

Highlights

- Genomic monomorphism on a scale not seen previously in eukaryotes is shown
- Deleterious variation and loss-of-function mutations are not purged
- Drift, without balancing selection, accounts for peaks of heterozygosity
- Persistence despite the absence of variation questions its role in small populations

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Genomic Flatlining in the Endangered Island Fox

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raphy 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 1A and 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 [6, 8, 9]. Each of these island populations represents a morphologically and genetically distinct subspecies, four of which (San Miguel, Santa Rosa, Santa Cruz, and Santa Catalina) have been listed as endangered under the US Endangered Species Act after catastrophic declines due to predation by non-native golden eagles (*Aquila chrysaetos*) and introduced canine distemper virus [10–12]. 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 ~13–23× coverage (Table S1).

Genome-wide autosomal heterozygosity is high for the mainland gray fox (12.0×10^{-4} /bp), whereas heterozygosity in island foxes is reduced by 3- to 84-fold ($4.08\text{--}0.142 \times 10^{-4}$ /bp; Figures 1A and S1). The most extreme reduction of heterozygosity is found in the San Nicolas population ($0.142\text{--}0.190 \times 10^{-4}$ /bp), which has a genome that is almost entirely monomorphic (Figure 2A). 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 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 [6, 8, 9]. 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 1A) [6]. 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.

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 demog-



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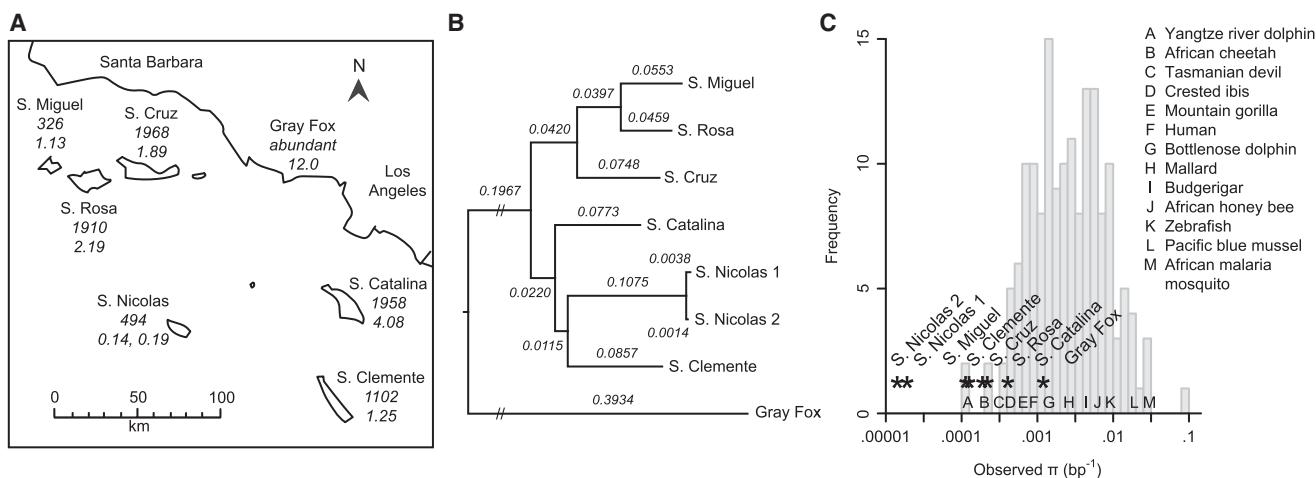


Figure 1. Island Foxes Show Exceptionally Low Heterozygosity

(A) Map showing island geography, estimated census size at time of sampling (upper number) [6], and genome-wide heterozygosity per 10 kb of autosomal sequence (lower number). See also [Figure S1](#).
 (B) Neighbor-joining tree constructed from a genome-wide pairwise distance matrix, displaying reciprocal monophly 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. See also [Figure S2](#).
 (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; see the [Supplemental Experimental Procedures](#)), with the position of island and gray fox heterozygosity values indicated by asterisks. See also [Data S1](#).

Indeed, an extreme population bottleneck most likely occurred in the San Nicolas population in the early 1970s [7, 13]. 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 the [Supplemental Experimental Procedures](#)). Based on previous genetic analyses [6–9, 14] and our inferred phylogenetic tree ([Figure 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 S2](#) and [S3](#)). 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 after 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 S3](#)). 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 [3–5, 15], 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 3A](#)). 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 [15, 18]. 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 3B](#)). 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 [16], are sufficient to explain the observed ratios ([Figure 3B](#)).

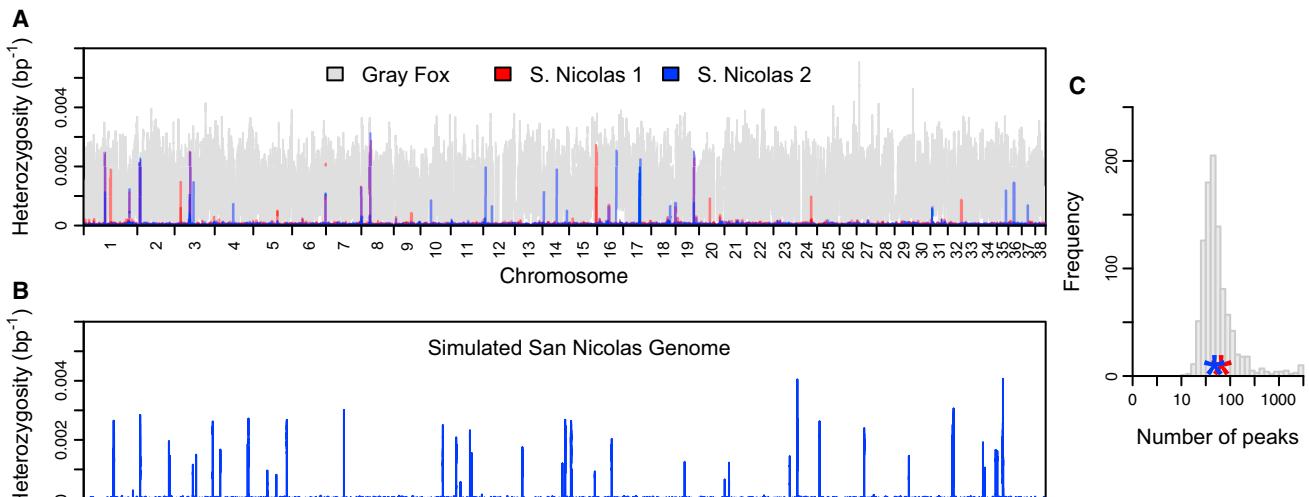


Figure 2. Distribution of Peaks of Heterozygosity across the Island and Mainland Fox Genomes

(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. See also [Figure S1](#).

(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. See also [Figure S3](#).

(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 ($p = 0.257\text{--}0.451$). See also [Data S2](#).

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 3C](#)). We utilized the prediction algorithm *sorting intolerant from tolerant* (*SIFT*) [17], which estimates whether 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 (deleterious; *SIFT* score < 0.05), whereas others are more likely to be tolerated (tolerated; *SIFT* score ≥ 0.05). We also cataloged loss-of-function mutations, as these mutations have a high probability of impacting fitness by reducing or eliminating gene functionality [19, 20]. 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 3C](#)). 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 is-

land populations than in the gray fox ($p < 0.007$; [Table S2](#)), 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 3C](#); [Table S2](#)), implying a substantial genetic load, particularly if loss-of-function variants are recessive [21, 22]. The general increase in loss-of-function alleles in all of 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 [7]. 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 2 SDs above the mean heterozygosity in a sliding window analysis across the autosomes were considered to be elevated ([Figures 2A](#) and [S1](#)). 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 S3](#)). In the two San Nicolas fox sequences and the mainland gray

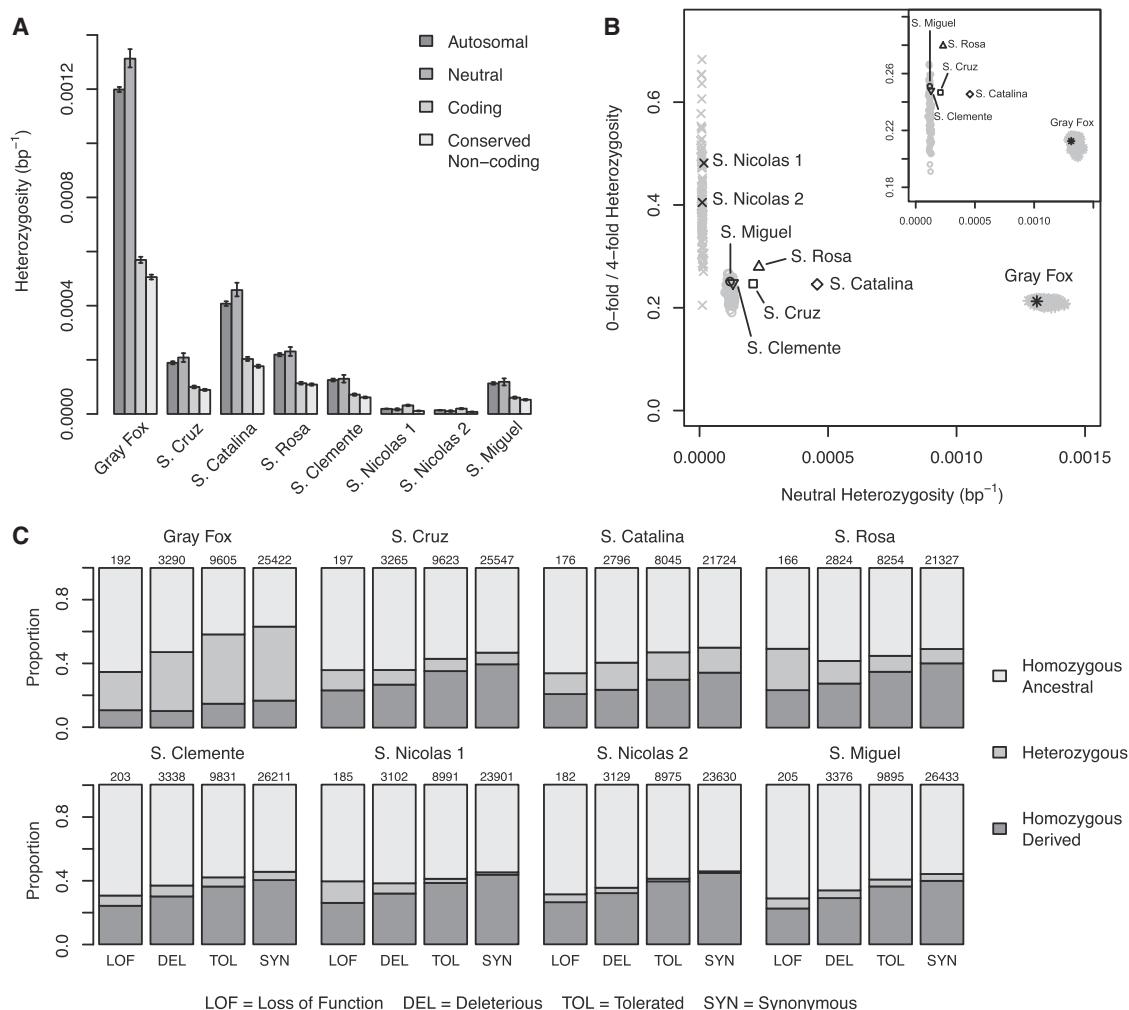


Figure 3. Extreme Drift in Island Foxes Reapportions Deleterious Genetic Variation

(A) In all foxes except San Nicolas individuals, autosomal and neutral diversity is higher than in regions putatively under purifying selection (coding and conserved non-coding sequences). Error bars represent 95% confidence intervals determined from bootstrap resampling. Individuals are ordered according to census sizes (Figure 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 [16] and our demographic models (Figure S3). A negative relationship between the zero-fold/four-fold heterozygosity ratio and neutral diversity also persists after excluding the San Nicolas foxes (inset). At an 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 [17]. See also Table S2.

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 [23–25].

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 2B and 2C). 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 whether 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 missense deleterious 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 to be healthy and 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 [26, 27]. 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 after population declines has resulted in apparent inbreeding depression, hampering recovery [28, 29]. 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 [30], 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 [31]. 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 [32, 33].

EXPERIMENTAL PROCEDURES

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

[8]. Samples for sequencing were selected to maximize DNA quality and quantity and were 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 S1.

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 ~13–23× coverage per individual (Table S1). We applied conservative ad hoc filters to minimize the inclusion of erroneous genotypes. We used the genomic coordinates identified in Freedman et al. [34] to extract coding, conserved non-coding, and putatively neutral sequences. In brief, 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 [35]. 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 [36]. Variant annotation was performed with VEP [37] running SiFT [17], 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 the [Supplemental Experimental Procedures](#).

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 2 SDs above the mean, calculated per genome (Figure S1). Coordinates of peaks of heterozygosity are given in Data S2. 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. GO enrichment analysis was performed on peak coordinates using gProfileR with the Ensembl *C. familiaris* annotation (release 79) [38].

Demographic Inference and Simulations

We used ABC to infer the demographic history of the San Nicolas, San Miguel, and mainland gray fox populations (Figures S3B and S3C). Demographic models and prior distributions of parameters were based on estimates from our own analyses (Figures 1B, S2, and S3A) and the literature [7, 14]. We conducted forward in time simulations with a modified version of the forward simulator used in [39] to jointly assess the effects of demography and purifying selection on levels of deleterious variation. We conducted simulations with MaCS [40] 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 the [Supplemental Experimental Procedures](#).

ACCESSION NUMBERS

Sequence data are available at NCBI's Sequence Read Archive under BioProject PRJNA312115.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, three tables, and two datasets and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.02.062>.

AUTHOR CONTRIBUTIONS

J.A.R. and R.K.W. conceived and designed the experiment. Sequence analysis was primarily carried out by J.A.R. with assistance from D.O.D.V., Z.F., B.Y.K., B.M.v.H., C.D.M., and K.E.L. Demographic inference and simulations were performed by D.O.D.V. The manuscript was written by J.A.R., D.O.D.V., K.E.L., and R.K.W., with input from all authors.

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