozygosity, we took all the mutations that were segregating in the most recent generation and sampled genotypes at each site using a multinomial distribution, where the probability of sampling a heterozygous genotype is equal to $2pq$. The value of heterozygosity for each simulation is computed by dividing the number of heterozygous genotypes sampled by the number of independent sites simulated, L .

We performed 100 simulations of neutral, zero-fold and four-fold sites for each of the three demographic models. In this analysis, we sampled 100 demographic parameters from their joint posterior distributions. For each of those 100 joint samples from the posterior distribution, we simulated $L = 10$ million independent neutral, zero-fold and four-fold sites using the same sampled demographic parameter values. The value of h was set to 0.5 in all simulations. The value of s was set to 0 in the neutral and four-fold sites. For zero-fold sites, we used the distribution of fitness effects inferred from human genetic variation data (Boyko et al. 2008), with appropriate re-scaling to account for the different population sizes in foxes versus humans. The mutation rates were set to 2.000×10^{-8} for the neutral sites, 2.468×10^{-8} for the four-fold sites and 2.059×10^{-8} for the zero-fold sites, as in Marsden et al. (2016).

The simulated values of heterozygosity at neutral sites and the ratio of zero-fold heterozygosity over four-fold heterozygosity match the observed data (Figure 1-6B). This suggests that the distribution of fitness coefficients at zero-fold sites, combined with our estimates of demography, can generate the negative relationship between the zero-fold/four-fold heterozygosity ratio and neutral heterozygosity. This finding suggests that our models, in conjunction with selection, can accurately recapitulate the observed patterns of variation within

these three individuals with different histories. Furthermore, it highlights the impact of the recent severe bottleneck in the San Nicolas population, which had a history generally similar to that of San Miguel prior to this event.

The results from the forward simulations are not used to support evidence of inbreeding depression, but rather to show the accumulation of weakly to moderately deleterious variants as a result of weakened selection/extreme drift through examining patterns of heterozygosity. We use a dominance coefficient of 0.5 in the simulations to model the accumulation of deleterious variants because this produced a good fit between our simulated and empirical results, which is expected, given that the distribution is specifically for heterozygote fitnesses (Boyko et al. 2008). Further, evidence suggests that many weakly deleterious alleles segregating in individual genomes are likely to have additive rather than recessive effects (Henn et al. 2015, 2016; Marsden et al. 2016).

Coalescent simulations to assess the distribution of peaks in the San Nicolas genome

As seen in Figure 1-3A, heterozygosity in the San Nicolas genome is very low along most of the genome, but there are a number of distinct regions with elevated heterozygosity. We identified peaks of heterozygosity within the empirical data through calculating heterozygosity in 100 kb windows with a 10 kb step size, and then extracting the coordinates of windows with heterozygosity greater than two standard deviations above the mean. 48 peaks were found in the San Nicolas 2 genome. We performed a simulation-based analysis to determine if this number of peaks is consistent with what would be expected under neutrality using our inferred demographic model of San Nicolas.

Specifically, we simulated 1,000 diploid genomes using the coalescent simulator *MaCS* (Chen et al. 2009). We sampled 1,000 independent values from the joint posterior distribution of the 4 demographic parameters present in the San Nicolas demographic model. Each genome contained 213 pairs of chromosomes of 10 Mb of sequence. The recombination rate per base pair of each simulated chromosome was randomly sampled from the recombination rate distribution described above (Wong et al. 2010), and was defined as constant along the chromosome. Then, we counted the number of high heterozygosity peaks in each simulated genome using the following procedure:

- 1) We sampled the first 19,360 windows of 100 kb with 10 kb step size from the 213 simulated chromosomes, mimicking the number of windows present in the San Nicolas data.
- 2) Since there is variation in the empirical number of genotype calls in each window of 100 kb, we randomly sampled the number of sites where a SNP could be called, S , from each window, based on the actual distribution of genotype calls on those respective windows along the actual San Nicolas genome, excluding windows where no genotype calls were made.
- 3) Based on the number of sites S sampled from each window, we took the first S simulated sites from each window. To calculate the heterozygosity value in each window, we counted the number of heterozygous sites found in the first S sites in each window and divided that number by S .
- 4) We estimated the mean and the standard deviation of heterozygosity along all windows along the simulated genomes.

- 5) We extracted windows containing heterozygosity in excess of the mean plus two times the standard deviation.
- 6) Overlapping windows were merged using the *merge* tool in *bedtools* (Quinlan and Hall 2010).

The distribution of the number of heterozygosity peaks from the 1,000 simulated genomes can be seen in Figure 1-3C. In 1,000 simulations, we obtain a higher number of peaks than in the empirical data 451 times. This result demonstrates that the observed peaks of heterozygosity within the San Nicolas genomes may be generated by genetic drift alone, assuming our inferred demographic model.

Estimates of π

Genomic estimates of π include a diverse array of 159 non-clonal eukaryotic species from review publications or species-specific accounts (sources denoted with † in the Bibliography of this chapter). The list includes 485 estimates of π from 137 animal, 11 plant, 8 fungus, and 3 protist species. For cases in which species had multiple estimates of π , the average value was used for constructing the histogram in Figure 1-1C.

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Chapter 2: Purging of deleterious alleles implicated in the absence of inbreeding depression in genetically depauperate island foxes

Abstract

The recovery and persistence of rare and endangered species is often threatened by genetic factors, such as the fixation and accumulation of deleterious mutations, the loss of adaptive potential, and inbreeding depression. Despite these genetic problems, the island fox (*Urocyon littoralis*) of California's Channel Islands has persisted in six small isolated populations for thousands of generations. Previous genomic analyses revealed that these foxes have exceptionally low levels of diversity and an elevated load of deleterious mutations. Nevertheless, they exhibit no obvious signs of inbreeding depression, and populations that recently experienced extreme bottlenecks swiftly recovered to pre-bottleneck levels under human management. Here, we combine morphological and genetic data with population genetic models to suggest that the absence of inbreeding depression in island foxes may be explained by the purging of strongly deleterious recessive mutations in small populations. Our study sheds light on the puzzle of island fox persistence, a unique success story that provides a model for persistence as species decline and become isolated due to increasing habitat loss and fragmentation.

Introduction

Based on theoretical and laboratory-based studies, the deleterious consequences of small population size and inbreeding are well established (Charlesworth and Charlesworth 1987; Lande 1994; Lynch et al. 1995; Frankham 1995; Bijlsma et al. 2000; Reed and Bryant 2000; Kirkpatrick and Jarne 2000). Examples such as the Florida panther (*Puma concolor coryi*), the

Illinois prairie chicken (*Tympanuchus cupido*), the gray wolves of Isle Royale (*Canis lupus*), and others have shown that inbreeding depression is associated with population declines in the wild (Roelke et al. 1993; Westemeier et al. 1998; Rääkkönen et al. 2009). Conversely, examples of species or populations declining to a single breeding pair, and then rebounding, are known. These examples include the golden hamster (*Mesocricetus auratus*), Chatham Island black robin (*Petroica traversi*), and Mauritius kestrel (*Falco punctatus*) (Frankham et al. 2002; Groombridge et al. 2000). Intentional inbreeding has long been used for the improvement of crops and livestock, although the possibility that this has resulted in a so-called “cost of domestication” has recently been demonstrated through population genomic studies showing an increased burden of deleterious mutations in domesticated plants and animals (Lu et al. 2006; Schubert et al. 2014; Renaut and Rieseberg 2015; Marsden et al. 2016). It has been suggested, however, that inbreeding depression may be worse in wild populations (Ralls et al. 1988; Crnokrak and Roff 1999), since captive populations experience a more benign environment, and managers of captive wildlife can intentionally avoid pairing closely related individuals or breeding unhealthy individuals. Thus, the response to inbreeding varies in wild and captive populations, confounding attempts to determine the conditions under which inbreeding depression is likely to occur. Moreover, evolutionary biologists remain challenged to reconcile the link between genetic diversity and genetic health when species vary greatly in genome-wide diversity (Romiguier et al. 2014; Corbett-Detig et al. 2015). The question remains, why do some species or populations appear resistant to the potentially deleterious effects of small population size and inbreeding, while others succumb to reduced fitness and edge closer to extinction?

Here, we investigate a unique system, which intriguingly displays no apparent evidence of inbreeding depression despite harboring exceptionally low levels of genetic diversity, and has

existed at small population sizes for thousands of generations (Collins 1982; Wayne et al. 1991; Coonan et al. 2010; Robinson et al. 2016). The island fox is the dwarfed descendant of the mainland gray fox (*U. cinereoargenteus*) found on six of California's Channel Islands. As a consequence of long-term isolation, each island contains a morphologically and genetically distinct subspecies (Collins 1982; Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Aguilar et al. 2004; Funk et al. 2016; Robinson et al. 2016). Four subspecies have experienced recent catastrophic declines due to the introduction of canine distemper on Santa Catalina (Timm et al. 2009) and predation by non-resident golden eagles (*Aquila chrysaetos*) on the three Northern Islands (San Miguel, Santa Rosa, Santa Cruz) (Roemer and Coonan et al. 2001). It is thought that canine distemper virus was accidentally introduced to Santa Catalina by a stow-away raccoon from the mainland. Golden eagles from the mainland colonized the three Northern Channel Islands and decimated the island fox populations following the decline of resident bald eagles (which typically do not prey on foxes) in the 1960s due to DDT exposure. Population sizes crashed to fewer than 100 individuals on each of these islands before they were protected under the Endangered Species Act (ESA), and captive breeding programs were initiated (USFWS 2004; Coonan et al. 2010). There is anecdotal evidence that the other two populations, San Clemente and San Nicolas, may also have experienced population bottlenecks during the 20th century due to unknown causes, though these populations were never listed as endangered (Laughrin 1980; Roemer and Coonan et al. 2001).

Even prior to these recent declines, the populations persisted at remarkably low population sizes (Wayne et al. 1991; Robinson et al. 2016). On San Nicolas Island, the smallest island, the population is nearly monomorphic across its entire genome, making its persistence on the island an enigma (Wayne et al. 1991; Funk et al. 2016; Robinson et al. 2016). In fact, the

rebound and subsequent de-listing of the four listed island fox populations is now the fastest recovery of any mammal under the ESA (USFWS 2016). In more than a decade of active management, no signs of inbreeding depression have been documented in island foxes (Coonan et al. 2010). This finding is in stark contrast with other endangered species that languished as a result of inbreeding depression, and sometimes required genetic rescue to recover (Johnson et al. 2010; Westemeier et al. 1998). The persistence and rapid recovery of the island foxes, particularly those on San Nicolas Island, therefore presents an enigma. Some possible explanations include: 1) purging of deleterious alleles on all islands due to long-term small population size; 2) undocumented inbreeding depression in San Nicolas following a recent, poorly documented population bottleneck; or 3) a relatively benign island habitat allowing long-term persistence despite reduced fitness in island foxes relative to mainland foxes. The possibility of undocumented fox migration between islands is unlikely, since genetic studies have consistently found that island foxes cluster according to population of origin, and show strong divergence between populations (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Aguilar et al. 2004; Funk et al. 2016; Robinson et al. 2016).

In this study, we first investigate the observation that island foxes do not appear to suffer from canonical signs of inbreeding depression. Specifically, a lack of visible anomalies does not preclude the presence of skeletal deformities that may signify inbreeding depression. A high frequency of deformities in the population would support the classic inbreeding depression scenario, and add justification for considering genetic rescue or assisted migration as part of future management efforts (Vellanoweth 1998; Levy 2010; Funk et al. 2016). Previous studies of inbred carnivoran skeletons have found a suite of pathologies linked to low genetic diversity. These defects often manifest in the skull and vertebral column, and include lumbosacral

transitional vertebrae (LSTV; malformed final lumbar vertebra with characteristics of a sacral vertebra) in domestic dogs and gray wolves, kinked tail vertebrae in Florida panthers, and dental anomalies in cheetahs (*Acinonyx jubatus*) (Morgan 1999; Rääkkönen et al. 2006, 2009; Roelke et al. 1993; Marker and Dickman 2004). Conversely, skeletal variations in the island foxes may be present and benign. Collins (1982) found skeletal variation between the islands with no fitness consequences, such as changes in the form of bregmatic bones and the number of caudal vertebrae, consistent with a high degree of neutral divergence between island fox populations due to genetic drift. This is the first study to investigate island fox skeletal morphology explicitly for canonical signs of inbreeding depression. Second, we address the impact, if any, of recent population bottlenecks on patterns of neutral and deleterious variation in island foxes, and if the genomic flatlining observed in the current population of San Nicolas pre-dates the purported bottleneck in the 1970s (Robinson et al. 2016). A critical issue is whether recent events have dramatically reduced variation, or whether it is a long-standing characteristic of the populations, pre-dating the arrival of Europeans.

Third, previous genomic analyses of individuals sampled in 1988 revealed that island foxes carry an increased load of putatively deleterious mutations, consistent with theoretical predictions that in small, isolated populations, deleterious mutations have an increased probability of fixation due to drift (Robinson et al. 2016; Kimura 1963). Alternatively, decreased population size and inbreeding may enhance the removal of strongly deleterious recessive alleles, as they are more frequently exposed to selection through increased homozygosity (Nei 1968; Hedrick 1994; Wang et al. 1999; Wang 2000; Glémin 2003). Inbreeding often results in the manifestation of Mendelian disorders through the inheritance of both copies of a recessive strongly deleterious allele present as a heterozygote in a common ancestor (Laikre and Ryman

1991; Ralls et al. 2000; Woods et al. 2006; Chong et al. 2012; Gao et al. 2015). These detrimental alleles can persist in large populations because they are immune to selection as long as they remain rare and are carried as heterozygotes. Although island foxes have previously been found to have higher levels of deleterious variation relative to gray foxes, much of this variation could be weakly deleterious, still allowing the possibility that strongly deleterious recessive alleles have been purged from the island populations (Robinson et al. 2016). We compared island fox genomes from 1988 and after 2000 to evaluate the impacts of recent short-term bottlenecks versus long-term small population size on levels of neutral and non-neutral variation.

Consequently, to address the likelihood of purging and the observed lack of inbreeding depression in island foxes, we conducted simulations under a variety of demographic models designed to assess the impacts of long-term and short-term demographic features on levels of deleterious variation. The history of island foxes remains somewhat unknown, but based on archaeological evidence it is thought that foxes first arrived on the Northern Channel Islands within the last 10,000 years, and that the Southern Channel Islands were colonized later (Collins 1991a; Vellanoweth 1998; Rick et al 2009; Hofman et al. 2015). About ~10,000 years before present (BP) the Northern Channel Islands constituted a single large landmass, Santarosae, that was separated from the mainland by a narrow channel less than 10km wide (Porcasi et al. 1999; Johnson 1978). If the colonization occurred at this time, then the effective size of the population may have increased beyond that which is observed today on each of the three separate islands. Moreover, although there is little evidence of gene flow today (Wayne et al. 1991; Goldstein et al. 1999; Hofman et al. 2015; Funk et al. 2016; Robinson et al. 2016), given the Northern islands were less well separated ~10,000 years ago, over-water dispersal may have allowed for higher levels of variation in island foxes early in their colonization history. Nonetheless, no

archaeological fox specimens pre-dating 7,160 years BP have been found on the islands (Wenner and Johnson 1980; Hofman et al. 2015). However, these early specimens are dwarfed, suggesting either that the foxes became dwarfed very rapidly after colonizing the islands, leaving little to no trace in the archaeological record, or that older larger fox remains from transitional individuals exist and simply have not been recovered (Rick et al. 2009; Hofman et al. 2015). A competing hypothesis states that humans, whose presence on the islands goes back at least 13,000 years, intentionally introduced foxes to the Northern Channel Islands, perhaps as semi-domesticated pets or for pest control (Collins 1991b; Johnson et al. 2002; Rick et al. 2009). In either case, foxes were almost certainly brought to the Southern Channel Islands by humans, as these islands are situated much further from the shore (32-98 km) and were never connected to each other or to the mainland (Johnson 1983; Collins 1991a, b). We explored the effects of small population size, bottlenecks, and inbreeding through simulations under models designed to mimic possible demographic factors that may have influenced variation within the island fox genomes.

In light of a recent severe drought and the loss of 41% of the San Nicolas fox population, the mechanism of persistence in this population should be a key consideration in future conservation action (Meyer 2016). Our results show a surprising lack of canonical indicators of inbreeding depression, which our simulation models suggest may be due to purging of the most deleterious variants, although the overall burden of deleterious variants in island genomes is higher than in mainland gray foxes. Consequently, island foxes provide a new paradigm for the long-term persistence and conservation of small isolated populations.

Methods

Morphological assay for inbreeding depression

Skulls (n=189) and complete postcrania (n=164) of adult *U. littoralis* and *U. cinereoargenteus* were placed in anatomical position and surveyed for skeletal abnormalities including irregular development, healed fracture, and bony growths (from infection, muscle traction or other causes). Vertebrae were identified and counted with the exception of distal caudal vertebrae, which were missing from many specimens, and also variable between islands (Collins 1982). We counted developmental pathologies, such as fused vertebrae, extra vertebrae, transitional vertebrae, and maloccluded teeth towards as “congenital.” We binned traumatic pathologies into a broad category of “trauma” and a more conservative category of trauma resulting from collision with a vehicle. Injury pathologies were broadly defined to include fracture, infection, osteoarthritis, osteophytes and evidence of abnormal muscle use (e.g. traction, roughened insertion). Only healed injuries were recorded, thus all foxes obtained as traffic fatalities were counted only if they had previously survived a collision. As such, our frequency of vehicle collision rates is an underestimate. Human impacts differ dramatically between the Northern and Southern Channel Islands; the Northern islands are undeveloped and constitute the Channel Islands National Park, but two of the Southern islands are Navy-owned (San Clemente and San Nicolas), and Santa Catalina is a popular tourist destination. We therefore expected differences in the rates of traumatic injury and vehicle collisions between islands. To identify probable vehicle collisions, we expanded the diagnosis of Harris (1977) in which multiple bones from the same side of the body were fractured, particularly in the hindlimb and ribs. In our analyses, evidence of muscular strain on the same side as multiple fractured elements were included as evidence of trauma to that side of the body, since we surveyed animals that survived collisions but Harris necropsied fresh traffic fatalities. Although many of the foxes examined by Harris had injuries to the tail, fractured caudal vertebrae alone were not counted toward multiple

fractures. Fisher's exact test was used to test for significant differences between the rates of congenital defects in island and gray foxes, and for differences between the prevalence of congenital and traumatic pathologies. A bootstrapped relative risk calculation was used to assess significant differences in the rates of traumatic injuries between populations.

Whole genome sequencing and analysis of neutral and non-neutral variation

We generated whole genome sequences of representative island foxes sampled in 2000-2009 from each population. Additionally, we sequenced DNA isolated from bone fragments from a San Nicolas island fox specimen (#15477, Donald R. Dickey Collection, University of California, Los Angeles) collected in 1929, as well as DNA from a gray fox from Golden Gate National Recreation Area in Northern California (Riley et al. 2004). Finally, we generated new higher quality sequence data from a 1988 Santa Rosa island fox, as the Santa Rosa genome from Robinson et al. (2016) was of relatively low quality compared to the other sequences. We combined the newly generated sequences with those of Robinson et al. (2016), yielding a total of 16 genomes at 13-23X coverage. Read alignment and processing followed the methods outlined in Robinson et al. (2016). Briefly, reads were aligned against the domestic dog reference genome, canFam3.1, followed by removal of duplicate and low quality reads, base quality score recalibration, joint genotyping with FreeBayes, and finally genotype filtering (McKenna et al. 2010; Garrison and Marth 2012). Further details about samples and sequencing are given in Appendix 2-I: Table S2-1.

We evaluated the genetic distance between individuals with principal components analysis and a maximum-likelihood phylogenetic tree, based on a set of 12,249 single nucleotide polymorphisms with no missing data, pruned for linkage disequilibrium (maximum=0.2), and

with a minimum minor allele frequency of 0.1. The PCA was conducted with SNPRelate (Zheng et al. 2012). The tree was generated in SNPhylo from 1,000 bootstrap replicates (Lee et al. 2014). We assessed genetic diversity by calculating heterozygosity, here defined as the number of heterozygous genotypes divided by the number of called sites within an individual. Heterozygosity was calculated for the entire autosomal genome and in 100 kb sliding windows with a 10 kb step size. Peaks of heterozygosity within a genome were defined as windows with heterozygosity greater than two standard deviations above the mean of the genome-wide distribution of per-window heterozygosity. Peaks were assessed for putative functional relevance by conducting a gene ontology enrichment analysis in gProfileR (Reimand et al. 2011). Levels of deleterious variation were evaluated by calculating the proportion of derived alleles per genome at synonymous and non-synonymous sites in coding regions. Coordinates of zero-fold degenerate sites (all mutations are amino acid changing) and four-fold degenerate sites (all mutations result in the same amino acid) were obtained from Marsden et al. (2016). Variant annotation was conducted with Ensembl's Variant Effect Predictor (VEP; McLaren et al. 2010) and the Sorting Intolerant From Tolerant (SIFT; Kumar et al. 2009) tool. SIFT classifies non-synonymous mutations at a site as likely to be deleterious or tolerated on the basis of amino acid conservation across taxa.

Assessing patterns of neutral and deleterious variation through simulation

We simulated neutral and deleterious variation under six different demographic models, each involving the establishment of a small island population (N=1,000) from a large mainland population (N=10,000) to evaluate the possibility of purging in island populations (Figure 2-1) Simulations were conducted with SLiM (Haller and Messer 2016). In the most basic model (Split

Model), an island population of 1,000 individuals is established through sampling with replacement of 1,000 individuals from the mainland population, such that the mainland population is unaffected by the formation of the island population. Island fox population census sizes range from a few hundred to just under 2,000 individuals (Wayne et al. 1991). This population remained at constant size for 10,000 generations. Recent estimates suggest the initial colonization of the islands by foxes at ~9,200 years BP, and we assume a one-year generation time (Hofman et al. 2015). The purpose of this model is to compare a small population against a larger one, without any additional complexities (e.g. inbreeding). The other five models are variations on this basic model, reflecting plausible elements of island fox history, specifically: 1) an ancient bottleneck to simulate a small founding population; 2) a recent bottleneck 30 generations ago, such as may have occurred in San Nicolas; 3) a serial bottleneck (at 10,000 and 2,000 generations ago) to mimic the possibility of serial colonization of the Northern and then the Southern Channel Islands; 4) a model in which the island population is initially large ($N=2,000$ for ~2,000 generations), as may have occurred if the foxes first colonized Santarosae or initially experienced high rates of migration among the Northern Channel Islands; and 5) a model with recent strong inbreeding, where individuals are twice as likely to mate with close relatives than non-kin. All bottlenecks consist of a starting population of ten individuals that doubles in size each generation until there are 1,000 individuals (seven generations).

Each simulated individual consists of a diploid 10 Mb genome, consisting of 2,000 “genes” carried on 38 chromosomes proportional to chromosome lengths in the dog genome. Each gene was represented by a contiguous 1 kb sequence that accumulated mutations at a rate of 1×10^{-8} per base pair (bp) per generation. 30% of these mutations were selectively neutral, and the remaining 70% were deleterious, with selection coefficients drawn from a gamma

distribution of fitness effects (Kim et al. 2017). Recombination was incorporated by including a single base pair in between each gene that did not accumulate mutations, but where crossovers occurred at a rate of 1×10^{-3} per site per generation. This was done to mimic intergenic regions 100 kb in length with a recombination rate of 1×10^{-8} per site per generation without simulating extraneous non-coding sequence. Each model was run for 10,000 generations following a 100,000-generation burn-in period. The average number of alleles carried by each individual was calculated for deleterious ($s < 0$) and neutral mutations ($s = 0$). We grouped deleterious mutations arbitrarily as strongly ($-1 \leq s < -0.01$), moderately ($-0.01 \leq s < -0.001$), and weakly deleterious ($-0.001 \leq s < 0$). To explore the dynamics under two different dominance regimes, we performed one set of simulations with recessive mutations ($h = 0$) and one set of simulations with additive mutations ($h = 0.5$). Twenty replicates were performed for each dominance value under each of the six models.

Results

Congenital defects are rare island and gray foxes

Congenital deformities were present but rare in all island fox subspecies ($n = 119$). Observed developmental pathologies included extra lumbar vertebrae, LSTV, and malocclusion from a shortened mandible (Figure 2-2, Appendix 2-I: Table S2-2). Among 43 San Nicolas specimens collected between 1929 and 2013, only one congenital defect was observed: a single specimen from 1984 possessed maloccluded incisors. For comparison, 58% of Isle Royale wolves exhibited a vertebral anomaly within 10 generations of the population's founding (Räikkönen et al. 2009). However, traumatic pathologies indicating prior injuries (primarily from suspected vehicular collisions) were common on all islands, particularly on San Nicolas, as

evidenced by specimens with fractured ribs, fractured radius and ulna, fractured metapodials, fractured caudal vertebrae and indication of infection (Figure 2-2B). Among 45 gray fox specimens, few pathologies were observed. Within this sample, congenital defects included one bilaterally symmetrical LSTV, one sacral-caudal transitional vertebra, and one case of achondroplastic dwarfism. In contrast to island foxes, traumatic pathologies were uncommon. One gray fox had a displaced fractured mandible and subsequent perforation of the palate by a molar, and two had severed forelegs. Given the location and evidence of subsequent infection, the latter two may have survived capture in a leg hold trap. Healed fractured ribs and fractured limbs consistent with vehicular impact were not observed in mainland grey foxes.

There was no significant difference ($p=0.6633$) in the proportion of congenital defects between the populations of the Channel Islands and the mainland gray fox (Figure 2-2A). There was a significant difference between the islands in the proportion of probable collision injuries ($p=0.0039$) (Figure 2-2B). A bootstrapped relative risk calculation of pathology was used to assess significant differences in collision injuries between populations. The relative risk of probable vehicle collisions showed San Nicolas, San Clemente and Santa Catalina foxes to be at significantly higher risk than those of San Miguel. No probable collisions were recorded in the Santa Cruz, Santa Rosa or mainland gray fox samples.

Minimal impact of recent demography on island fox genomes

Phylogenetic relationships among individuals showed that island fox genomes cluster by population of origin, and Southern and Northern island populations define distinct clusters, consistent with prior studies (Figure 2-3) (Gilbert et al. 1990; Wayne et al. 1991; Goldstein et al. 1999; Aguilar et al. 2004; Funk et al. 2016; Robinson et al. 2016). Interestingly,

we found no evidence that the Northern California gray fox is closer to the island foxes based on the set of pruned SNPs, but a PCA generated from SNPs not pruned for linkage disequilibrium (Appendix 2-II: Figure S2-1) showed that there is a closer affinity between island foxes and the Northern California gray fox. Based on mitochondrial genome sequences, Hofman et al. (2015) found that island fox haplotypes clustered within the clade formed by Northern California gray fox haplotypes, which the authors attributed to possible northward post-glacial range shifts among California gray fox populations after the colonization of the Channel Islands, a finding later supported by Goddard et al. (2015).

Overall, we did not find significant changes in genomic heterozygosity between island foxes sampled from the same population across a population bottleneck. Island fox genomes were little changed between 1988 and after the year 2000 (Figure 2-3B, C). Among the four populations that crashed in the 1990s, two genomes showed slightly higher autosomal heterozygosity than in 1988 (Santa Catalina, Santa Cruz), whereas two showed slightly lower heterozygosity (San Miguel, Santa Rosa). Similarly, in San Nicolas and San Clemente, genome-wide heterozygosity was unchanged since 1988. These minor differences before and after bottlenecks are not statistically significant (Wilcoxon signed rank test, $p=1$), and may be expected due to small inter-individual differences in heterozygosity, such as between the two 1988 San Nicolas foxes. Genome-wide heterozygosity differs between islands, but not across time. This likewise holds for the 1929 San Nicolas individual. Robinson et al. (2016) hypothesized that the genomic flatlining of San Nicolas foxes may have resulted, in part, from a severe bottleneck in the 1970s. However, the 1929 genome bears incredibly low diversity (1.33×10^{-5} heterozygotes per bp), on par with the genomes sampled in 1988 and 2000 (mean= 1.35×10^{-5} heterozygotes per bp) (Figures 2-3 and 2-4). The flatlining in the San Nicolas

island fox population is therefore a long-standing phenomenon, underscoring the mystery concerning the persistence of this population with extremely low genetic diversity.

Previous analysis of the remnant peaks of heterozygosity in the San Nicolas genomes found strong enrichment for olfactory function (*KEGG:04740*) (Robinson et al. 2016). We examined the new San Nicolas genomes to assess whether their peaks were also enriched for olfactory receptor genes. Across all four San Nicolas genomes, 56% of the peaks of heterozygosity were shared among two or more individuals, and 44% of peaks were unique to one individual (Figure 2-4). The 1929 individual was the most divergent, with 59% of its peaks not shared with other individuals, suggesting variability in peaks of heterozygosity over time. Nonetheless, we found that peaks in the 1929 and 2000 San Nicolas genomes are strongly enriched for olfactory receptor genes (1929 $p=3.57 \times 10^{-16}$, 2000 $p=3.79 \times 10^{-20}$). Although olfactory receptor genes are the largest gene family within mammals, this does not explain their statistical enrichment, as the analysis controls for the abundance of olfactory receptor genes. As previously reported, peaks of heterozygosity in the Southern California gray fox genome were enriched for olfactory receptor genes ($p=1.43 \times 10^{-16}$). Interestingly, we found this result holds in the Northern California gray fox genome as well ($p=4.98 \times 10^{-3}$). It is likely that olfactory receptor genes are highly variable within gray foxes, as they are in mammals generally, and therefore these hotspots of diversity also have above average variability in island genomes (Buck and Axel 1991; Niimura and Nei 2007). Thus, these genes may be among the last to lose all remnant diversity as drift erodes heterozygosity in the island genomes over time. The high heterozygosity of olfactory receptor genes in the gray fox suggests that, as in other mammals, these genes have an elevated rate of evolution (Rouquier et al. 1998; Nielsen et al. 2005; Niimura and Nei 2007; Alonso et al. 2008). Only one other individual was found with olfactory receptor

enrichment in peaks of heterozygosity: the 2005 Santa Catalina fox ($p=3.64 \times 10^{-25}$). Other island fox genomes were not found to be enriched for olfactory receptor genes, but were enriched for a variety of ontological terms, including cytoskeletal filaments (GO:0005882, GO:0045095) and plasma membrane elements (GO:0005886, GO:0007186, GO:0016021) (Appendix 2-I: Table S2-3). However, no functional terms were as strongly enriched as the olfactory receptor genes.

Levels of non-neutral variation in island foxes are largely similar between genomes sampled at different times from the same population. Comparison of heterozygosity at zero-fold and four-fold degenerate sites showed that island fox genomes have a high ratio of amino acid-changing to non-amino acid-changing mutations relative to the gray fox, along with a reduction in neutral diversity, demonstrating the consequences of enhanced drift relative to selection on the islands (Figure 2-5). Neutral diversity and the ratio of zero-fold to four-fold heterozygosity was essentially unchanged between genomes from 1988 and after 2000. Similarly, all San Nicolas genomes showed a strongly elevated ratio of heterozygosity at zero-fold degenerate sites relative to four-fold degenerate sites, although the small number of heterozygous sites in San Nicolas produces high variance in this statistic, as seen previously (Robinson et al. 2016). We also interrogated annotated variants in coding regions to examine synonymous, deleterious missense, tolerated missense, and loss of function mutations (Figures 2-6 and 2-7). Likelihood ratio tests were used to assess the significance of differences in homozygosity and in the total number of derived alleles between mainland and island foxes (Appendix 2-I: Table S2-4). Across all populations, island foxes had increased homozygosity of derived alleles relative to the gray fox (Figure 2-6). Island fox genomes had 2.15 times the number of homozygous putatively deleterious alleles ($p<10^{-16}$), and 2.02 times the number of homozygous loss of function alleles compared to gray foxes ($p=1.75 \times 10^{-10}$). This increase in

homozygosity would be predicted to drastically reduce fitness if deleterious mutations are recessive. Further, island genomes also contain an excess total number of deleterious alleles per genome relative to mainland gray foxes (Figure 2-7). We calculated that island fox genomes from 1988 have 2.3% more derived deleterious missense alleles per genome and 4.9% more loss of function alleles per genome relative to the gray foxes (deleterious, $p=1.11 \times 10^{-10}$; loss of function, $p=2.43 \times 10^{-3}$). Island fox genomes from after 2000 contained 1.7% more derived deleterious missense alleles per genome ($p=4.68 \times 10^{-9}$) and 3.3% more loss of function alleles per genome relative to the gray foxes, although the difference for loss of function mutations was not significant below a threshold of 0.05 ($p=0.056$). There is evidence that genomes after 2000 have slightly fewer deleterious and loss of function mutations relative to genomes from 1988 (Wilcoxon signed rank test, $p=0.0313$). This result should be interpreted with caution, as it is potentially due to higher error rates in the genomes from 1988, which have lower mean coverage (17.0X) than the genomes sequenced for this study after 2000 (21.4X). Nonetheless, our results clearly show an elevated burden of deleterious variants in the islands, such that even under an additive model, genetic load is predicted to be higher in island foxes relative to the mainland.

Genetic variation in islands versus mainland under simulated models

We hypothesized that the absence of inbreeding depression in island foxes may be attributed to the purging of strongly deleterious recessive mutations in the island populations. To test this hypothesis, we conducted simulations under a battery of demographic models (Figure 2-1), and then counted the number of strongly (Figure 2-8A, B), weakly (Figure 2-8C, D), moderately deleterious (Figure 2-8E, F), and neutral (Figure 2-8G, H) mutations per individual. Results were strikingly consistent across all six variations of the island model, indicating that the

predominant factor driving levels of variation is the long-term small population size on the islands relative to the mainland. As in our empirical data, moderately and weakly deleterious mutations (Figure 2-8C-F) accumulated and had a higher rate of fixation on the islands. Considering additive mutations, island genomes had 2.32 times as many moderately deleterious alleles, and 1.32 times as many weakly deleterious alleles than in the mainland. 77% of moderately deleterious alleles and 92% of weakly deleterious alleles were fixed on the islands, compared with only 1.0% and 6.7% fixed in the mainland population, respectively. Similar results were obtained when all mutations were recessive, except that the genomic burden of recessive moderately deleterious mutations was higher across all populations than when these mutations were additive (Figure 2-8D, F).

Strongly deleterious mutations, however, showed a qualitatively different pattern relative to more weakly deleterious mutations. Unlike weakly and moderately deleterious mutations, the number of strongly deleterious additive mutations did not differ between the mainland and the islands (one-way ANOVA, $p=0.783$), with a mean number of ~ 0.30 mutations per individual (Figure 2-8A). This result is expected, given that these mutations sharply reduce fitness and are immediately exposed to strong selection and likely removed from the population, regardless of population size. Moreover, across all simulations, strongly deleterious recessive mutations were the only mutations that were more abundant in mainland genomes than in island genomes (Figure 2-8B) (one-way ANOVA, $p < 1 \times 10^{-7}$). Mainland genomes contained 61-69% more strongly deleterious recessive mutations (5.00-6.72 mutations per individual) than island genomes (0.82-4.83 mutations per individual). This result was consistent across all island models; pairwise comparisons among the six island models showed no significant differences ($p=0.061-1.00$). There were no simulations in which the number of strongly deleterious recessive

alleles was higher in island genomes than in mainland genomes, consistent with the purging of strongly deleterious recessive mutations, which are more frequently exposed to selection through increased homozygosity in the smaller island populations.

Discussion

We confirmed the lack of canonical signs of inbreeding depression in island foxes, and, through simulations, demonstrated that this lack of inbreeding depression, along with the elevated burden of weakly and moderately deleterious mutations in island fox genomes, may be explained by long-term small population size. Our empirical and simulated results confirmed that weakly and moderately deleterious variants accumulate in small populations due to the effect of strong drift, making selection ineffective. In contrast, strongly deleterious recessive alleles, which can persist in large populations where they are infrequently exposed to selection as heterozygotes, cannot persist in small populations, and would have been rapidly purged as homozygosity increased following the colonization of the islands. Our models predict that island populations may have 61-69% fewer strongly deleterious variants per genome. We note that, although all island subspecies share low levels of congenital skeletal defects, this finding does not preclude the presence of soft tissue traits such as syndactyly and reproductive anomalies occurring in the population (Wildt et al. 1983; Barone et al. 1994; Räikkönen et al. 2009). However, these traits have not previously been reported in island foxes, despite over a decade of intensive management involving several years of captive breeding programs and necropsy of road killed mortality (Coonan et al. 2010; Snow et al. 2011). Thus, we could not link the exceptionally low levels of diversity in island foxes with deficiencies in fitness-related traits,

although the loss of variability due to inbreeding has led to inbreeding depression in other small populations, as discussed above.

Potentially, the purging of strongly deleterious alleles in island foxes may have enabled their long-term persistence and prevented their potential demise during the recent severe bottlenecks. Another isolated canid population in Isle Royale National Park has not been so fortunate. Founded less than a hundred years ago, the population of gray wolves on Isle Royale has succumbed to inbreeding depression and is bound for extinction (Räikkönen et al. 2009; Hedrick et al. 2014). Evidence suggests that purging with persistence is more likely when the rate of inbreeding is slow, such as in a chronically small population, rather than fast, as in a sudden extreme bottleneck (Ehiobu et al. 1989; Latter et al. 1995; Fu et al. 1998; Wang et al. 1999; Reed and Bryant 2001; Day et al. 2003). The Channel Islands support fox populations of several hundred to over a thousand individuals, and may therefore exemplify the slow inbreeding scenario, although strictly speaking, island foxes are not thought to inbreed under normal circumstances (Roemer and Smith et al. 2001). Additionally, the initial founder population could have consisted of many individuals if Native Americans intentionally introduced foxes to the islands. In contrast, Isle Royale is only large enough to support a few dozen wolves, and was likely founded by a single breeding pair from the mainland (Mech 1966, Peterson and Page 1988). Isle Royale wolves may therefore represent the fast inbreeding scenario. The different demographic histories of these two systems may explain their contrasting responses to recent bottlenecks and inbreeding. Large outbred populations, such as the mainland source population for Isle Royale wolves, likely carry a greater hidden load of recessive deleterious mutations, leaving a population at much greater risk of inbreeding depression following a rapid decline. Our

morphological and simulated results suggest that island foxes may have a lower burden of strongly deleterious recessive alleles, reducing their risk for inbreeding depression.

An important distinction between island foxes and some other populations with inbreeding depression, such as Isle Royale wolves, is that island foxes likely do not inbreed, as they typically form monogamous breeding pairs of unrelated individuals (Roemer and Smith et al. 2001). Breeding of related individuals was essentially unavoidable in the San Miguel and Santa Rosa captive breeding programs where the number of founders was small (eight and thirteen individuals, respectively) (Coonan et al. 2010). Nonetheless, we did not observe decreased heterozygosity in the post-bottleneck populations as predicted. We recommend deeper sampling of pre- and post-bottleneck populations to investigate possible changes in allelic diversity, which are undetectable in our analysis of representative individuals, to complement the results presented here. However, the rapid expansion of the bottlenecked population appears to have preserved heterozygosity. Additional analysis of historic and ancient island fox genomes would allow more detailed study of fluctuations in neutral and deleterious variation over time. If genetic variation was already low, even a severe bottleneck may not have had much effect on allelic diversity. Overall, the recent decline and rapid recovery of the endangered fox populations has not had a significant impact on heterozygosity or the burden of deleterious variants within island fox genomes.

We caution that intentionally inbreeding or maintaining populations at small size to induce purging of strongly deleterious recessive alleles is extremely unwise, as it carries a high risk of extinction while population sizes are small, and leads to the accumulation of weakly and moderately deleterious mutations even if strongly deleterious mutations are eliminated (Wang et al. 1999; Wang 2000; Glémin 2003). This practice has unwittingly led to the high proportion of

deleterious variants present in many domesticated plant and animal genomes, and has been dubbed the “cost of domestication” (reviewed in Moyers et al. 2017). The progressive fixation and accumulation of weakly and moderately deleterious mutations over time is expected to ultimately lead to mutational meltdown and extinction, once fitness declines to the point at which death rates exceed birth rates (Lynch et al. 1995). However, this process may take many thousands of generations, and depends critically upon the interaction of various demographic and genetic parameters, such as population size, fecundity, and the fitness effects of mutations (Lynch et al. 1995, Garcia-Dorado 2003). Whether or not the accumulation of weakly and moderately deleterious mutations leads to extinction in the wild has been debated, since non-genetic factors are also usually implicated (Lande 1988). However, a recent study posits that the extinction of an island-bound mammoth was the result of mutational meltdown (Rogers and Slatkin 2017). It is unclear whether the elevated burden of deleterious variants in island foxes may eventually lead to their extinction. However, the rapid recovery of island fox populations following recent bottlenecks suggests that these mutations have not seriously compromised their fitness or capability to rebound from population crashes.

Perhaps a greater concern for the future persistence of island foxes is the depletion in their genetic variability, which may compromise their capacity to adapt to changing environmental conditions or their ability to respond to parasites and disease (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003; Spielman et al. 2004; Willi et al. 2006; Hoffman et al. 2014). The genetic monomorphism on San Nicolas is particularly concerning, since the population has no reservoir of diversity to respond to the introduction of a novel pathogen. The Santa Catalina fox population was decimated following exposure to canine distemper virus, and foxes on other islands are plagued by high parasite loads and disease (Garcelon et al. 1992; Timm et al. 2009;

Crooks et al. 2001; Gaffney et al. 2016). Foxes on Santa Catalina are now vaccinated for CDV, but a more recent threat to this population is a fatal ear cancer, thought to be induced by ear mite infection (Vickers et al. 2015). Foxes on other islands with the ear mites do not develop ear cancer, suggesting a genetic predisposition underlying cancer in response to mites in Catalina foxes, a hypothesis that is currently being investigated. Although the ratcheting of deleterious mutations over time may gradually decrease fitness, the lack of diversity in island fox populations leaves them susceptible to sudden catastrophe if they cannot withstand disease outbreaks.

The observation that island foxes have persisted until now with low variation and increasing genetic load may be attributed to a historically benign island environment. Unlike gray foxes, island foxes face no predation pressure (except by non-natives) and only two populations (Santa Cruz, Santa Rosa) coexist with a competitor: the island spotted skunk (*Spilogale gracilis amphiala*) (Crooks and Van Vuren 1995). Meanwhile, gray foxes on the mainland face high rates of interspecific competition and predation; in Southern California, it is estimated that coyotes and bobcats cause more than 90% of gray fox mortalities (Farias et al. 2005). Nonetheless, island foxes are clearly impacted by anthropogenic threats. The near extinction of foxes on the Northern Channel Islands due to non-resident golden eagle predation and the near extinction of foxes on Santa Catalina due to introduced canine distemper virus were both indirectly precipitated by recent human activities on the islands (Roemer and Coonan et al. 2001; Timm et al. 2009).

From 2012 to 2015, the San Nicolas population declined by 41% due to as yet unknown reasons (Meyer 2016), but possibly in response to California's extreme drought. This recent decline has reignited the debate over whether genetic rescue, or human-assisted migration,

should be employed in the management of island foxes. Much of the justification for this intervention rests on the premise that the initial introduction of island foxes to the Channel Islands was likely facilitated by Native Americans, who also may have moved foxes between islands, facilitating gene flow that may have temporarily staved off genetic deterioration (Vellanoweth 1998; Levy 2010; Funk et al. 2016). However, all populations are genetically distinct with no evidence of appreciable recent or historic gene flow (Wayne et al. 1991; Goldstein et al. 1999; Hofman et al. 2015; Funk et al. 2016; Robinson et al. 2016). Currently, there is no indication that the recent rapid decline on San Nicolas has a genetic basis, especially given the long-term persistence of this population with very low diversity. If the recent decline is not the result of inbreeding depression, but is rather due to environmental stressors such as drought or human activity on the islands, genetic rescue may not materially enhance the likelihood of population persistence and would compromise the genetic distinction of the population. It is possible that lack of diversity has compromised the resilience of the population to environmental fluctuation, such as drought, indirectly contributing to the recent decline, and highlighting the island fox's elevated extinction risk. The suddenness of this recent decline underscores the importance of continued monitoring of island fox populations, regardless of their status in relation to the ESA.

The overall higher burden of deleterious variants in island fox genomes, which our simulations suggest is composed of weakly and moderately deleterious mutations, does not appear to have jeopardized their persistence thus far, and may not threaten their well being for generations to come, if ever. The impacts of these weakly deleterious mutations are unclear, but they do not cause canonical signs of inbreeding depression in island foxes. The future longevity of island fox populations likely depends on their resilience to non-genetic threats. For example,

our morphological analysis shows that vehicle collisions pose a greater threat to the island foxes than congenital skeletal deformities. Traumatic pathologies were most frequent on San Nicolas, San Clemente and Santa Catalina islands, the three islands with paved roads and significant human habitation (Santa Catalina) or naval bases (San Clemente, San Nicolas). On San Clemente Island, the annual survival of foxes that regularly cross roads declines from 0.97 to 0.76 (Snow et al. 2011). Unlike the rare congenital malformations we observed, vehicular impacts likely compromise mobility and contribute to early mortality.

In sum, our results provide the basis for several recommendations pertaining to the management of island foxes going forward. The successful recovery of all four ESA-listed island populations in record time is a testament to the efforts of the dedicated managers engaged in the recovery. It is also a testament to the island fox's inherent capacity to rebound from drastic population declines, perhaps due to the reduced burden of strongly deleterious recessive alleles in island populations. The island fox is clearly not spiraling into an "extinction vortex," the process by which population sizes continuously fall due to the synergistic effects of genetic deterioration, demographic stochasticity, and environmental stressors (Gilpin and Soulé 1986). Instead, island fox populations have effectively evolved to persist at small population sizes in isolation, as they have done for thousands of generations, even if they were moved between islands by Native Americans at one time. As such, genetic rescue is currently not merited, and would erase the unique genetic legacy of the populations, which are more threatened by environmental rather than genetic factors, and may even be locally adapted (Funk et al. 2016). Instead, we advocate for the continued close monitoring of island fox populations to ensure that a swift response can be mounted in the event of a catastrophic decline. Any signs of inbreeding

depression should also be watched for and reported if found, as this would challenge the recommendations we make here.

Despite a longstanding interest in purging, and whether it may be used as a management technique for the purposes of conservation, few examples are known (Ballou 1997; Crnokrak and Barrett 2002). Purging as a result of long-term reduced effective population sizes was recently invoked to explain the proportionally lower number of loss of function mutations in endangered mountain gorillas (Xue et al. 2015). Conversely, examples of inbreeding depression abound in both wild and captive environments (Ralls and Ballou 1983; Crnokrak and Roff 1999). The island fox provides a model of persistence in a goldilocks-type scenario in which population sizes are small enough to facilitate purging of strongly deleterious recessive alleles, but large enough to avoid extinction during the process. At carrying capacity, each island fox population numbers a few hundred to almost two thousand individuals, which may be the minimum number sufficient for selection to remove the most deleterious mutations without resulting in sudden extinction. This is likely a key distinction between the island fox and other endangered populations, which are often too small for selection to remove strongly deleterious mutations without causing a downward spiral in population size through the extinction vortex. Thus, our results demonstrate that, in spite of reduced genetic diversity and an increased burden of weakly deleterious variants, purging of strongly deleterious recessive mutations occurs in small populations of a few hundred individuals, potentially allowing persistence over thousands of generations. Our results suggest a model for the conservation of species for which habitat fragmentation and loss dooms them to isolation and small population sizes (as is the case for many large mammals). Our results stress the need to keep population sizes large enough for

selection to be effective on severely deleterious mutations (generally hundreds of individuals), and accepting some accumulation of less severe variants in the short term.

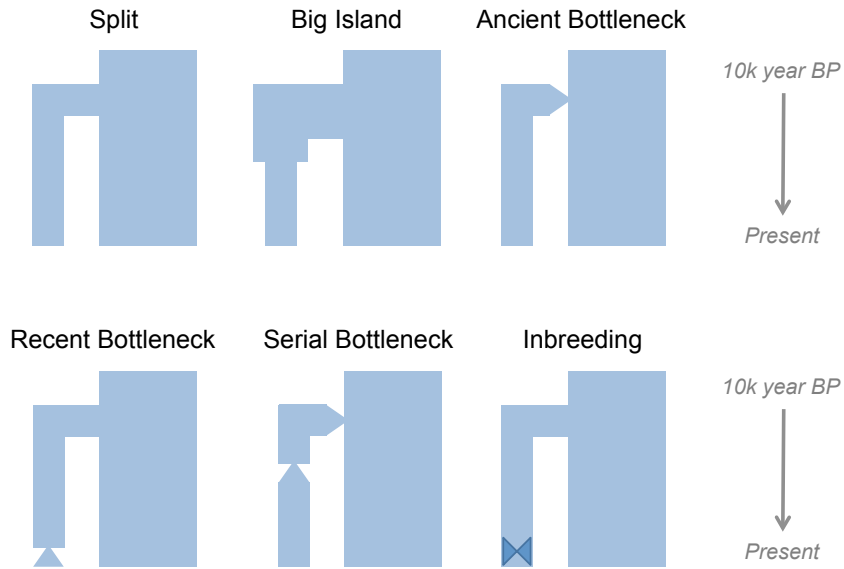


Figure 2-1. Pictorial depictions of demographic models used in simulations. Each simulation begins with a 100,000-generation burn-in (not shown) with only the large mainland population ($N=10,000$), before the establishment of the smaller island population. The generation time is one year. Models are not to scale.

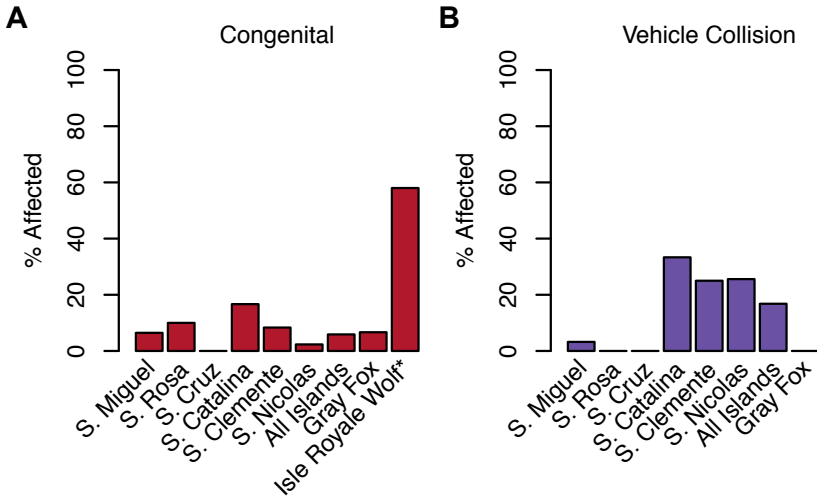


Figure 2-2. Prevalence of skeletal pathologies in island and gray foxes. Sample sizes: S. Miguel, n=31, S. Rosa, n=10; S. Cruz, n=5; S. Catalina, n=6; S. Clemente, n=24; S. Nicolas, n=43; all island foxes, n=119; gray fox, n=45; Isle Royale wolf, n=36. (A) Incidence of congenital defects in gray and island foxes (this study), compared with those of Isle Royale wolves (*Räikkönen et al. 2009). (B) Incidence of traffic-related pathologies in gray and island foxes.

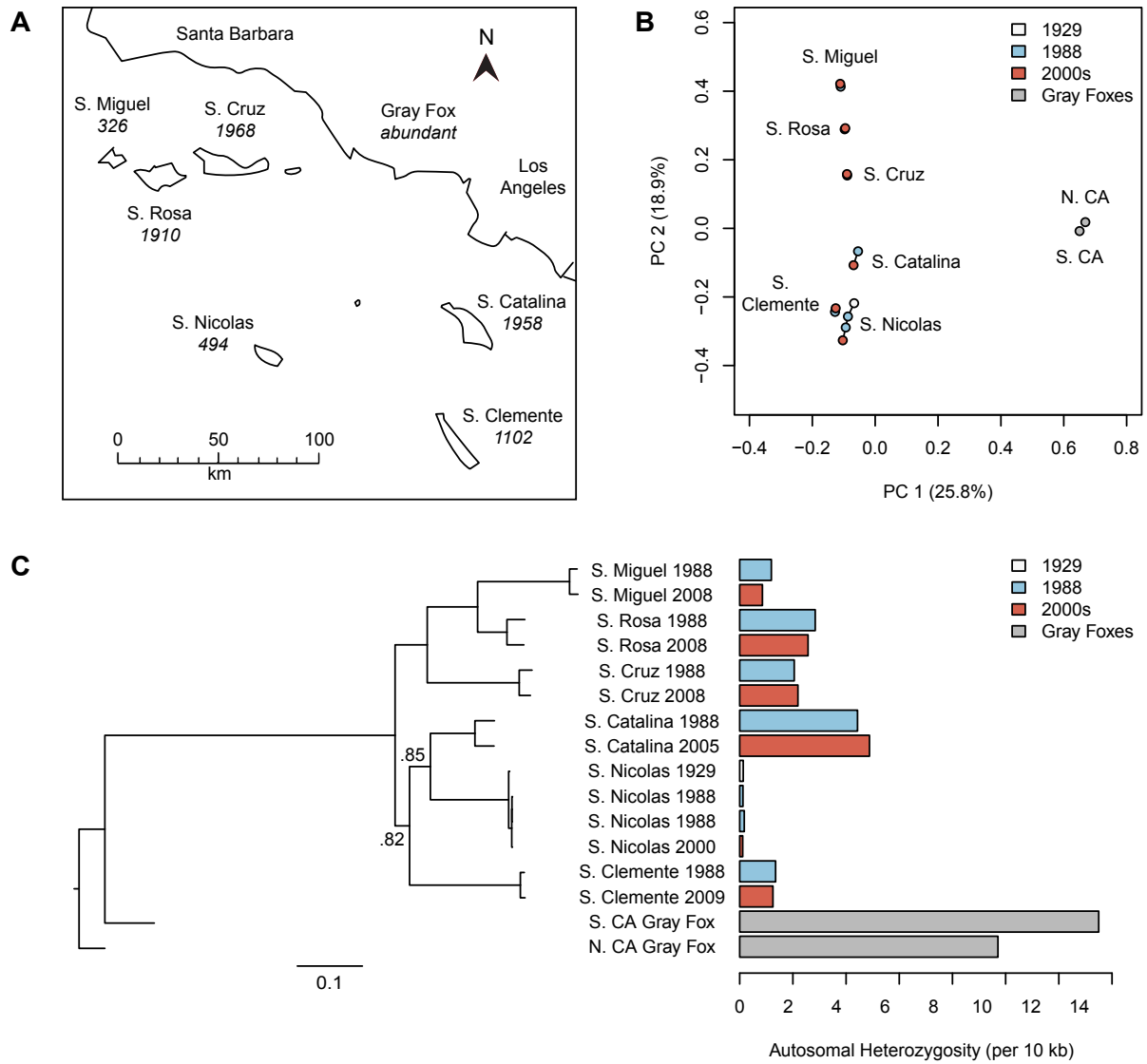


Figure 2-3. (A) Map of the Channel Islands with estimated island fox census sizes (Wayne et al. 1991). (B) PCA showing genetic distance between individuals, based on >12,000 SNPs pruned for LD. Solid black lines connect points from the same population. (C) Left: Maximum-likelihood tree based on >12,000 SNPs pruned for LD. All nodes have 100% bootstrap support except where noted. Right: Mean per-site heterozygosity across the autosomal genome.

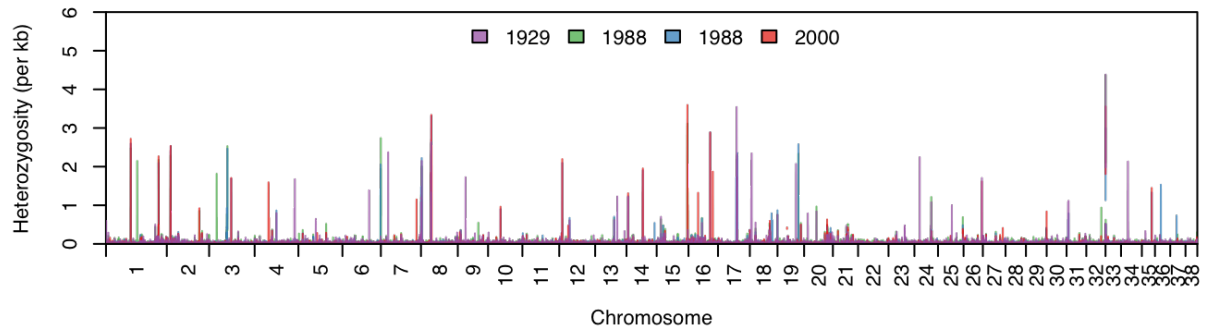


Figure 2-4. Per-site heterozygosity in 100 kb windows with a 10 kb step size across the autosomal genome in four San Nicolas foxes, showing the genomic flatlining characteristic of this population punctuated by occasional peaks of heterozygosity. Gaps indicate windows with too few callable sites.

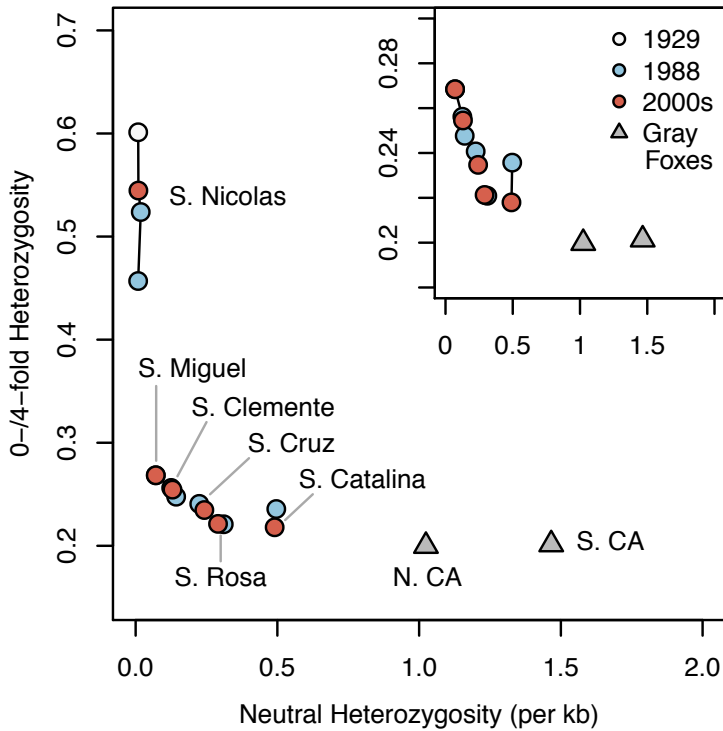


Figure 2-5. Plot of the negative relationship between neutral genetic diversity (proxy for effective population size) and the ratio of heterozygosity at zero-fold degenerate relative to four-fold degenerate sites (proxy for the efficacy of selection). Points for individuals from the same population are connected by solid black lines. Inset: The relationship holds for island foxes even when removing San Nicolas.

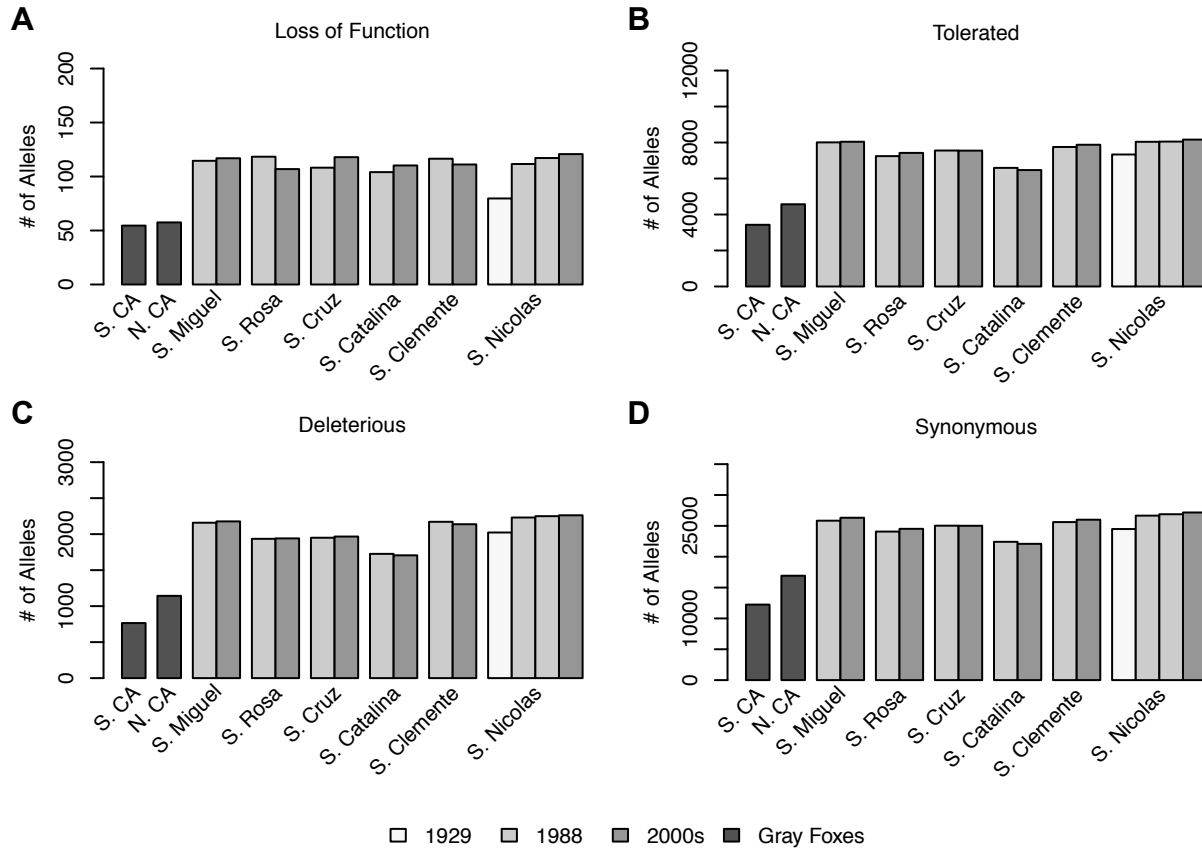


Figure 2-6. The number of derived alleles contained in homozygous genotypes in each individual, classified by annotation type by VEP and SIFT. Island foxes have higher homozygosity of all variant types. (A) Loss of function mutations are those that encode premature stop codons. (B, C) Tolerated and deleterious mutations are missense mutations categorized by SIFT according to whether they are predicted to be damaging.

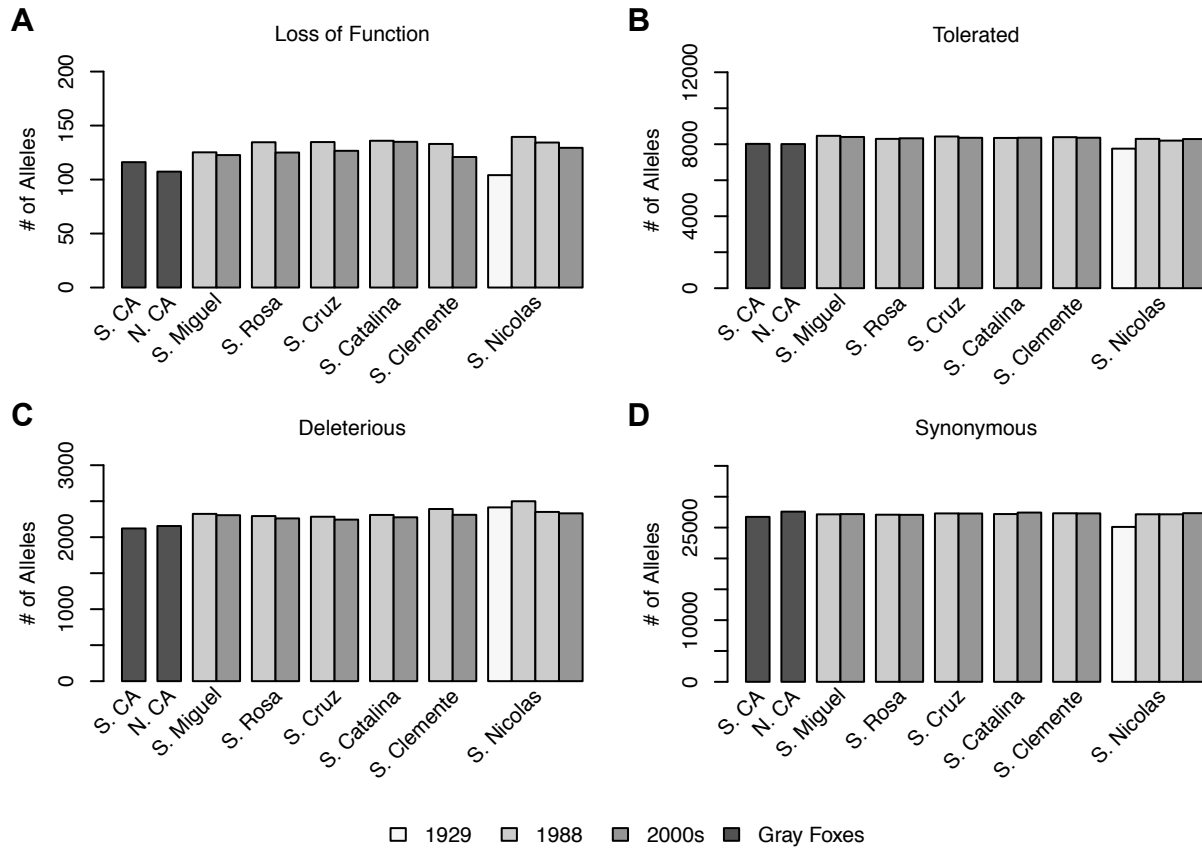


Figure 2-7. The total number of derived alleles contained in each individual, classified by annotation type by VEP and SIFT. Island foxes have a higher total number of loss of function and deleterious mutations. (A) Loss of function mutations are those that encode premature stop codons. (B, C) Tolerated and deleterious mutations are missense mutations categorized by SIFT according to whether they are predicted to be damaging.

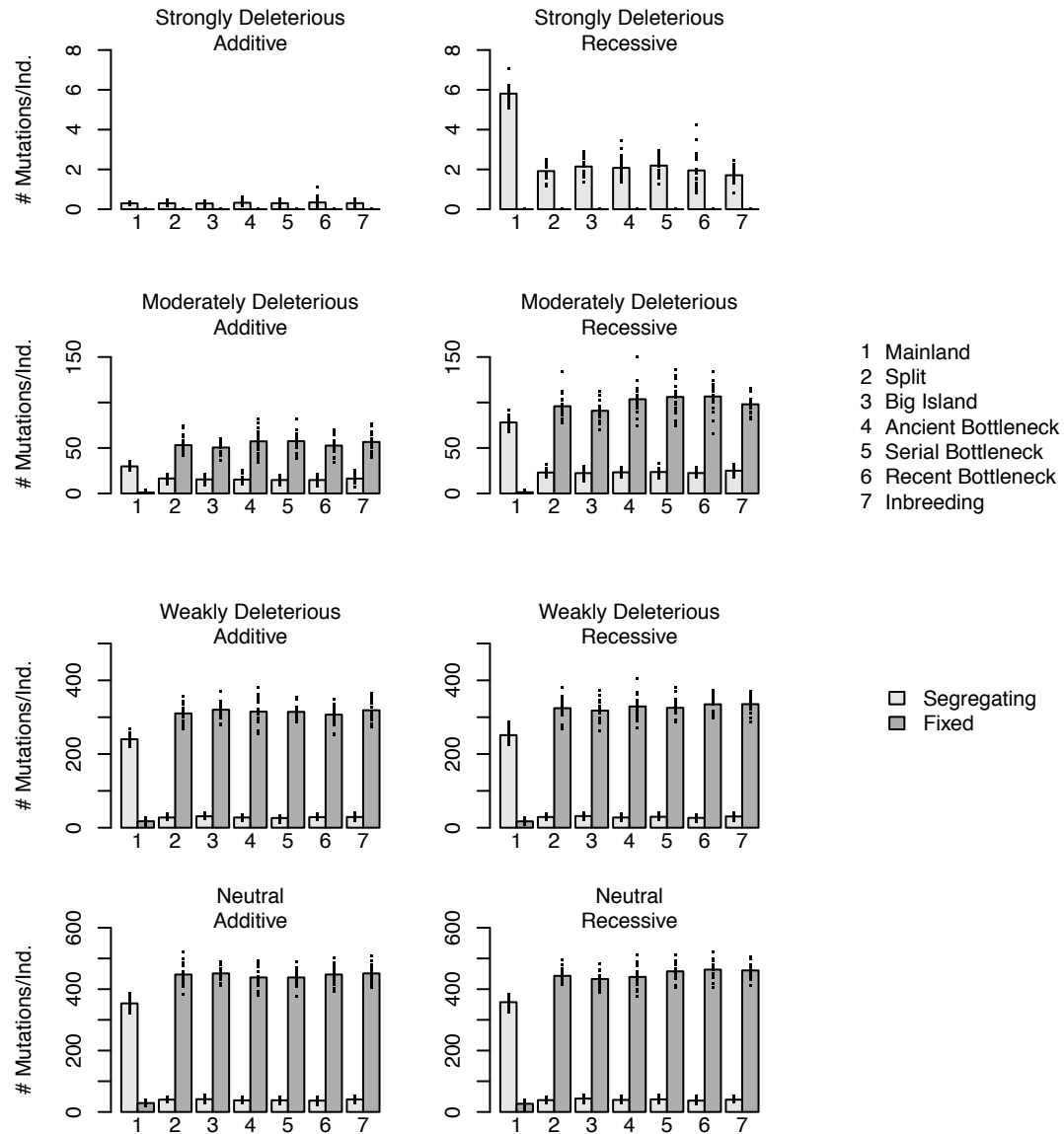


Figure 2-8. Simulation results showing the number of mutations per individual according to dominance and selection strength. Each dot represents the mean number of mutations per individual within a single replicate, while bar height represents the mean across all 20 replicates. Selection strengths are as follows: (A, B) strongly deleterious, $-1 \leq s < -0.01$; (C, D) moderately deleterious, $-0.01 \leq s < -0.0001$; (E, F) weakly deleterious, $-0.0001 \leq s < 0$; (G, H) neutral: $s = 0$. At the end of the simulations, the mainland population has a size of 10,000 individuals, and the islands each have 1,000 individuals.

Appendix 2-I: Supplemental Tables

Table S2-1: Sample information and metrics for genome sequences.

Species*	Location	Sample #	Sex	Year Sampled	Platform	Base Pairs (Gbp)	# of reads aligned, post-filtering (x 10 ⁶)	Mean depth of coverage (X)	Source
ULI	Santa Catalina	SCA16/ RKW4644	F	1988	HiSeq2000, 2x100bp	82.5	311	12.8	Robinson et al. 2016
ULI	San Clemente	SCLV4/ RKW4045	F	1988	HiSeq2000, 2x100bp	73.2	450	18.7	Robinson et al. 2016
ULI	Santa Cruz	SCZ05/ RKW12331	M	1988	HiSeq2000, 2x100bp	77.9	348	14.5	Robinson et al. 2016
ULI	San Miguel	SMI15/ RKW12354	F	1988	HiSeq2000, 2x100bp	80.5	559	23.2	Robinson et al. 2016
ULI	San Nicolas	SNI05/ RKW4038	F	1988	HiSeq2000, 2x100bp	66.1	339	13.8	Robinson et al. 2016
ULI	San Nicolas	SNI41/ RKW12349	F	1988	HiSeq2000, 2x100bp	84.3	328	13.6	Robinson et al. 2016
ULI	Santa Catalina	RKW11697	M	2005	HiSeq4000, 2x100bp	70.8	526	22.3	This study
ULI	San Clemente	RKW13704	F	2009	HiSeq4000, 2x100bp	69.5	442	18.7	This study
ULI	Santa Cruz	RKW8695	F	2008	HiSeq4000, 2x100bp	72.3	511	21.7	This study
ULI	San Miguel	RKW11655	M	2008	HiSeq4000, 2x100bp	69.6	516	21.9	This study
ULI	San Nicolas	RKW12297	M	2000	HiSeq4000, 2x100bp	73.0	492	20.9	This study
ULI	San Nicolas	15477	F	1929	HiSeq4000, 1x100bp	118.5	478	15.8	This study
ULI	Santa Rosa	SRO13/RKW 12355	F	1988	HiSeq4000, 2x100bp	76.0	527	22.2	This study
ULI	Santa Rosa	RKW10660	M	2008	HiSeq4000, 2x100bp	78.0	534	22.7	This study
UCI	SMMNRA**	GFO41	F	2012	HiSeq2000, 2x100bp	67.5	409	17.0	Robinson et al. 2016
UCI	GOGANRA***	GFO30	M	1993	HiSeq4000, 2x100bp	70.6	446	18.8	This study

* ULI: *Urocyon littoralis*, UCI: *Urocyon cinereoargenteus*

** SMMNRA: Santa Monica Mountains National Recreation Area

*** GOGANRA: Golden Gate National Recreation Area

Table S2-2: Catalog of skeletal pathologies observed in morphological assessment of island and gray fox specimens from museum collections. LACNMH: Los Angeles County Museum of Natural History; SBMNH: Santa Barbara Museum of Natural History; Dickey: Donald Ryder Dickey Bird and Mammal Collection at University of California, Los Angeles.

Population	Year	Collection	Specimen	Type	Description
San Clemente	1939	LACMNH	M1413	Trauma (collision)	2 fractured rib shafts, OA R costal facet of T12, R wing of sacrum fused to ilium, R fibula rough at lateral malleolus, infection of L ankle joint with roughened surfaces of L astragalus and calcaneum
San Clemente	1939	LACMNH	M1373	Trauma (collision)	2 fractured proximal ribs, fractured caudal vertebra
San Clemente	1939	LACMNH	M1374	Trauma (collision)	Origin L gracilis m. expanded and rough, mid caudal vertebrae fused following fracture, fractured 20th caudal
San Clemente	1939	LACMNH	M1375	Congenital, Trauma (collision)	Malocclusion-underbite, R radius+ulna fused following radial fracture, R fibula fractured distally with malunion, R fused with OA, R ribs 1+3+4 fractured mid-shaft
San Clemente	1972	SBMNH	836	Congenital, Trauma (collision)	Congenital fused S3+caudal 1, dysplasia L femoral head and formation of new joint surface, R sacral wing fused to ilium, L pes septic, L MT1 fractured, T9 postzygapophyses wrapped around T10, C7+T1 fused following fracture
San Clemente	1976	LACMNH	52293		Traction spur L ilium dorsal to acetabulum
San Clemente	1976	SBMNH	1999	Trauma	T9-11 roughened zygapophyses, OA T1+associated rib head, C7 osteophytes on vertebral centrum, axis osteophytes on vertebral centrum
San Clemente	1976	SBMNH	2001		OA L lateral distal femur+tibial head. Young animal.
San Clemente	1981	SBMNH	2891		Roughened L MC2+3, bone spur acromion L scapula, L styloid process of radius fractured and healed with upward displacement
San Clemente	1982	SBMNH	2548	Trauma (collision)	Fractured distal caudal vertebrae, rough calcaneal surface, 2 fractured L distal ribs, roughened sternbrae
San Clemente	1982	SBMNH	2627		T10-11 ankylosing spondylitis, osteophytes on associated rib heads
San Miguel	1939	LACMNH	M1361		Severe OA MT 2-5, MT 1 fused to neighbor, cuboid articular surface is excessively worn
San Miguel	1939	LACMNH	M1362	Congenital, Trauma (collision)	Congenital fused S3+ caudal 1, 3 rib fractures at angle (1 displaced when healed), evidence of infection on carpals+phalanges, fractured styloid process of ulna
San Miguel	1939	LACMNH	M1363		Fractured distal caudal
San Miguel	2011	SBMNH	9187	Congenital	Extra lumbar vertebrae- 8 lumbar, 13 thoracic, 7 cervical
San Nicolas	1929	Dickey	15.476	Trauma (collision)	3 adjacent ribs fractured at midshaft
San Nicolas	1929	Dickey	15.477	Trauma	Caudal vert. 3+4 fused following trauma; L2 has asymmetrical prezygapophyses; neural spines of thoracic vertebrae expanded vs other individuals.
San Nicolas	1929	Dickey	15.495		Asymmetrical thoracic vertebrae, especially caudal articular process; slight asymmetry of atlas wings (may not be pathology); proximal metatarsal rough at

					insertion digital flexor muscle.
San Nicolas	1939	LACMNH	31011		Osteoarthritis of phalanges
San Nicolas	1940	LACMNH	M1500	Trauma	Fractured caudal vertebrae
San Nicolas	1974	SBMNH	856	Trauma (collision)	Pyogenic arthritis of L knee (distal femur+head tibia affected), fractured L fibula
San Nicolas	1974	SBMNH	F-1074	Trauma (collision)	Evidence of infection on R astragalus+calcaneum, fractured R fibula, R ilium rough at origin quadratus femoris, L humerus rough at spinodeltoid insertion
San Nicolas	1974	SBMNH	F-837	Trauma	Single fractured rib
San Nicolas	1980	SBMNH	F-2268		Bone spur R distal fibula
San Nicolas	1982	SBMNH	2547	Trauma	Fractured distal fibula
San Nicolas	1982	SBMNH	M-2163	Trauma (collision)	Infection distal MT3+adjacent MT2+associated phalanges, mid-caudal fracture with pseudoarthrosis, 1 fractured distal rib
San Nicolas	1984	SBMNH	3135	Trauma	2 metatarsals affected by trauma or nearby infection
San Nicolas	1984	SBMNH	3137	Congenital	Maloccluded incisors, metatarsal has smooth bony growth (possible trauma)
San Nicolas	1984	SBMNH	3434	Trauma	Fractured caudal vertebrae
San Nicolas	1984	SBMNH	3707	Trauma	Fractured L fibula, 2 locations. OA 2 L metatarsals
San Nicolas	1985	SBMNH	3281	Trauma (collision)	Fractured caudal vertebrae, R radius and ulna fractured with radius shortened
San Nicolas	1985	SBMNH	3713	Trauma (collision)	Infection L+R calcanea and pes, evidence of infection on L distal radius and ulna, L knee infection affecting distal femur and tibial head, L tibia and fibula roughened by infection, fractured caudal vertebrae.
San Nicolas	1986	SBMNH	3708	Trauma (collision)	Fractured distal caudal vertebrae, 1 rib callused from partial break
San Nicolas	1986	SBMNH	3711	Trauma	Rough distal R radius+ulna, 3 proximal metatarsals show infection w/pyogenic OA
San Nicolas	1986	SBMNH	3714	Trauma	Fractured distal caudal vertebrae
San Nicolas	1987	SBMNH	3982	Trauma (collision)	Fractured distal rib, OA 3 phalanges and proximal MT2, expanded insertion Achilles' tendon, fractured caudal vertebra
San Nicolas	1987	SBMNH	M-2954	Trauma	Irregular dorsal scapular margin, fractured first rib
San Nicolas	2008	SBMNH	9151	Trauma	Fractured distal R ulna
San Nicolas	2012	SBMNH	TTX 261	Trauma (collision)	Periostitis R femoral midshaft, 3 R distal ribs fractured
San Nicolas	2013	SBMNH	PWC 5648	Trauma (collision)	Fractured distal caudal vertebrae, fractured and fused C7+T1, L fibula fractured and fused to tibia mid-shaft
San Nicolas	NA	SBMNH	3712	Trauma (collision)	Evidence of infection on L tibia posterior surface, L phalangeal fracture w/non-union, L fibular fracture w/non-union, caudal vertebra fractured
Santa Catalina	1942	LACMNH	M1496	Trauma (collision)	Distal fracture R femur with osteophytes on both surfaces of tibiofemoral joint, R fibular head fractured and fused to tibia, fractured and fused caudal vertebrae
Santa Catalina	1986	LACMNH	74997	Congenital	Congenital fused sacrum+caudal 1, premaxillary incisors widely spaced, anterior spine of axis roughened
Santa Catalina	1986	LACMNH	74999		Osteoarthritis at articulation of calcaneus and cuboid, hypomineralized tooth enamel
Santa Catalina	1986	LACMNH	75000	Trauma	Fractured caudal vertebrae 8-9, fractured neural spines

				(collision)	T4-5, broken ribs 2-5, hypomineralized teeth throughout upper and lower jaw
Santa Cruz	1929	Dickey	13.85		Asymmetry of articular surface of CV2+3, CV2 bone spur on margin of centrum
Santa Cruz	1939	LACMNH	M1385	Trauma	Fractured L radius w/non-union, outgrowth from associated ulna
Santa Cruz	1967	LACMNH	28229		Hypomineralized teeth
Santa Rosa	1929	Dickey	13.452		Metacarpal fused to phalanx, likely trauma
Santa Rosa	1939	LACMNH	M1372		Rough posterior surface of femur without sign of infection-direct trauma? Associated tibia and pelvis unaffected.
Santa Rosa	1976	SBMNH	872	Congenital	LSTV sacralized L side L7, fractured R distal tibia
Santa Rosa	1976	SBMNH	927		Osteoarthritis of proximal MC 2-4
Gray Fox	1929	Dickey	13.845	Congenital	LSTV Bilateral sacralized L7
Gray Fox	1929	Dickey	16.312	Congenital	LSTV sacralized caudal 1, R side only
Gray Fox	1989	LACMNH	90921	Congenital	Achondroplastic dwarf, slight tooth crowding at full eruption, epiphyses fully fused.
Gray Fox	1929	LACMNH	15.914	Trauma	Fractured R mandible, perforation L palate
Gray Fox	1974	LACMNH	833		Fractured R mandible, perforation L palate
Gray Fox	1978	LACMNH	1753	Trap	Trap- R radius and ulna severed midshaft, fusion of remaining bone, total destruction of cubital joint
Gray Fox	1978	LACMNH	1935		Fused radius and ulna at single midshaft point, no evidence of trauma
Gray Fox	1978	LACMNH	1954	Trap	Trap- L radius and ulna severed midshaft, fusion of remaining bone. R radius and ulna fused with no evidence of trauma
Gray Fox	1978	LACMNH	1962		Fused radius and ulna at single midshaft point, no evidence of trauma

Table S2-3: Gene ontology enrichment results for peaks of heterozygosity in island and gray fox genomes.

Individual	Enrichment p-value	GO Term	Description
S. CA Gray Fox	1.29E-16	GO:0050911	Detection of chemical stimulus involved in sensory perception of smell
	1.46E-11	GO:0007186	G-protein coupled receptor signaling pathway
	6.84E-11	GO:0005886	Plasma membrane
	0.00576	GO:0045095	Keratin filament
	0.0163	GO:0000786	Nucleosome
	0.00556	GO:0005549	Odorant binding
	1.29E-16	GO:0004984	Olfactory receptor activity
	0.0103	HP:0005356	Decreased serum complement factor I
	0.0432	KEGG:04610	Complement and coagulation cascades
1.43E-16	KEGG:04740	Olfactory transduction	
N. CA Gray Fox	0.014	GO:0018101	Protein citrullination
	0.00108	GO:0071944	Cell periphery
	3.90E-13	GO:0005882	Intermediate filament
	0.00317	GO:0016021	Integral component of membrane
	0.014	GO:0004668	Protein-arginine deiminase activity
	0.0209	GO:0060089	Molecular transducer activity
	0.00498	KEGG:04740	Olfactory transduction
S. Catalina 1988	0.0477	GO:0098662	Inorganic cation transmembrane transport
	0.0331	GO:0045095	Keratin filament
	0.000906	GO:0016021	Integral component of membrane
S. Catalina 2005	1.23E-29	GO:0050906	Detection of stimulus involved in sensory perception
	0.00278	GO:1900543	Negative regulation of purine nucleotide metabolic process
	1.65E-05	GO:0005882	Intermediate filament
	1.32E-13	GO:0005886	Plasma membrane
	4.66E-06	GO:0005549	Odorant binding
	1.52E-29	GO:0004984	Olfactory receptor activity
	0.00587	GO:0004064	Arylesterase activity
	3.64E-25	KEGG:04740	Olfactory transduction
0.00371	KEGG:04610	Complement and coagulation cascades	
S. Clemente 1988	NA	NA	NA

S. Clemente 2009	NA	NA	NA
S. Cruz 1988	0.00413 0.0295	KEGG:04924 KEGG:04713	Renin secretion Circadian entrainment
S. Cruz 2008	0.0222 0.027 0.0143	GO:0007399 GO:0048856 GO:0008146	Nervous system development Anatomical structure development Sulfotransferase activity
S. Miguel 1988	NA	NA	NA
S. Miguel 2008	5.56E-06 0.0454 0.0428 0.0106 0.0129 0.00157 0.001 0.042 0.00157 0.042 0.000385 0.0227 0.0221	GO:0045095 HP:0010669 HP:0002253 HP:0004428 HP:0000796 HP:0100025 HP:0002623 HP:0010780 HP:0001361 HP:0100817 HP:0007720 HP:0000015 HP:0002183	Keratin filament Hypoplasia of the zygomatic bone Colonic diverticula Elfin facies Urethral obstruction Overfriendliness Overriding aorta Hyperacusis Nystagmus-induced head nodding Renovascular hypertension Flat cornea Bladder diverticulum Phonophobia
S. Nicolas 1929	2.55E-07 5.38E-17 5.59E-17 0.00578 3.57E-16	GO:0007186 GO:0007608 GO:0004984 GO:0005549 KEGG:04740	G-protein coupled receptor signaling pathway Sensory perception of smell Olfactory receptor activity Odorant binding Olfactory transduction
S. Nicolas 1988 (1)	0.0229 5.61E-31 1.15E-43 3.79E-12 0.000464 2.36E-05 0.00943 0.0126 2.94E-43	GO:0035095 GO:0007186 GO:0050907 GO:0016021 GO:0004252 GO:0003823 GO:0004497 GO:0016705 GO:0004984	Behavioral response to nicotine G-protein coupled receptor signaling pathway Detection of chemical stimulus involved in sensory perception Integral component of membrane Serine-type endopeptidase activity Antigen binding Monooxygenase activity Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen Olfactory receptor activity

	0.0259	GO:0005549	Odorant binding
	8.42E-31	KEGG:04740	Olfactory transduction
S. Nicolas 1988 (2)	8.05E-19	GO:0007608	Sensory perception of smell
	0.0036	GO:0035095	Behavioral response to nicotine
	0.00871	GO:0016021	Integral component of membrane
	0.021	GO:0005886	Plasma membrane
	1.16E-18	GO:0004984	Olfactory receptor activity
	2.21E-09	GO:0005149	Interleukin-1 receptor binding
	0.0241	GO:0004252	Serine-type endopeptidase activity
	0.0496	HP:0002654	Multiple epiphyseal dysplasia
	2.78E-15	KEGG:04740	Olfactory transduction
S. Nicolas 2000	0.00689	GO:0051716	Cellular response to stimulus
	7.45E-26	GO:0050907	Detection of chemical stimulus involved in sensory perception
	6.62E-19	GO:0007186	G-protein coupled receptor signaling pathway
	0.0253	GO:0031224	Intrinsic component of membrane
	7.46E-09	GO:0005549	Odorant binding
	0.000212	GO:0004252	Serine-type endopeptidase activity
	7.43E-25	GO:0004984	Olfactory receptor activity
	3.79E-20	KEGG:04740	Olfactory transduction
S. Rosa 1988	0.00932	KEGG:00590	Arachidonic acid metabolism
S. Rosa 2008	0.018	GO:0044763	Single-organism cellular process
	0.0218	GO:0051216	Cartilage development
	0.0389	GO:0048149	Behavioral response to ethanol
	0.000344	GO:0005887	Integral component of plasma membrane
	0.0329	GO:0004499	N,N-dimethylaniline monooxygenase activity
	0.000802	KEGG:04080	Neuroactive ligand-receptor interaction

Table S2-4. Comparisons of the proportion of homozygous derived genotypes and the total number of derived alleles per individual between gray and island foxes. In null models, there is a single mean value for all foxes. In the alternative models, the island fox mean may differ from the gray fox mean. Log-likelihood values for the null and alternative models were used to calculate the likelihood ratio test statistic, $\Lambda = -2(\log\text{-likelihood}_{\text{null}} - \log\text{-likelihood}_{\text{alternative}})$. Asymptotically, Λ is χ^2 distributed with one degree of freedom. This distribution was used to calculate P -values. For allele tests, the null model assumes the ratio of p_i to p_g found at synonymous SNPs. This is a conservative test of whether the difference in the proportion of derived alleles between island and mainland foxes is greater than that seen at synonymous SNPs, which should be equivalent under neutrality, but were found to differ, reflecting possible technical biases such as the under-calling of heterozygotes. Significant P -values (<0.05) are in bold.

Type	Null model MLE	Alternative model MLE	Likelihood ratio test
Proportion of Homozygous Derived Genotypes (Gray Foxes v. 1988-2009 Island Foxes)			
Synonymous	$p_i = p_g = 0.320$	$p_i = 0.339, p_g = 0.196$	$\Lambda = 6693.60, P < < 10^{-16}$
Tolerated	$p_i = p_g = 0.280$	$p_i = 0.298, p_g = 0.157$	$\Lambda = 2480.40, P < < 10^{-16}$
Deleterious	$p_i = p_g = 0.209$	$p_i = 0.224, p_g = 0.104$	$\Lambda = 808.78, P < < 10^{-16}$
Loss of function	$p_i = p_g = 0.230$	$p_i = 0.251, p_g = 0.124$	$\Lambda = 40.73, P = 1.75 \times 10^{-10}$
Proportion of Derived Alleles (Gray Foxes v. 1988-2009 Island Foxes)			
Tolerated	$p_i = 1.002435p_g, p_g = 0.326$	$p_i = 0.328, p_g = 0.315$	$\Lambda = 31.60, P = 1.89 \times 10^{-8}$
Deleterious	$p_i = 1.002435p_g, p_g = 0.251$	$p_i = 0.254, p_g = 0.234$	$\Lambda = 32.72, P = 1.06 \times 10^{-8}$
Loss of function	$p_i = 1.002435p_g, p_g = 0.282$	$p_i = 0.289, p_g = 0.247$	$\Lambda = 6.76, P = 9.33 \times 10^{-3}$
Proportion of Derived Alleles (Gray Foxes v. 1988 Island Foxes)			
Tolerated	$p_i = 1.001165p_g, p_g = 0.325$	$p_i = 0.328, p_g = 0.315$	$\Lambda = 30.20, P = 3.90 \times 10^{-8}$
Deleterious	$p_i = 1.001165p_g, p_g = 0.252$	$p_i = 0.254, p_g = 0.234$	$\Lambda = 40.56, P = 1.91 \times 10^{-10}$
Loss of function	$p_i = 1.001165p_g, p_g = 0.285$	$p_i = 0.289, p_g = 0.247$	$\Lambda = 8.53, P = 3.50 \times 10^{-3}$
Proportion of Derived Alleles (Gray Foxes v. 2000-2009 Island Foxes)			
Tolerated	$p_i = 1.003915p_g, p_g = 0.324$	$p_i = 0.328, p_g = 0.315$	$\Lambda = 25.40, P = 4.66 \times 10^{-7}$
Deleterious	$p_i = 1.003915p_g, p_g = 0.246$	$p_i = 0.254, p_g = 0.234$	$\Lambda = 18.24, P = 1.95 \times 10^{-5}$
Loss of function	$p_i = 1.003915p_g, p_g = 0.271$	$p_i = 0.289, p_g = 0.247$	$\Lambda = 3.67, P = 5.56 \times 10^{-2}$

Appendix 2-II: Supplemental Figures

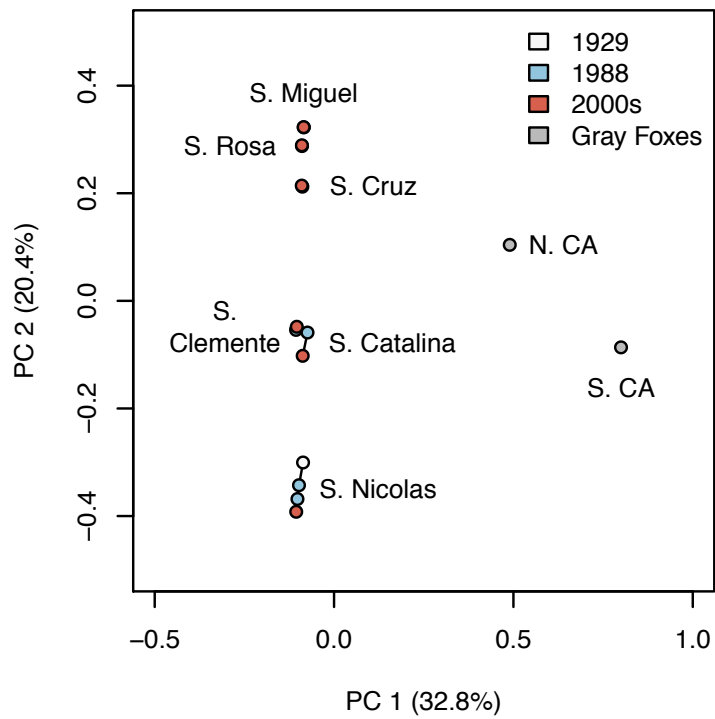


Figure S2-1. PCA showing genetic distance between individuals, based on 3,905,988 SNPs not pruned for LD. Solid black lines connect points from the same population.

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Chapter 3: Genomic signatures of inbreeding in Isle Royale wolves, a population on the threshold of extinction

Abstract

The observation that small, isolated populations often suffer reduced fitness as a result of inbreeding depression has guided conservation theory and practice for decades. However, investigating the genome-wide dynamics associated with inbreeding depression in natural populations is only now feasible with the accessibility of relative inexpensive sequencing technology and well assembled and annotated reference genomes. To characterize the genome-wide effects of intense inbreeding, we sequenced complete genomes from a highly inbred and isolated wild population of gray wolves (*Canis lupus*) on Isle Royale in Lake Superior. Through comparison with other wolf genomes from a variety of demographic backgrounds, we found that neither the level of heterozygosity nor the number of deleterious variants per genome were reliable predictors of inbreeding depression. This is consistent with the hypothesis that inbreeding depression is the result of increased homozygosity of strongly deleterious recessive mutations, which are actually more prevalent in historically large source populations. Our results affirm the conventional wisdom of conservation biology that it is important to minimize inbreeding, but also imply that smaller populations that are still large enough for selection to remove strongly deleterious mutations that have accumulated in large ancestral populations may be able to persist as long as they avoid inbreeding.

Introduction

Under increasing human population pressure, many species with once continuous ranges have been reduced to small, fragmented populations (Crooks et al. 2017). Higher levels of

inbreeding in such small populations place them at risk of extinction though inbreeding depression. Large carnivore species are very susceptible to this threat since many populations are highly isolated and their density is far lower than the herbivores they prey on. Additionally, they often need extensive natural areas to persist and are persecuted because of the threat they pose to humans and livestock (Ceballos and Ehrlich 2002). Some well-known examples of inbreeding depression in the wild have been observed in large carnivores, such as Florida panthers (*Puma concolor*, Roelke et al. 1993) and gray wolves (Liberg et al. 2005; Raikkonen et al. 2006, 2009).

Despite being a subject of study for more than a century (Darwin 1876), and a central concern of conservation genetics, the underlying genetic basis of inbreeding depression remains elusive. Inbreeding depression is associated with a reduction in genetic diversity, suggesting two potential mechanisms: 1) inbreeding leads to increased homozygosity of recessive deleterious alleles (partial dominance hypothesis); or 2) inbreeding leads to a decrease of heterozygotes, which are more fit than homozygotes (overdominance hypothesis). These mechanisms are not mutually exclusive, as they may each operate at different loci within the genome. The predominant mechanism affecting the fitness of inbred organisms is unclear, though previous studies largely support the partial dominance model (reviewed in Charlesworth and Willis 2009).

With the persistent reduction in costs for whole genome sequencing, it is now feasible to study the genome-wide burden of deleterious variation (genetic load) (eg. Lu et al. 2006; Lohmueller et al. 2008; Fu et al. 2014; Schubert et al. 2014; Simons et al. 2014; Renaut and Rieseberg 2015; Xue et al. 2015; Do et al. 2015; Marsden et al. 2016; Henn et al. 2016). However, recent studies have primarily dealt with the effects of long-term reduced population size or ancient bottlenecks, such as in non-African human populations or those in domesticated species. Additionally, studies have focused on the excess of deleterious variants associated with

expanding populations (Peischl et al. 2013; Peischl and Excoffier 2015). Generally, small increases in genetic load due to ancient bottlenecks or small population size have been observed, but the genomic effects of severe inbreeding have not been well studied and may have a distinct impact on the load of deleterious variation.

The adverse consequences of small population size have been debated, in part because theory predicts that small populations may have an enhanced capacity for purging strongly deleterious recessive mutations (Fu et al. 1998; Wang et al. 1999; Bataillon and Kirkpatrick 2000; Wang 2000; Crnokrak and Barrett 2002; Glémin 2003; Boakes et al. 2007). Such mutations may persist at low frequencies in large populations, since they will primarily be hidden from selection as heterozygotes within carrier individuals. These mutations may be purged following a population decline in two ways: 1) they may be lost by drift during a population contraction; and 2) if they are not lost, they have a higher chance of becoming homozygous due to inbreeding or the increase in frequency due to drift, thereby exposing them to selection. Although purging has been observed or inferred only rarely, evidence of inbreeding depression in small populations is common (Ralls et al. 1988; Crnokrak and Roff 1999; Keller and Waller 2002).

Some examples of hereditary defects in inbred carnivores associated with inbreeding include heart defects in Florida panthers (Roelke et al. 1993), cryptorchidism (undescended testes) in maned wolves (*Chrysocyon brachyurus*) and Florida panthers (Burton and Ramsay 1986; Roelke et al. 1993), and eye deformities in snow leopards (*Panthera uncia*) (Gripengburg et al. 1985). Studies of inbred wolves in the wild and in captivity have found elevated rates of blindness, cryptorchidism, heart and kidney defects, dental anomalies, and vertebral malformations (Laikre and Ryman 1991; Laikre et al. 1993; Räikkönen et al. 2013). Further,

studies have also found that inbred carnivores have reduced reproductive fitness, such as depressed reproduction and litter size in Arctic foxes (*Vulpes lagopus*) and brown bears (*Ursus arctos*) (Nordrum 1994; Laikre et al. 1996), as well as high levels of abnormal sperm in cheetahs (*Acinonyx jubatus*), lions (*P. leo*), and Florida panthers (Wildt et al. 1983; Wildt et al. 1987; Roelke et al. 1993). In gray wolves, inbreeding is associated with decreased reproduction, litter size, body weight, and longevity (Laikre and Ryman 1991; Liberg et al. 2005; Åkesson et al. 2016). It was once argued that carnivores, and wolves in particular, might be resistant to inbreeding depression due to their relatively small effective population sizes (Shields 1983; Mech 1995). However, the overwhelming evidence that inbreeding is nearly always detrimental, including in carnivores, proves otherwise, and shows that such small population size often does not lead to increased fitness through purging of deleterious alleles (see reviews by Crnokrak and Roff 1999; Laikre 1999; Crnokrak and Barrett 2002).

In this study, we present results from complete genome sequencing of an inbred very small wolf population on Isle Royale in Lake Superior that has been under annual observation almost since its founding, serving as a model system for the study of ecological and behavioral dynamics, as well as conservation genetics, for decades (eg. Allen and Mech 1963; Jordan et al. 1967; Wayne et al. 1991; Post et al. 1999; Peterson et al. 2014). The island was first colonized by wolves in the 1940s by a single breeding pair that crossed frozen Lake Superior from the mainland, establishing a population on Isle Royale that, at its peak, numbered as many as 50 individuals (Mech 1966; Peterson and Page 1988). Following a harsh winter and a disease outbreak in 1982, the population crashed to fourteen individuals, and has failed to rebound, despite an abundance of prey and a reported genetic rescue by a migrant wolf from the mainland in 1997 (Peterson et al. 1998; Adams et al. 2011; Hedrick et al. 2014).

The population is known to be extremely inbred, and has continued to wane while exhibiting signs of severe inbreeding depression (Räikkönen et al. 2009; Hedrick et al. 2014). A pair of old wolves, both brother-sister and father-daughter, descended from a legacy of repeated close inbreeding events, are the only individuals that remain. No successful reproduction has occurred during the past two years (Peterson and Vucetich 2017), and the population is destined to disappear without the introduction of wolves from the mainland, a move that is currently under consideration (National Park Service 2016). Although previous investigation of Isle Royale wolves has focused on inbreeding using genetic assays (Wayne et al. 1991; Adams et al. 2011; Hedrick et al. 2014), and on suspected inbreeding depression using morphological assessment (Räikkönen et al. 2009), this is the first study to combine both approaches and use complete genome sequence data.

Our results show that both inbreeding and long-term small population size lead to reduced heterozygosity, but generate starkly different patterns of diversity across the genome, providing insight into the mechanism of inbreeding depression. In the past, levels of diversity at small number of loci have been used as to make inferences about the demographic history and genetic health of populations. These methods may be misleading, as our results suggest that the relationship between heterozygosity and inbreeding depression is not straightforward, but depends on the nature of segregating deleterious variation, which is influenced by demographic history. With the low cost of sequencing, it is now feasible to use complete genomes or high density SNP genotyping to more accurately infer demographic history and reveal whether inbreeding is occurring. This insight may be used to more effectively manage populations to enhance their persistence. Our findings support the hypothesis that inbreeding depression is caused by increased homozygosity of strongly deleterious recessive mutations generated as a

result of recent inbreeding, rather than an overall increase in genetic load due to long-term small population size. These results have implications for understanding the genetic basis of inbreeding depression and for the effective management of small isolated populations, particularly those recently derived from large outbred populations, as is the case for many species threatened by habitat fragmentation and loss.

Results

Data Set

We obtained genetic samples from eleven Isle Royale wolves collected between 1988 and 2012 for whole genome sequencing and analysis. Over this period the population size on the island was estimated to number 8-30 individuals (Peterson and Vucetich 2017). A pedigree, adapted from Hedrick et al. (2014), shows the relationships of the sequenced wolves (where known), as well as inbreeding events between close relatives (Figure 3-1A). Based on this pedigree, the wolves in our dataset include inbred and putatively non-inbred individuals, with inbreeding coefficients ranging from 0 to 0.375 (Table 3-1, Figure 3-1B).

We supplemented the Isle Royale wolf genome sequences with publicly available and newly generated genomes from other wolves and their sister taxa (see Table 3-2 for genome sources). In addition to the Isle Royale wolves, our complete dataset comprised six mainland wolves from nearby Minnesota, nine gray wolves from elsewhere in North America, six Eurasian gray wolves, and a single genome from each of the following species: red wolf (*C. rufus*), coyote (*C. latrans*), African golden wolf (*C. anthus*), Ethiopian wolf (*C. simensis*), and gray fox (*Urocyon cinereoargenteus*) (Figure 3-2, Table 3-2). All genomes were aligned, genotyped, and annotated with respect to the domestic dog reference genome (canFam3.1), yielding mean

genome-wide coverage values of 9-49X after read filtering. This dataset spans the Holarctic range of the gray wolf and contains individuals derived from a variety of demographic histories that feature recent inbreeding, long-term small population size, isolation, and admixture (Table 3-2). To our knowledge, Isle Royale and Mexican wolves are the only populations we sampled that suffer from documented inbreeding depression (Fredrickson et al. 2007; Rääkkönen et al. 2009).

Phenotypic evidence of inbreeding depression in Isle Royale wolves

The canonical wolf spinal column is composed of seven cervical (C1–7), thirteen thoracic (T1–13), seven lumbar (L1–7), three fused sacral (S1–3) vertebrae from atlas to sacrum and ~20 coccygeal vertebrae (Co1–22) in the tail (Gilbert 1997). A 2009 study found a high prevalence (58%) of vertebral defects in Isle Royale wolves between 1964 and 2007, including extra vertebrae, and defects such as thoracolumbar, lumbosacral, and sacrococcygeal transitional vertebrae (exhibiting characteristics of two different types of vertebrae), as well as vertebrae with severe asymmetries. More recently, skeletal remains collected post-mortem from 6 of the 11 sequenced wolves were assessed for signs of congenital malformations, specifically in the spine and rib cage. None of these specimens were part of the 2009 study. Consistent with the results of that study, however, the incidence of anatomical defects in these six individuals was high, and only one individual was free of aberrations, whereas the remaining individuals possessed 1-4 abnormalities each (Table 3-1). Observed anomalies in the six Isle Royale specimens included transitional vertebrae, extra vertebrae, and extra ribs. Three of the six specimens also exhibited severe bone spurs (osteophytes), which, although not congenital, may form as a result of abnormal vertebral anatomy and alignment. Malformations like these, rarely observed in large,

outbred wolf populations, were previously shown to have steadily increased in prevalence during more than six decades of isolation and inbreeding in Isle Royale wolves (Räikkönen et al. 2009). For example, Räikkönen et al. (2006) found that that incidence of lumbosacral transitional vertebrae in outbred wolf populations in Finland and historic Scandinavia was 0-1%, compared to 58% in Isle Royale wolves and 10% in modern inbred Scandinavian wolves. The fitness impact of the vertebral defects observed in Isle Royale wolves is not clear, but lumbosacral transitional vertebrae in dogs have been implicated in cauda equina syndrome, which can cause severe pain, incontinence, gait problems and paralysis (Morgan et al. 1993).

Genome-wide patterns of variation shaped by demographic history

Generations of isolation and close inbreeding have reduced genome-wide heterozygosity in Isle Royale wolves relative to outbred mainland wolves (Figure 3-3). Genome-wide heterozygosity in Isle Royale wolves (0.94-1.43 heterozygotes per kb) is 11-41% lower than the mean heterozygosity of wolves from nearby mainland Minnesota (1.60 heterozygotes per kb). We calculated per-site heterozygosity in non-overlapping 1 Mb windows to assess spatial patterns of diversity in the genome of each individual (Figure 3-4). The genomes can be qualitatively divided into three groups: 1) genomes with high heterozygosity throughout (Figure 3-4A, B); genomes with low heterozygosity throughout (Figure 3-4C, D), and genomes with a sawtooth-like pattern characterized by regions of high heterozygosity interspersed by long runs of homozygosity devoid of variation (Figure 3-4E, F). We observed high heterozygosity across the genome in individuals derived from outbred populations with large long-term effective size, such as lowland Chinese (Xinjiang) and Minnesota wolves (Gray et al. 2009; vonHoldt et al. 2011, 2016; Fan et al. 2016). Conversely, low genome-wide heterozygosity is associated with

populations with a history of isolation and small long-term effective size, such as Ethiopian and Tibetan wolves (Gottelli et al. 1994, 2004; Zhang et al. 2014; Fan et al. 2016). The sawtooth-like pattern is characteristic of individuals with a history of recent inbreeding, as in the Isle Royale and Mexican wolves (Hedrick et al. 2014; Fredrickson et al. 2007).

Inbreeding generates runs of homozygosity (ROH), regions that are identical by descent (IBD), which result from an individual inheriting two copies of a chromosomal segment from a common ancestor. The advent of whole genome sequencing has recently revealed that the proportion of the genome contained within ROH (F_{ROH}) is a more reliable metric than pedigree-based inbreeding coefficients (F_{PED}) for inferring the degree of inbreeding in an individual's history (Kardos et al. 2015). The reasons for this observation include technical factors, such as incomplete knowledge of the pedigree leading to underestimates of the level of inbreeding, as well as biological factors, such as variance due to recombination and chromosome segregation during gamete formation (Broman and Weber 1999; Knief et al. 2015; Hedrick et al. 2016). We compared F_{PED} values obtained from the pedigree of Hedrick et al. (2014) to F_{ROH} in Isle Royale wolves to assess their correlation (Figure 3-1B). The pedigree is limited, as it only includes Isle Royale wolves genotyped between 1998-2013, and furthermore, our dataset includes several individuals for which we have no genealogical records (F25, F55, M61, F67). Among the pedigreed wolves, the correlation between F_{PED} and F_{ROH} was 0.607 ($P=0.024$), and F_{ROH} was always higher than F_{PED} (intercept=0.297, $P=9.95 \times 10^{-5}$), consistent with expectation. The slope of the regression line was shallow (0.426, $P=0.0238$), but this result should be interpreted with caution due to the small number of samples. We calculated that 23-47% of the Isle Royale wolf genomes were contained within ROH greater than 100 kb in length, which is significantly greater than F_{ROH} in Minnesota wolf genomes (12-24%; Mann-Whitney U (MWU) test, $P=3.23 \times 10^{-4}$).

Thus, recent severe inbreeding in Isle Royale wolves has sharply increased homozygosity across the genome, beyond the expected values yielded by the pedigree.

Without continuous inbreeding, ROH are broken down into shorter segments over time by recombination. Thus, long ROH are the hallmark of recent inbreeding, and shorter ROH are the traces of ancient or historic inbreeding, or bottlenecks (McQuillan et al. 2008; Gray et al. 2009; Kirin et al. 2010; vonHoldt et al. 2011). Isle Royale wolf genomes harbor many extremely long ROH (>10 Mb), but few shorter ROH, consistent with their recent descent from mainland wolves, which bear very few ROH of any length and have high heterozygosity across their entire genomes (Figures 3-3 and 3-5). Isle Royale wolves contain 14-35 of these long ROH per individual, whereas Minnesota wolves contain just 1-9 per individual (Figure 3-5C). Long ROH are also prevalent in the genomes of the inbred Mexican wolf and the Ellesmere Island wolf, the latter suspected but not previously known to be inbred (Carmichael et al. 2008). In contrast, the Tibetan and Ethiopian wolf genomes contained many shorter ROH, resulting from their long-term isolation and small effective population sizes, but few long ROH, suggesting no recent inbreeding (Figure 3-5A, B). Neither the Tibetan wolf nor the Ethiopian wolf is known to suffer from inbreeding depression. In fact, Ethiopian wolves are thought to practice inbreeding avoidance (Sillero-Zubiri et al. 1996; Randall et al. 2007). However, both the Ethiopian and Tibetan wolves exist in small isolated populations (Gottelli et al. 1994, 2004; Zhang et al. 2014; Fan et al. 2016). The Ethiopian wolf genome contained the highest number (514) of medium-sized ROH (1-10 Mb) (Figure 3-5A), and the Tibetan wolf contained the highest number (2,381) of short ROH (0.1-1 Mb) (Figure 3-5B). Since the Tibetan and Ethiopian wolves are not thought to suffer from inbreeding depression, these results imply that the intensity and timing of inbreeding are key factors modulating the risk of inbreeding depression.

The genetic basis of inbreeding depression in Isle Royale wolves

Although the distribution of ROH number and length within a genome are informative about the extent and timing of past inbreeding, it is still unclear how the genomic landscape of heterozygosity impacts fitness. To explore this relationship, we focused on protein-coding regions of the genome, which are more likely to directly affect fitness, and are also more amenable to functional interpretation. We found that as the proportion of the genome contained within ROH increases, the amount of coding sequence contained within ROH increases linearly ($R^2=0.994$, $P<2.20 \times 10^{-16}$) (Figure 3-6). Thus, ROH are not enriched for coding regions beyond that expected for their genome-wide distribution, suggesting it is not an increase in the proportion of coding regions in ROH that causes inbreeding depression. However, compared to outbred wolves, Isle Royale wolves had a higher fraction of the genome in ROH, and thus a higher proportion of coding sequence in ROH (0.23-0.47). Nonetheless, Tibetan and Ethiopian wolves had higher proportions of coding sequence within ROH (0.53 and 0.90, respectively), further demonstrating that the risk of inbreeding depression is not simply a function of increasing homozygosity in coding regions.

Since the overall homozygosity of a genome or its coding regions is not a reliable predictor of inbreeding depression, this suggests that the homozygosity of particular variants has a disproportionate effect on fitness. To assess the putative biological effects of particular mutations, we annotated variants in coding regions with respect to their impact on the encoded amino acid (eg. synonymous, non-synonymous, etc.) using the Variant Effect Predictor (VEP, McLaren et al. 2010), and further classified non-synonymous SNPs as likely to be deleterious or tolerated with the Sorting Intolerant From Tolerant (SIFT, Kumar et al. 2009) algorithm, which

predicts whether missense mutations are likely to be deleterious or tolerated on the basis of amino acid conservation at a site across taxa. We then classified all variants as putatively damaging or benign. Here, synonymous and tolerated missense mutations comprise the benign group, whereas deleterious missense mutations, and mutations that disrupt splice sites or start or stop codons comprise the damaging group.

To gauge how inbreeding on Isle Royale has impacted patterns of variation in coding regions, we compared the Isle Royale genomes to those of the mainland Minnesota wolves, which have a shared history until the recent founding of the Isle Royale population. Both damaging and benign variants in Isle Royale wolves have shifted from the heterozygous to the homozygous state (Figure 3-7A, B). The proportion of homozygous derived genotypes was 4.07% higher (MWU $P=3.23 \times 10^{-4}$) in Isle Royale compared to the mainland for damaging mutations, and 3.84% higher (MWU $P=6.46 \times 10^{-4}$) for tolerated mutations (Figure 3-7A). However, the total proportion of derived alleles per genome across the Minnesota and Isle Royale wolves was unchanged for both damaging and benign mutations (MWU $P>0.52$) (Figure 3-7C). This is consistent with population genetic theory, as inbreeding distorts genotype frequencies, rather than allele frequencies. Further, because the founding of the Isle Royale population was very recent, it would not be expected to result in an accumulation of new deleterious variants entering the population through mutation or extensive drift of existing weakly deleterious variants. In other words, inbreeding depression in Isle Royale wolves cannot be explained by an increase in additive genetic load, which may be proportional to the number of derived deleterious variants per genome (Simons and Sella 2016). Rather, it must be accounted for by the increased homozygosity of deleterious variants.

Even strongly deleterious recessive alleles are expected to be present in standing genetic variation, segregating at low frequencies in large populations where drift is minimal and inbreeding is rare. In contrast, variants that are at high frequency in large populations of outbred wolves are not likely to be strongly deleterious. Thus, in the absence of appreciable gene flow with the mainland, we predicted that strongly deleterious recessive alleles carried in the founder genomes could have attained high frequency within the Isle Royale population due to drift. This phenomenon has been observed in the increased prevalence of rare genetic disorders in founder populations of humans (reviewed by Sheffield et al. 1998) and purebred dogs (reviewed by Sutter and Ostrander 2004). We compared the frequencies of segregating variants in Isle Royale wolves and mainland Minnesota wolves to those of outbred North American wolves by constructing two-dimensional allele frequency spectra, and performing linear regression to assess correlations between populations (Figure 3-8, Table 3-3). We found that variants that are low frequency in outbred wolves are also typically at low frequency in the Minnesota wolves, consistent with weak drift and efficient selection in the mainland population (Figure 3-8C, D, Table 3-3). In contrast, mutations in Isle Royale wolves had higher frequencies, due to the effects of isolation and high relatedness among individuals (Figure 3-8A, B). Further, for both damaging and benign variants, derived allele frequencies in Isle Royale were less correlated with allele frequencies in outbred wolves than were derived allele frequencies in Minnesota wolves, consistent with our prediction that the founder effect and inbreeding in Isle Royale wolves allowed damaging variants to attain high frequency ($P < 2.2 \times 10^{-16}$, Table 3-3).

We predicted that these damaging variants might account for the high incidence of vertebral anomalies observed in Isle Royale wolves. We used the following criteria to identify candidate deleterious variants underlying the phenotypes in our dataset: 1) variants should be

homozygous and located within ROH in the affected individuals (F65, F75, M152, M175, F189) but heterozygous or absent in the unaffected individual (M61); and 2) should also be at low frequency (<10%) among outbred wolves. 784 genes containing such mutations were found in at least one affected individual, while eight genes were found in all five affected individuals: *RTTN*, *AFM*, *SCUBE2*, *NDUFAF3*, *PLEKHM2*, *NCAPG*, *ENSCAFG00000001422*, and *ENSCAFG000000032301*. Only two of these genes, *RTTN* and *NDUFAF3*, have known associations with abnormal phenotypes. Mutation of *NDUFAF3* is associated with a fatal mitochondrial disease in humans (Saada et al. 2009), whereas *RTTN* (rotatin) notably affects vertebral development. *RTTN* is a large, highly conserved gene with 49 exons, spanning 144 kb on chromosome 1 of the dog genome.

Using a mouse model, researchers have found that *RTTN* plays an essential role in early embryonic development, specifically in left-right specification, embryo turning, and notochord formation (Faisst et al. 2002). Embryos with *RTTN* knocked out were inviable, but developed normally with one functional gene copy. The five Isle Royale wolves with vertebral malformations are homozygous for a C to T transition that converts a leucine residue to phenylalanine in exon 11 of *RTTN*. The SIFT score for this mutation is 0, indicating very strong conservation at this site, and that this mutation is therefore predicted to have a deleterious effect. No other homozygotes for this mutation were present in our dataset, whereas three other Isle Royale and two Minnesota wolves were heterozygotes. Of the 784 candidate genes we identified in the affected individuals, 22 are associated with the Human Phenotype Ontology term “abnormality of the vertebral column” (HP:0000925), but were not shared across all five affected individuals. Morphogenesis is a complex process, and the variation in phenotypes within the Isle Royale wolves suggests the involvement of multiple genes.

Testing models for the mechanistic basis of inbreeding depression

We hypothesized that the reason inbreeding depression afflicts Isle Royale wolves, but not other populations with a long-term history of small population size and isolation, may be due to differences in the prevalence of severely deleterious recessive alleles combined with recent inbreeding in Isle Royale. Theory predicts that the majority of strongly deleterious alleles segregating in large populations must be recessive, and recent work in *Arabidopsis* suggests deleterious mutations tend to be more recessive on average compared to less deleterious mutations (Huber et al. 2017). We conducted simulations in SLiM (Haller and Messer 2016) under a two-population model incorporating estimates of the long-term effective population sizes of outbred North American wolves ($N=17,300$) and Tibetan wolves ($N=2,500$), and the estimated split time between Old and New World wolves of 12,500 years (Figure 3-9A) (Fan et al. 2016). We simulated diploid individuals containing genomes of 1,000 “genes” that accumulated neutral and deleterious mutations, in order to compare the number of mutations per genome in each population. We conducted one set of simulations in which all mutations were additive ($h=0.5$), and one in which all were recessive ($h=0$), to explore the impact of dominance. Here, the North American wolf population represents the source population for Isle Royale wolves, which only became isolated fewer than one hundred years ago. The Tibetan population represents a population with long-term small size. We predicted that this larger population would contain more strongly deleterious recessive mutations per individual relative to the Tibetan population. These mutations in particular would severely compromise fitness in an individual that inherits two copies from a common ancestor through inbreeding.

Our simulations confirmed that strongly deleterious recessive alleles were more prevalent in the large North American population (4.09 per individual) than in the smaller Tibetan population (1.57 per individual) (MWU, $P=1.58 \times 10^{-14}$) (Figure 3-9B). However, moderately and weakly deleterious alleles accumulated and were more homozygous in the smaller population (MWU, $P=1.58 \times 10^{-14}$). These results are consistent with the prediction that selection is less effective at removing deleterious alleles when population sizes are small, even though strongly deleterious recessive alleles in particular are removed more effectively through increased homozygosity (Ohta 1973; Wang et al. 1999; Bataillon and Kirkpatrick 2000; Wang 2000; Glémin 2003). The number of strongly deleterious additive mutations did not differ between the simulated North American and Tibetan populations, since these mutations are immediately exposed to strong negative selection in populations of any size. Finally, we evaluated the average age of segregating mutations to confirm that strongly deleterious recessive alleles may persist in large populations for a longer time. Segregating strongly deleterious recessive alleles in the North American population were, on average, 2.38 times older than these mutations in the Tibetan population ($P<2.2 \times 10^{-16}$). In sum, we found that a smaller population, such as Tibetan wolves, has fewer strongly deleterious recessive alleles, and that these mutations can persist in large populations, such as North American wolves. Thus, inbreeding is expected to produce more individuals homozygous for strongly deleterious mutations in individuals drawn from a large population compared to individuals drawn from a smaller population. Thus, because the Isle Royale wolves were recently founded from a large population of wolves in the Great Lakes region, and then experienced extensive inbreeding, our simulations suggest they should have more strongly deleterious recessive mutations in the homozygous state, resulting in increased inbreeding depression.

Finally, we considered whether the overdominance model might account for inbreeding depression in Isle Royale wolves. Although we cannot explicitly reject this model given the small number of individuals in our dataset, the overdominance model is unlikely to be the basis of inbreeding depression in Isle Royale wolves for at least four reasons. First, Isle Royale wolves were not the least heterozygous among the wolves in this dataset, either at the level of the whole genome or specifically in coding regions (Figures 3-3 and 3-6). Second, there was no obvious relationship between spinal malformation and heterozygosity in the phenotyped Isle Royale wolves. For example, the only wolf free of any defects (M61) actually had the lowest heterozygosity among the six phenotyped individuals (Table 3-1). Admittedly, our sample size is too small to draw strong conclusions about the relationship between heterozygosity and vertebral defects in the phenotyped wolves. Third, a corollary of the overdominance model is that the loci involved in inbreeding depression are subject to ongoing balancing selection in healthy populations, including populations with long-term small size such as the Tibetan and Ethiopian wolves. Like other forms of selection, balancing selection is less effective when population sizes are reduced and genetic drift dominates. Finally, as stated above, inbreeding depression typically results in the manifestation of rare Mendelian disorders. The genes underlying these diseases, which negatively affect development, reproduction, and survival, are not likely to be under balancing selection, but are highly conserved under strong purifying selection (Kimura 1968; Domazet-Loso and Tautz 2008; Cai et al. 2009).

Discussion

Wolves on Isle Royale were once used as evidence that a very small population in isolation may persist, and even thrive, without succumbing to genetic deterioration (Mech and

Cronin 2010; Harding et al. 2016). During the past few decades, however, Isle Royale wolves have experienced a precipitous decline across generations of inbreeding and physical degeneration (Räikkönen et al. 2009; Hedrick et al. 2014). Although inbreeding depression was not the sole determinant, it has certainly played a role in the collapse of the population, along with stochastic demographic and environmental events, such as periodic disease outbreaks, severe winters, and the drowning of three wolves in a flooded abandoned mine shaft in 2011 (Hedrick et al. 2014). The genomes of Isle Royale wolves bear the hallmarks of their extreme demographic history, characterized by extensive ROH, in some cases spanning whole chromosomes, leading to a marked increase in the homozygosity of deleterious variants without increasing the total number of deleterious variants per genome.

This increase in homozygosity has had profound effects on the morphology of Isle Royale wolves, including malformed vertebrae, extra vertebrae, and extra ribs. Other abnormalities have also been observed in Isle Royale wolves, including syndactyly, cataracts, a shortened tail, an unusual “rope tail” phenotype, and anomalous fur phenotypes (Räikkönen et al. 2009, Peterson and Vucetich 2015). Although reduced longevity and reproduction have not been linked to inbreeding in Isle Royale wolves, studies from inbred captive wolves have found that a 10% increase in inbreeding results in a 10.8% decrease in longevity and a 6.8% decrease in the number of pups per litter (Laikre and Ryman 1991). In wild Scandinavian wolves, a 10% increase in inbreeding led to a 17.6% reduction in the number of pups surviving to their first winter (Liberg et al. 2005). The individual with the highest homozygosity among our sequenced Isle Royale wolves, M141 ($F_{ROH}=0.47$), lived only two years and did not reproduce (Hedrick et al. 2014). A female wolf, F75 ($F_{ROH}=0.42$), died at four years of age while giving birth to a litter of pups presumably sired by her own father (Hedrick et al. 2014). The pedigree-based inbreeding

coefficient of this litter was 0.375, and on examination it was found that all 8 pups had congenital vertebral changes, and all but one had extra ribs (Hedrick et al. 2014). Reproduction within the population has ceased, and has fallen from 30 individuals to only two over the past eleven years (Peterson and Vucetich 2017). The population of moose on Isle Royale, the wolves' main prey, has ballooned from 450 to 1600 individuals over the same period (Peterson and Vucetich 2017). Thus the demise of wolves on Isle Royale cannot be attributed to lack of available prey. The observation that the Arctic wolf from Ellesmere Island had a genome remarkably similar to those of Isle Royale wolves in terms of its ROH content suggests that severe inbreeding depression does occur in wild populations, and that Isle Royale is not a singular exceptional case.

Notably, wolves from other populations with more homozygous genomes, but with shorter ROH, are not afflicted with inbreeding depression. This can be explained by the fact that long ROH indicate recent inbreeding, and may therefore harbor newly generated homozygous strongly deleterious recessive mutations previously hidden from selection as heterozygotes. ROH that have been broken down into shorter segments by recombination have been exposed to selection over a longer time period, and are therefore less likely to contain these strongly deleterious alleles (Szpiech et al. 2003). This is consistent with our simulated results, which suggest that the risk of inbreeding depression is higher for genomes recently originating from a historically large population, as they carry a greater burden of strongly deleterious recessive mutations. These mutations are carried as heterozygotes within the founders, but become homozygous in the island population, resulting in inbreeding depression. Taken together, our empirical and simulated results strongly support the partial dominance hypothesis of inbreeding depression.

It has been suggested that the number of derived deleterious alleles is proportional to the additive genetic load and is thus a useful statistic for quantifying the genetic load of a population (Simons et al. 2014; Do et al. 2015; Simons and Sella 2016). In our study, we found no difference between the number of derived deleterious alleles in the Isle Royale wolf population compared to the mainland Minnesota population. This would imply no difference in additive genetic load between the populations. Clearly, however, the Isle Royale population has reduced fitness due to inbreeding depression. Our findings suggest that the additive genetic load may be the same between both populations, but the reduction in fitness on Isle Royale is due to recessive mutations, which is supported by other lines of evidence as we discuss. Alternatively, it is possible that additive genetic load is truly higher on the Isle Royale population, but we are unable to detect this increased load due to the difficulties of determining which amino acid changing variants are truly deleterious (Pedersen et al. 2017). In either scenario, our findings suggests that populations with a similar number of derived deleterious alleles and similar heterozygosity may still differ in their genetic load, and additional metrics should be used to quantify the load across populations.

A key statistic of interest is the burden of strongly deleterious recessive alleles within a genome. Currently, there is no method for obtaining this value for a wild population or inferring the fitness of an organism based solely on its genomic content. We indirectly infer that inbreeding depression in Isle Royale wolves is caused by strongly deleterious recessive alleles, but genotype and allele frequencies from a large sample would be required to confirm whether a putatively deleterious mutation remains rare and is likely to be recessive in outbred wolves. In humans, it has been estimated that each diploid genome carries ~1-2 recessive lethal mutations, but the number of recessive sub-lethal mutations that nonetheless compromise fitness is likely to

be higher (Gao et al. 2015). Although our simulations affirm that purging may take place in smaller populations, purging has not protected the Isle Royale wolf population. In fact, the purging of strongly deleterious mutations in Isle Royale wolves is likely to have contributed to their demise by further reducing the population size. Others have explored the impact of the rate of inbreeding, and found that the risk of extinction is higher under “fast” inbreeding relative to “slow” inbreeding (Ehiobu et al. 1989; Latter et al. 1995; Fu et al. 1998; Wang et al. 1999; Reed and Bryant 2000; Day et al. 2003). The wolf population on Isle Royale typifies the fast inbreeding regime, and its unfavorable consequences, while the Tibetan and Ethiopian wolves may have undergone slow inbreeding, in which selection eliminates strongly deleterious alleles gradually without causing extinction.

Thus, the development of long ROH must be avoided to minimize the risk of inbreeding depression, particularly for species with historically large population sizes. An estimate of the diversity within a population and thus its historic effective size can now readily be deduced from even a single genome, given the availability of heterozygosity estimates in a wide array of species, and with existing and emerging tools (Romiguier et al. 2014; Corbett-Detig et al. 2015; Li and Durbin 2011). In this study, we found that the distribution of heterozygosity across the genome of each wolf revealed a clear signature of the population’s demographic history. Even for a species with a historically large population size, the minimization of ROH formation could be accomplished with tailored management actions, for either wild or captive populations. In many cases, captive populations of threatened species are managed to reduce inbreeding by keeping detailed studbooks and pairing individuals with low kinship (Ballou et al. 2010). This practice may now be augmented through sequencing the complete genomes of potential breeders, enabling the selection of individuals based on their genomic profiles. When a detailed pedigree is

unavailable, genome sequences or high density SNP information can be used to calculate the realized inbreeding within a genome, and to construct a pedigree if desired. Though pedigrees provide the expected values of kinship and IBD, genomic methods provide the realized values, which this study and others have shown is typically higher than the expected values (Broman and Weber 1999; Kardos et al. 2015; Knief et al. 2015). Individuals can be screened directly for ROH, and to assess the pairwise genomic distance between potential mates, essentially maximizing the diversity within any breeding pair and thus, its offspring. In this way, inbreeding may even be reversed under careful management, provided the remaining individuals retain some variability.

In wild populations, establishing or maintaining corridors is critical for permitting gene flow in a fragmented landscape to offset the detriment of inbreeding (Couvét 2002). Previous genetic analysis of Isle Royale wolves revealed that undetected migration may have been occurring in years when the winter was cold enough for an ice bridge to form (Adams et al. 2011; Hedrick et al. 2014). However, warmer winters over the past several decades have resulted in a dramatic reduction in the formation of ice bridges, a trend that is likely to continue in a warming climate (Hedrick et al. 2014). The reported genetic rescue of the Isle Royale wolf population by a single migrant from the mainland in 1997 also appears to have been short-lived (Hedrick et al 2014). This male wolf was such a successful breeder that his genome effectively swamped the population, leading to intense inbreeding in his descendants within two generations (Figure 3-1A). A similar episode occurred in the inbred Scandinavian wolf population, resulting in the same outcome (Wabakken et al. 2001; Vilà et al. 2003; Liberg et al. 2005; Bensch et al. 2006). A higher rate of gene flow between the mainland and the island may potentially have staved off inbreeding depression, but the effective rate of gene flow into Isle Royale was clearly

insufficient. Assisted gene flow may therefore be the only option for the persistence of wolves on Isle Royale.

Consideration of life history traits should also be taken into account to determine the best course of action to reduce the risk of inbreeding. For example, wolves typically avoid mating with close relatives through the exchange of individuals between different packs, which are usually familial units made up of a breeding pair and its offspring (Smith et al. 1997). Previously, Isle Royale was home to 3-4 wolf packs, hence the estimate that the long-term effective population size of wolves on Isle Royale was a mere 3.8 individuals (Peterson et al. 1998). Results from simulations under models incorporating ecological and demographic stochasticity found that the mean time to extinction for social organisms, specifically wolves, is strongly tied to the number of social groups rather than the number of individuals (Vucetich et al. 1997). Thus, a minimum number of wolves to maintain multiple packs, and therefore maximize the number of breeding individuals, is essential.

A vigorous ongoing discussion concerns whether wolves should be reintroduced to the island (Gore et al. 2011; Vucetich et al. 2012; Mech et al. 2017). The fate of a reintroduced population is grim, given the inevitability of inbreeding on Isle Royale and its proven detrimental outcome. On the other hand, wolf predation is an important top-down influence on Isle Royale, and its absence threatens the stability of the island ecosystem (Peterson et al. 2014). The ethics and wisdom of wolf reintroduction to Isle Royale are beyond the scope of this paper, but our study provides the basis for several recommendations for a reintroduction that may improve its outcome, should it take place. First, reintroduction should involve the release of multiple unrelated wolves, not a single breeding pair, as in the initial founding, to establish a more diverse population at the outset. Genetic diversity within the population will inevitably decline due to

genetic drift, however an introduction of many wolves rather than a small number will delay the onset of severe inbreeding. Thereafter, the population must remain large enough to sustain multiple packs. New individuals should periodically be introduced to bolster the population and to mitigate the otherwise inevitable decline of genetic diversity and fitness. Our study reiterates the critical importance of maintaining effective population sizes large enough to allow selection to remove deleterious variants. It is now too late to resurrect a population from the lone remaining pair of wolves on Isle Royale, but the demise of the population provides numerous lessons for a potential future wolf reintroduction program, and guidance for the management of other species or populations to minimize the risk of inbreeding depression.

Materials and Methods

Samples and Sequencing

DNA from Isle Royale wolves was extracted from blood samples archived at Michigan Technological University. DNA from Minnesota and Canadian Arctic wolves was extracted from blood and tissue samples from the archive of Dr. Robert Wayne that were used in previous studies (vonHoldt et al. 2011, Schweizer et al. 2016). Sequencing was performed on Illumina HiSeq machines. The pipeline used to convert raw sequence data into high quality genotypes was based on the methods described in Robinson et al. (2016). Briefly, raw reads were aligned to the domestic dog genome using bwa MEM (Li 2013), before removal of PCR duplicates and low quality reads. Base quality score recalibration and genotyping were performed with the Genome Analysis Toolkit (GATK, McKenna et al. 2010). Genotypes were filtered for quality and depth, leaving only high quality biallelic SNPs, and annotated using the Ensembl Variant Effect Predictor and the Sorting Intolerant From Tolerant (SIFT) program (Kumar et al. 2009; McLaren

et al. 2010). SIFT determines whether a non-synonymous mutation is likely to be damaging or benign on the basis of amino acid conservation at a site across taxa.

Calculation of Heterozygosity and Identification of Runs of Homozygosity

In this study, we define heterozygosity as the number of heterozygous genotypes divided by the total number of called genotypes. We calculated heterozygosity for the entire autosomal genome, as well as in non-overlapping 1 Mb windows across the autosomes. Windows with more than 80% missing data were excluded. ROH were identified using VCFtools (Danecek et al. 2011). ROH spanning regions with fewer than 50 variant sites were excluded. Genes contained within ROH were identified using gProfileR (Reimand et al. 2011).

Simulations of neutral and deleterious variation

Simulations were carried out in SLiM (Haller and Messer 2016) under a divergence model with parameters estimated by Fan et al. (2016). Each simulated individual consisted of a diploid 1 Mb genome, with a simple architecture of 1,000 “genes” carried on 38 chromosomes proportional to chromosome lengths in the dog genome. Each gene consisted of a contiguous 1 kb sequence that accumulated mutations at a rate of 1×10^{-8} per site per generation. Selection coefficients for deleterious mutations were drawn from the distribution of fitness effects inferred by Kim et al. (2017). 70% of mutations were deleterious, and the remaining 30% were selectively neutral. Each simulation began with a burn-in period of 450,000 generations to allow the ancestral population to reach equilibrium. Recombination was permitted at single base positions between each gene at a rate of 1×10^{-3} per site per generation, to simulate the effective rate of crossing over that would occur in 100 kb noncoding regions between each gene. At the

end of each simulation, the average number of alleles per individual and the average age of segregating mutations were calculated for strongly ($-1 \leq s < -0.01$), moderately ($-0.01 \leq s < -0.001$), weakly deleterious ($-0.001 \leq s < 0$), and neutral mutations ($s=0$). We performed 25 replicates in which mutations were additive ($h=0.5$) and 25 replicates in which mutations were completely recessive ($h=0.0$), to examine the effects of dominance.

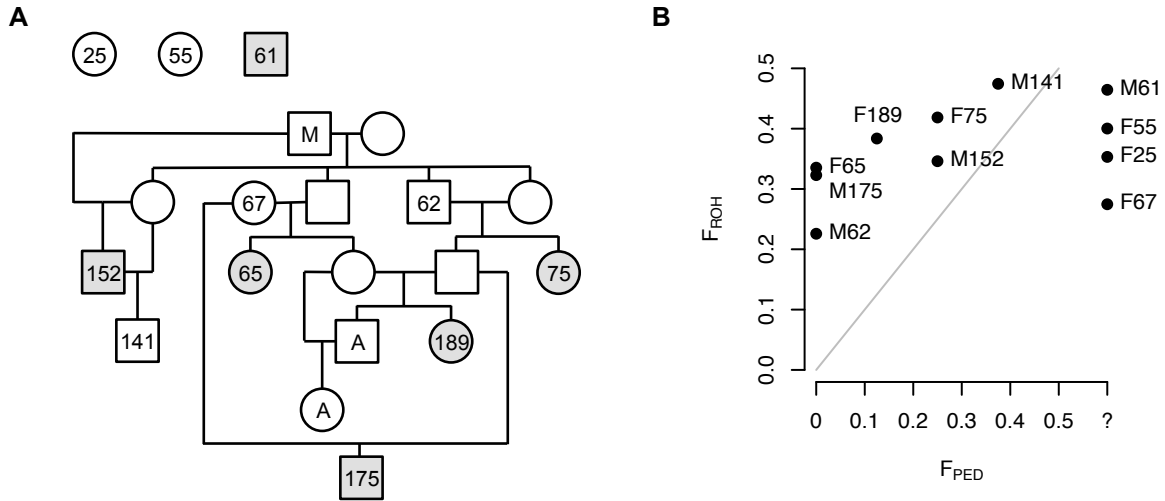


Figure 3-1. (A) Pedigree of Isle Royale wolves sequenced in this study, adapted from Hedrick et al. (2014). Circles represent females and squares represent males. Relationships were inferred from genotypes at 18 microsatellite loci. Shaded individuals were examined for the presence of vertebral abnormalities for this study (Table 3-1). The ancestry of F25, F55, M61, and F67 is unknown. “M” was a wolf that migrated from the mainland in 1997 (Adams et al. 2011). The “A” individuals are the last two wolves alive on Isle Royale as of 2017 (Hedrick et al. 2014; Peterson and Vucetich 2017). (B) The relationship between pedigree-based inbreeding coefficients (F_{PED}) and the proportion of the genome contained within ROH ≥ 100 kb (F_{ROH}). The grey line shows the diagonal.

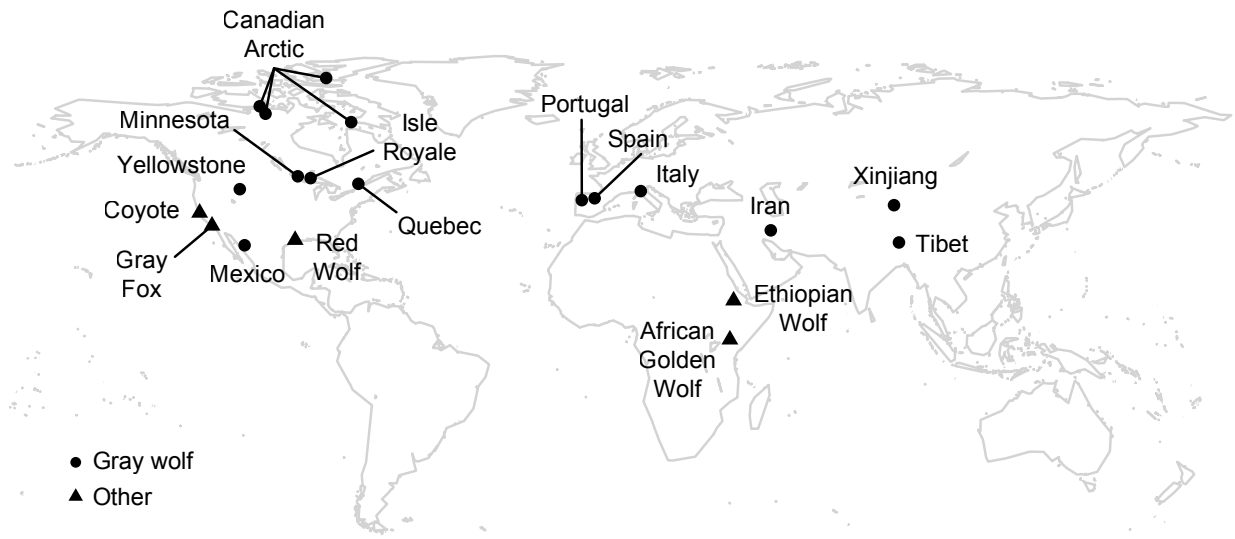


Figure 3-2. Map showing approximate origins of all individuals included in this study. See Table 3-2 for further sample information.

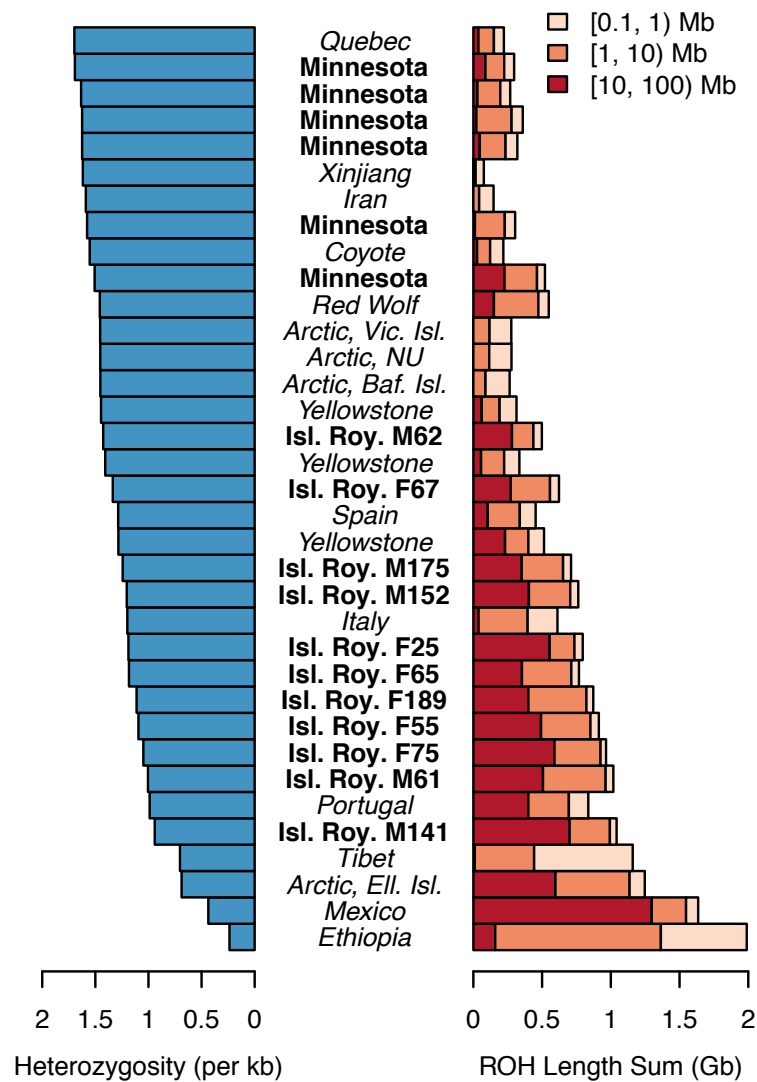


Figure 3-3. Left: Per-site autosomal heterozygosity across the autosomal genome. Samples are ordered by decreasing heterozygosity from top to bottom. Right: Sum of the lengths in megabases for short ($0.1 \text{ Mb} \leq \text{ROH} < 1 \text{ Mb}$), medium ($1 \text{ Mb} \leq \text{ROH} < 10 \text{ Mb}$), and long ($10 \text{ Mb} \leq \text{ROH} < 100 \text{ Mb}$) ROH.

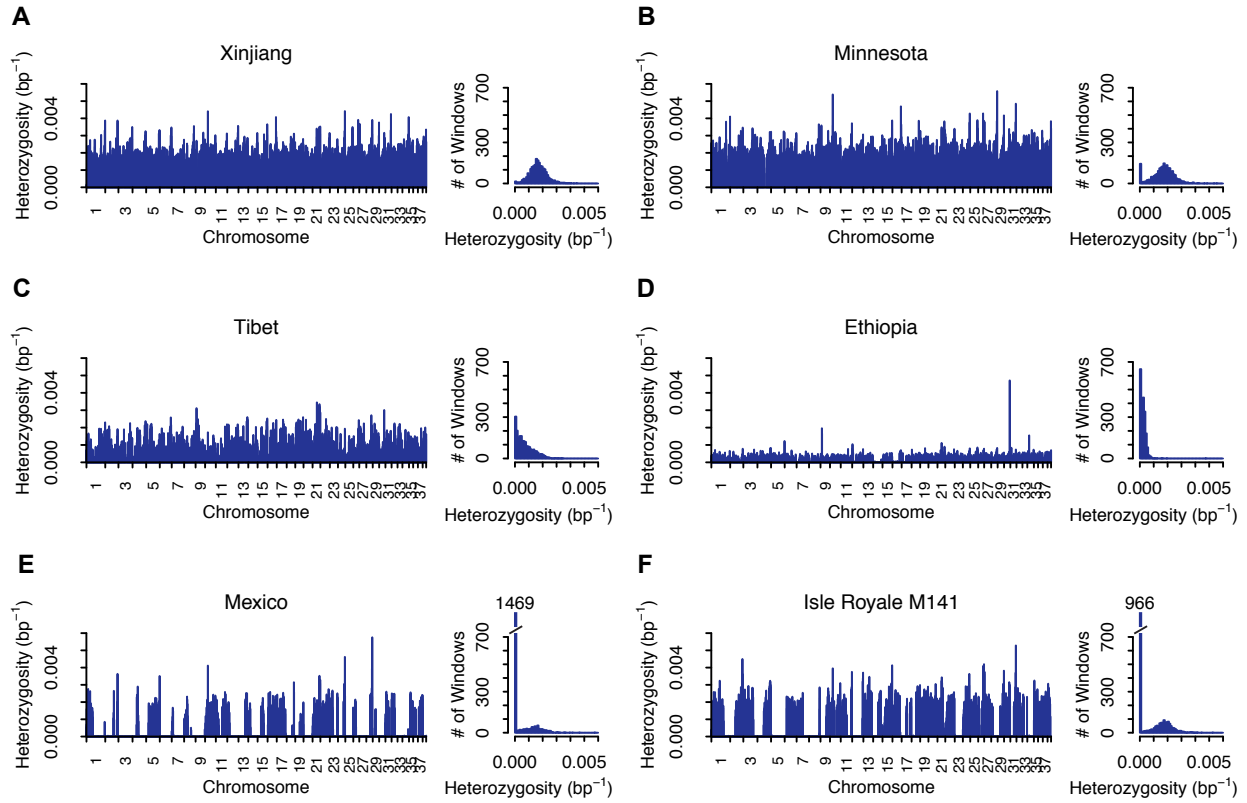


Figure 3-4. Example barplots showing per-site heterozygosity in non-overlapping 1 Mb windows across the autosomal genome, with histograms of per-window heterozygosity shown on the right. (A, B) The Xinjiang and Minnesota wolves represent large, outbred populations. (C, D) The Tibetan and Ethiopian wolves represent small, isolated populations. (E, F) The Mexican and Isle Royale wolves represent populations with recent inbreeding.

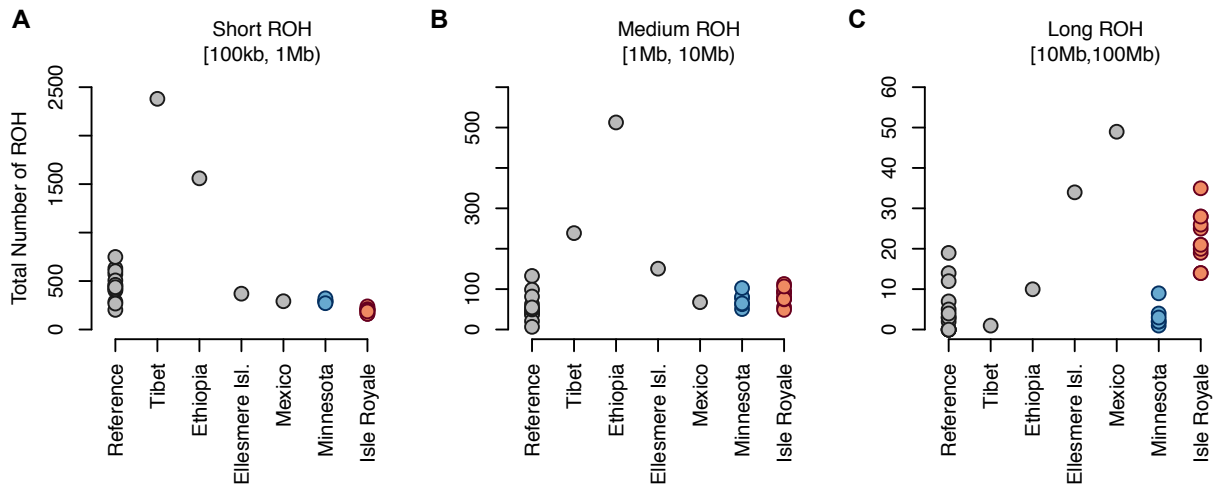


Figure 3-5. The number of ROH in various size classes is indicative of demographic history. (A) Short ROH indicate ancient inbreeding, as in the Tibetan wolf. (B) Medium ROH indicate ancient and historic inbreeding, as in the Ethiopian wolf. (C) Long ROH indicate recent inbreeding, as in the Mexican, Isle Royale, and Ellesmere Island wolves.

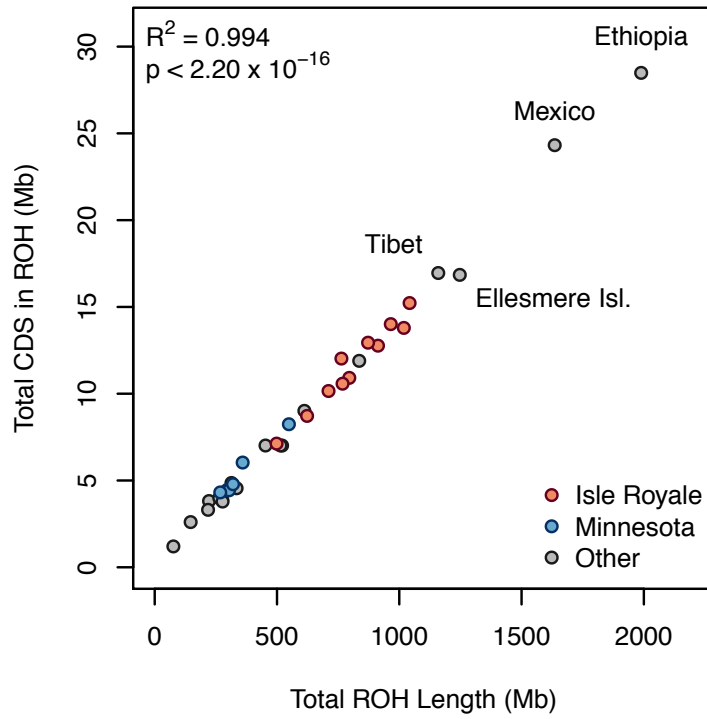


Figure 3-6. There is a strong linear correlation between the total amount of the genome contained within ROH and the total amount of coding DNA sequence (CDS) contained within ROH.

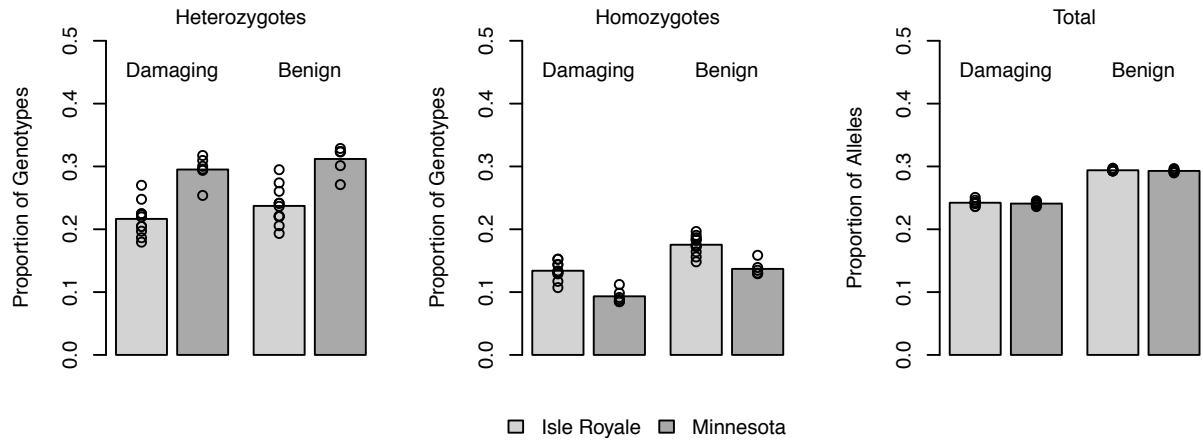


Figure 3-7. (A) Inbred Isle Royale wolves contained significantly fewer heterozygotes and, (B) significantly more homozygotes than mainland Minnesota wolves, for both damaging and benign SNPs. (C) The total number of derived alleles is unaffected by recent inbreeding.

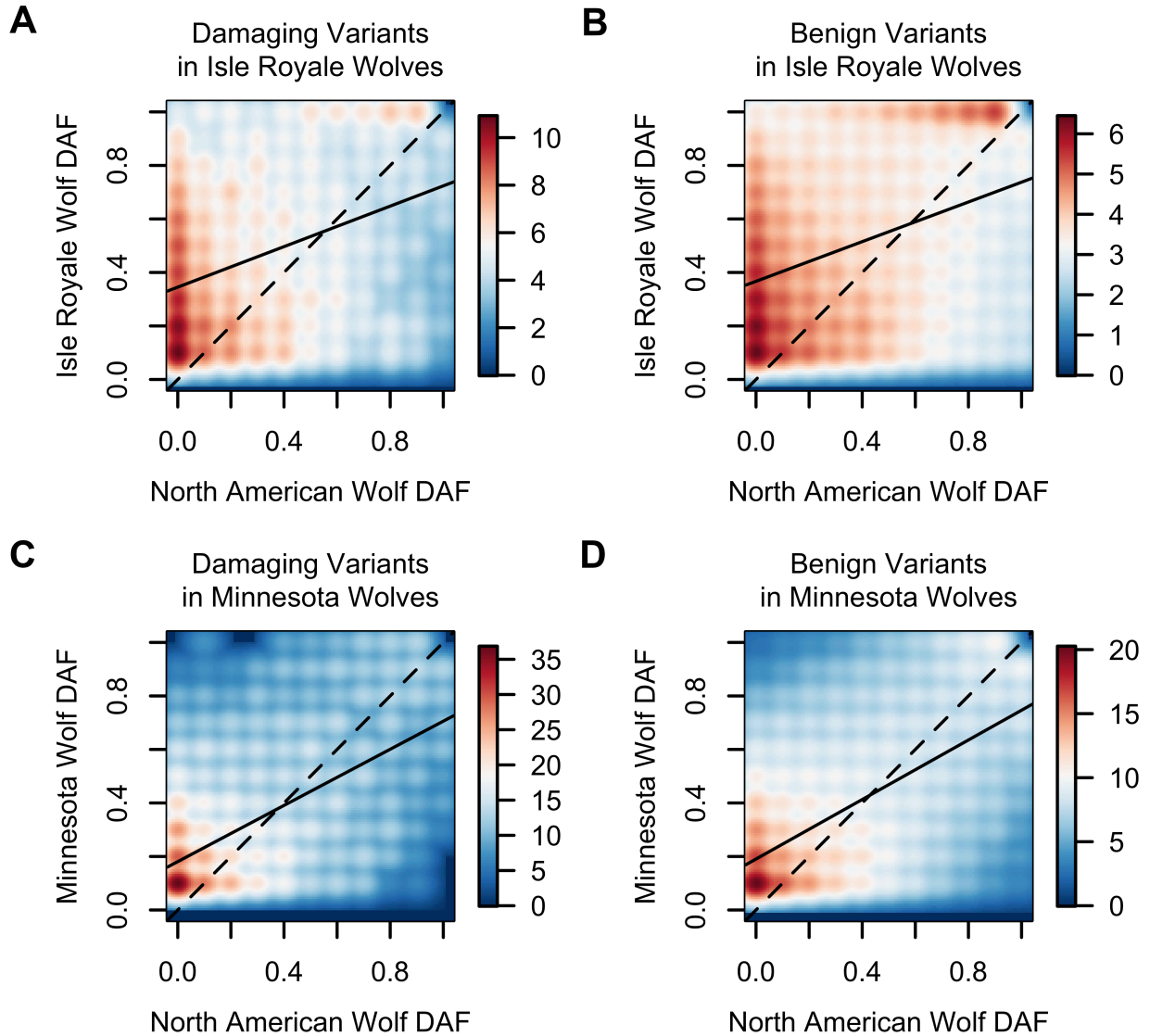


Figure 3-8. Two-dimensional allele frequency spectra showing the correlation in derived allele frequencies (DAF) between North American wolves (Quebec, Yellowstone, Canadian Arctic excluding Ellesmere Island) and Isle Royale or Minnesota wolves, for variants present in Isle Royale or Minnesota wolves. All sites were downsampled to include exactly five individuals from each group. Color represents the density of points (see legends). The dashed line represents the diagonal, whereas the solid line represents the linear regression line (see Table 3-3). (A, B) The frequency of derived alleles is higher in Isle Royale wolves than in outbred North American wolves, for both damaging and benign SNPs, relative to mainland Minnesota wolves (C, D).

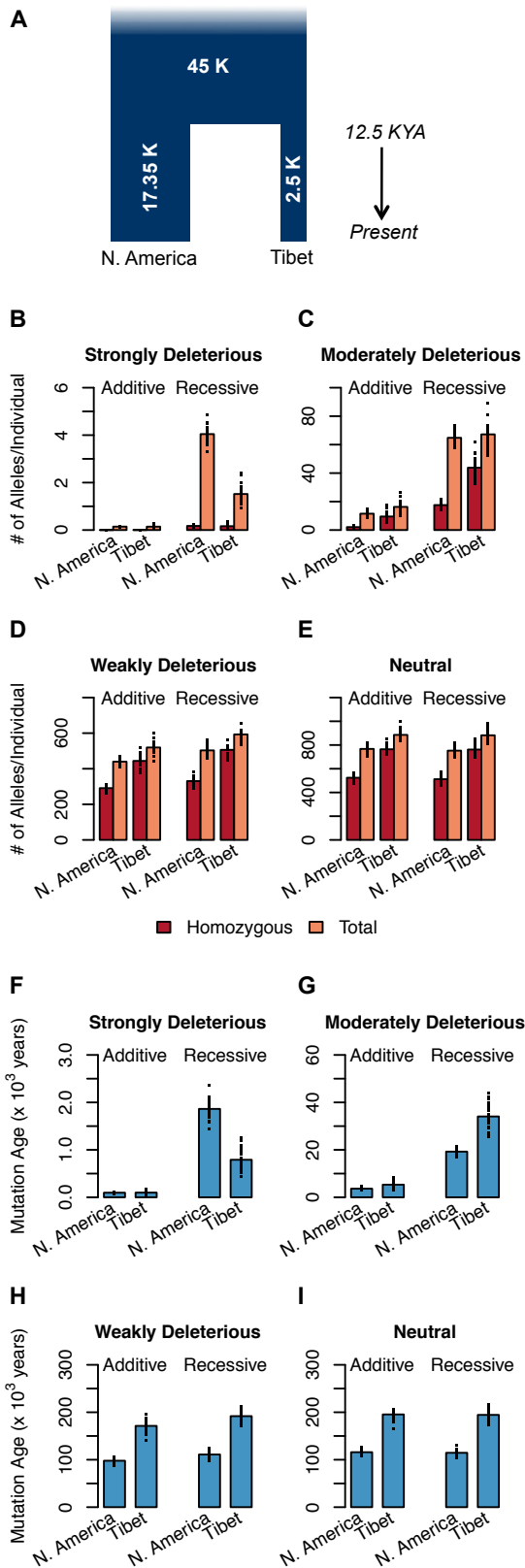


Figure 3-9. (A) Demographic model used to simulated the expected number of mutations in a large population (N. America, 17.35×10^3 individuals) versus a small population (Tibet, 2.5×10^3 individuals). Both populations derive from a large ancestral population (45×10^3 individuals) 12.5×10^3 years ago (3-year generation time). Population sizes and split time from Fan et al. (2016). (B-I) Results from simulations, grouped according to dominance and selection coefficients. Additive: $h=0.5$, recessive $h=0$; strongly deleterious, $-1 \leq s < -0.01$; moderately deleterious, $-0.01 \leq s < -0.0001$; weakly deleterious, $-0.0001 \leq s < 0$; neutral: $s = 0$. (B-E) The average number of homozygous and total mutations per individual. (F-I) The average ages (in years) of segregating alleles in each population.

Table 3-1. Isle Royale wolves sequenced for this study and their inbreeding coefficients. F_{PED} is the pedigree-based inbreeding coefficient (Figure 3-1), and F_{ROH} is the proportion of the genome contained within runs of homozygosity (Figure 3-1B). Six individuals were examined for vertebral defects, and their phenotypes are listed. LSTV: lumbosacral transitional vertebra, SCTV: sacrococcygeal transitional vertebra, TLTV: thoracolumbar transitional vertebra.

ID	Year Sampled	F_{PED}	F_{ROH}	Vertebrae examined	Phenotype
F25	1988	?	0.35	N/A	N/A
F55	1998	?	0.40	N/A	N/A
M61	2001	?	0.46	Atlas-Sacrum	No abnormalities
M62	2001	0	0.23	N/A	N/A
F65	2003	0	0.34	Atlas-T12,L1-Co3	LSTV
F67	2003	?	0.27	N/A	N/A
F75	2007	0.25	0.42	Atlas-Co16	LSTV, TCTV, SCTV, +2 ribs, osteophytes
M141	2009	0.375	0.47	N/A	N/A
M152	2009	0.25	0.35	Atlas-Sacrum	TCTV, SCTV, +1 rib, osteophytes
M175	2009	0	0.32	Atlas-Co9	LSTV, TCTV, SCTV, osteophytes
F189	2012	0.125	0.38	Atlas-Co2	TCTV, SCTV, +1 vertebra, cervical intrasegmental transitional & asymmetry

Table 3-2. Complete set of genomes used in this study, and information about their demographic history from the literature.

Species	Origin	N	Description	Genome Source	References
North American gray wolf (<i>Canis lupus</i>)	Isle Royale NP, Michigan, US	11	Small insular population, naturally colonized by wolves from nearby mainland, inbred	This study	Mech 1966; Peterson & Page 1988; Gray et al. 2009; vonHoldt et al. 2011
	Minnesota, US	6	Large mainland population near Isle Royale with history of coyote admixture	Fan et al. 2016, this study	Gray et al. 2009; vonHoldt et al. 2011, 2016
	Quebec, Canada	1	Large mainland population with history of coyote admixture	This study	Gray et al. 2009; vonHoldt et al. 2011, 2016
	Canadian Arctic (Baffin Isl., Ellesmere Isl., Nunavut, Victoria Isl.)	4	Large, outbred population, some indication of reduced diversity in Ellesmere Island population	This study	Carmichael et al. 2007, 2008; Musiani et al. 2007; Gray et al. 2009
	Yellowstone NP, Wyoming, US	3	Reintroduced population of wolves derived from Canada	Fan et al. 2016, Schweizer et al. <i>in prep</i>	vonHoldt et al. 2008; Gray et al. 2009
	Mexico	1	Highly endangered wolf, captive management, inbred	Fan et al. 2016	Hedrick et al. 1997; Kalinowski et al. 1999; Fredrickson et al. 2007
Old World gray wolf (<i>Canis lupus</i>)	Portugal	1	Small isolated population, recent bottleneck	Fan et al. 2016	Sastre et al. 2011; Fan et al. 2016
	Spain	1	Large population with recent decline	Fan et al. 2016	Ramirez et al. 2006; Gray et al. 2009; Fan et al. 2016
	Italy	1	Isolated population, historic bottleneck	Fan et al. 2016	Lucchini et al. 2004; Fabbri et al. 2007; Gray et al. 2009; Pilot et al. 2006; Fan et al. 2016
	Iran	1	Large, outbred population	Fan et al. 2016	Fan et al. 2016
	Xinjiang, China	1	Large, outbred population	Zhang et al. 2014	Zhang et al. 2014; Fan et al. 2016
	Tibet, China	1	Historically small, isolated population	Zhang et al. 2014	Zhang et al. 2014; Fan et al. 2016
Ethiopian wolf (<i>Canis simensis</i>)	Ethiopia	1	Historically isolated, fragmented population in decline	Unpublished, from Dr. Thomas Gilbert, University of Copenhagen	Gottelli et al. 1994, 2004
Coyote (<i>Canis latrans</i>)	California, US	1	Large expanding population, no history of wolf admixture	Fan et al. 2016	Roy et al. 1994
Red wolf (<i>Canis rufus</i>)	Southeastern US	1	Endangered coyote-like canid, likely hybrid origin, captive management	Fan et al. 2016	Roy et al. 1994; Kalinowski et al. 1999; vonHoldt et al. 2011, 2016
African golden wolf (<i>Canis anthus</i>)	Kenya	1	Outgroup to polarize ancestral v. derived alleles	Koepfli et al. 2015	Koepfli et al. 2015
Gray fox (<i>Urocyon cinereoargenteus</i>)	California, US	1	Outgroup to polarize ancestral v. derived alleles	Robinson et al. 2016	Robinson et al. 2016

Table 3-3. Table showing the parameters of linear regressions performed to assess derived allele frequency correlations between North American wolves and Isle Royale or Minnesota wolves (Figure 3-8).

Populations	Type	Intercept	Slope	R²	P-value
Isle Royale wolves v. North American wolves	Damaging	0.345	0.379	0.131	<2.2e-16
	Benign	0.367	0.370	0.138	<2.2e-16
Minnesota wolves v. North American wolves	Damaging	0.181	0.525	0.353	<2.2e-16
	Benign	0.190	0.556	0.400	<2.2e-16

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