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Adaptive divergence despite strong genetic drift: genomic analysis of the evolutionary mechanisms causing genetic differentiation in the island fox (*Urocyon littoralis*)

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Abstract

The evolutionary mechanisms generating the tremendous biodiversity of islands have long fascinated evolutionary biologists. Genetic drift and divergent selection are predicted to be strong on islands and both could drive population divergence and speciation. Alternatively, strong genetic drift may preclude adaptation. We conducted a genomic analysis to test the roles of genetic drift and divergent selection in causing genetic differentiation among populations of the island fox (*Urocyon littoralis*). This species consists of six subspecies, each of which occupies a different California Channel Island. Analysis of 5293 SNP loci generated using Restriction-site Associated DNA (RAD) sequencing found support for genetic drift as the dominant evolutionary mechanism driving population divergence among island fox populations. In particular, populations had exceptionally low genetic variation, small N_e (range = 2.1–89.7; median = 19.4), and significant genetic signatures of bottlenecks. Moreover, islands with the lowest genetic variation (and, by inference, the strongest historical genetic drift) were most genetically differentiated from mainland grey foxes, and vice versa, indicating genetic drift drives genome-wide divergence. Nonetheless, outlier tests

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identified 3.6–6.6% of loci as high F_{ST} outliers, suggesting that despite strong genetic drift, divergent selection contributes to population divergence. Patterns of similarity among populations based on high F_{ST} outliers mirrored patterns based on morphology, providing additional evidence that outliers reflect adaptive divergence. Extremely low genetic variation and small N_e in some island fox populations, particularly on San Nicolas Island, suggest that they may be vulnerable to fixation of deleterious alleles, decreased fitness and reduced adaptive potential.

Keywords: conservation genomics, divergent selection, effective population size, genetic drift, population divergence

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Introduction

Islands are global centres of biodiversity and endemism (Stuart *et al.* 2012), but the evolutionary mechanisms generating this diversity are typically poorly understood (with some notable exceptions, e.g., Losos *et al.* 1998; Grant & Grant 2002; Jordan & Snell 2008). Isolation is a common feature associated with divergence and speciation among island populations (Grant 1998), but ultimately, the processes of genetic drift and/or divergent selection acting on standing genetic variation and new mutations is required for differences to accumulate (Fisher 1930; Wright 1931, 1951). To date, most research on island biodiversity has largely focused on those examples where divergent selection has generated striking cases of adaptive evolution (Losos *et al.* 1998; Grant & Grant 2002). However, much less attention has been given to the role of genetic drift in generating differences among isolated islands (Jordan & Snell 2008). Thus, investigating the joint roles of genetic drift and divergent selection is key for understanding how island populations diverge, thereby generating island biodiversity and endemism.

Genetic drift is expected to be strong in island populations for several reasons. First, many island populations are founded by a small number of individuals whose genetic composition may differ significantly from the source (often continental) population due to random chance (Martínez-Solano & Lawson 2009; Kolbe *et al.* 2012). Second, island populations often have small effective population sizes (N_e). Especially if an island is limited in size, it may have a low carrying capacity and, as a result, a small N_e (Frankham 1998; Eldridge *et al.* 1999). The third reason is bottlenecks. Like many populations, island populations are likely to experience fluctuations in population size in the course of their history, sometimes resulting in significant population size reductions or bottlenecks (Frankham 1998; Heber *et al.* 2013). Yet unlike continental populations, isolated island populations may not receive an infusion of genetic variation through gene flow after bottlenecks, resulting in a permanent reduction in genetic variation (or at least a long-term reduction, as mutation may eventually partially replenish lost genetic variation; Eldridge *et al.* 1999).

Divergent selection is also expected to be strong among island populations due to high environmental heterogeneity among islands and between islands and the mainland (Weigelt *et al.* 2013). One potential source of environmental variation on islands is climate, including temperature, precipitation and fog (Fischer & Still 2007). The specific position of islands relative to ocean currents can have profound effects on their climate (Spalding *et al.* 2007), as do differences in elevation and topography. Another source of environmental variation among islands is community composition. Island biogeography theory makes the simple but important prediction that larger islands have more species (MacArthur & Wilson 1967), but even adjacent islands that are the same size may have different species due to climate, microhabitat availability, and random chance of which species end up on which islands (Burns 2007). Thus, both genetic drift and divergent selection can be strong in island populations, which means either or both processes could drive genetic differentiation and population divergence among islands. However, if drift is strong, it may overwhelm selection, precluding adaptive divergence (Wright 1931, 1951).

One of the most iconic island species in the world is the island fox (*Urocyon littoralis*), a species whose origin, evolution and divergence has fascinated evolutionary biologists for decades (Grinnell *et al.* 1937; Gilbert *et al.* 1990; Wayne *et al.* 1991; Collins 1993; Goldstein *et al.* 1999; Aguilar *et al.* 2004). This species is a diminutive, endemic fox sister to the mainland grey fox (*Urocyon cinereoargenteus*) and is found on six of the eight Channel Islands off the coast of southern California (Fig. 1) (Coonan *et al.* 2010). Recent archeological and mitogenomic studies have demonstrated that island foxes diverged from their mainland progenitor ~9200–7100 years ago and have been on the northern Channel Islands (San Miguel [SMI], Santa Rosa [SRI], and Santa Cruz [SCI]) for at least 7100 years and the southern

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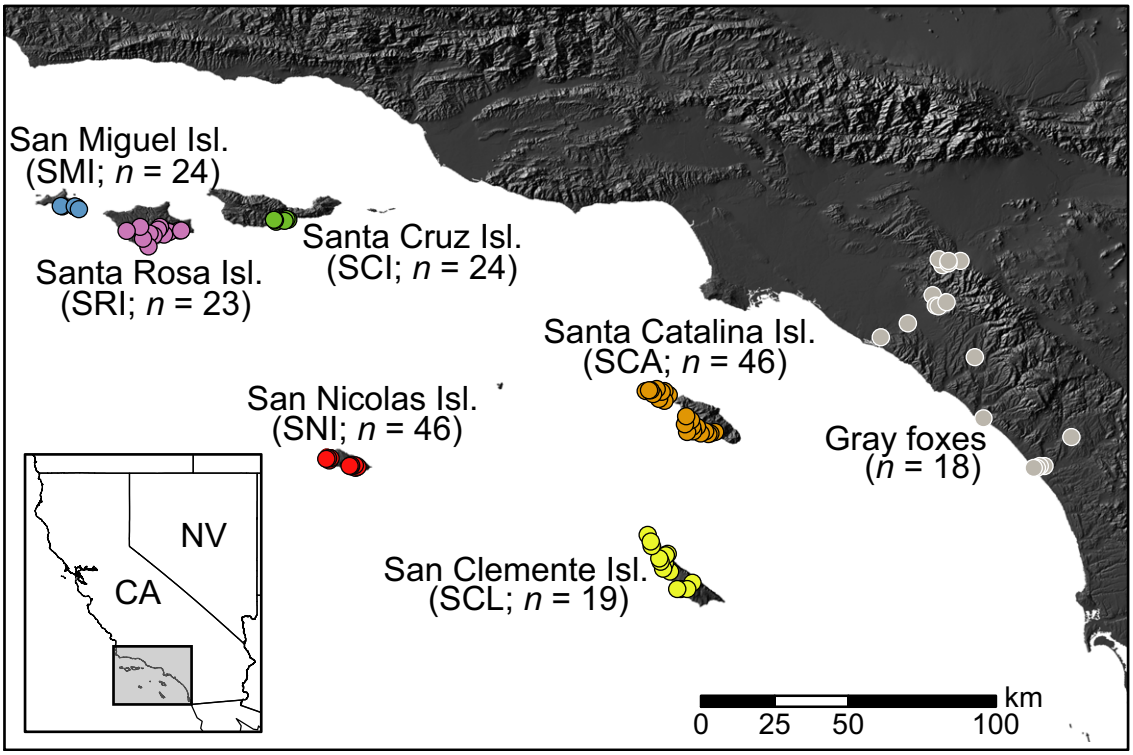


Fig. 1 Map of island fox and grey fox individuals included in genomic analyses. Abbreviations and sample sizes are shown in parentheses. Inset shows location of study area in southern California, USA.

Channel Islands (Santa Catalina [SCA], San Clemente [SCL], and San Nicolas [SNI]) for at least 5000 years (Rick *et al.* 2009; Hofman *et al.* 2015). Foxes may have been transported to the Channel Islands by rafting on debris flows or by Native Americans as part of ritual practices, as they are found in formal cemeteries and burials, for their pelts which were used as clothing, and for helping reduce pests (e.g., mice populations) (Rick *et al.* 2009; Hofman *et al.* 2015). These studies also suggest a prehistoric, human translocation of island foxes from the northern to the southern Channel Islands based on AMS radiocarbon dates. Earlier morphological and genetic studies described island foxes on each island as a separate subspecies (Grinnell *et al.* 1937; Gilbert *et al.* 1990; Wayne *et al.* 1991; Collins 1993; Goldstein *et al.* 1999). Although high levels of genetic differentiation have been documented among island fox populations (Aguilar *et al.* 2004), there is no evidence to date that observed morphological differences among islands are genetically based or adaptive.

Solving the puzzle of high genetic differentiation among island fox populations requires an understanding of their recent population crashes and recovery. In the late 1990s, subspecies on four islands went through severe population bottlenecks (Table 1), leading to their listing as endangered in 2004 under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2004). The three northern island populations crashed because of predation by golden eagles (*Aquila chrysaetos*), which had colonized the islands due to abundant food provided by feral pigs and sheep (Roemer *et al.* 2001; Coonan *et al.* 2010). On SCA, declines were caused by canine distemper virus (CDV), likely introduced by raccoons (*Procyon lotor*) or dogs (Timm *et al.* 2009). San Nicolas Island foxes may have dropped to as low as 20 individuals in the 1970s for unknown reasons (Coonan *et al.* 2010). The only island that has not experienced a recent bottleneck based on intensive island fox population monitoring efforts on all islands is SCL. Populations on each of the northern islands and SCA have recovered over the past decade as the result of intensive and rapid management efforts, including removal of golden eagles, feral pigs, and ungulates, CDV vaccinations, and separate captive breeding programs on each of these islands (Table 1) (Coonan *et al.* 2010). However, the SNI population of island foxes is small and currently declining (Table 1; Coonan 2015).

Strong genetic drift caused by founder effects and the bottlenecks described above could play an important role in causing high genetic differentiation among island fox populations. In addition, island fox populations are exposed to varying environmental conditions

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Table 1 Population bottleneck year and sizes, current population sizes, sample sizes (*n*) before and after SNP quality filters for mainland grey foxes and each island fox population

Site	Bottleneck year	Bottleneck size	Current size in 2014	*n* (total sampled)	*n* (after filters)
Grey foxes	NA	NA	Unknown	18	16
SMI	1999–2000	15	470	24	21
SRI	1999–2000	15	826	23	23
SCI	1999–2000	50–60	2466	24	24
SCA	1999	(>90% decline)	1624	46	43
SCL	NA	NA	1230	19	17
SNI	1970s	20?	263	46	44

Island fox bottleneck year and bottleneck population size estimates from Coonan *et al.* (2010). No estimate of the population size is available for Santa Catalina Island during its 1999 bottleneck, but this population is estimated to have declined by >90%. San Nicolas island foxes may have dropped to as low as 20 individuals in the 1970s. Current adult population size estimates for 2014 from Coonan (2015).

across the Channel Islands archipelago, likely generating divergent selection on top of genetic drift. In particular, SMI and SRI are substantially cooler and wetter than SCI, SCA and SCL because they are nested within different marine ecoregions (Spalding *et al.* 2007). In addition, island foxes on different islands have significantly different prey bases and diets (Cypher *et al.* 2014). Finally, exposure to pathogens varies among islands, e.g., CDV in the late 1990s on SCA (Timm *et al.* 2009). Genetic drift and divergent selection therefore could both be important drivers of divergence among island fox populations, or genetic drift may overwhelm selection, preventing adaptation.

Determining the roles of genetic drift and divergent selection in causing genetic differentiation in island foxes also has direct bearing on conservation and management of this high-profile species of conservation concern. First, understanding the relative importance of these mechanisms is directly relevant to their current legal designation and management as distinct subspecies. Under most definitions of subspecies and Evolutionarily Significant Units (ESUs)—which can receive legal protection under the U.S. Endangered Species Act (ESA), as in the case of island foxes on SMI, SRI, SCI and SCA—adaptive differences, in addition to genome-wide genetic differentiation and phenotypic differences, are required (Ryder 1986; Crandall *et al.* 2000; Funk *et al.* 2012; Robertson *et al.* 2014). Thus, knowing whether or not there are adaptive differences among island fox populations is directly relevant to their legal protection as subspecies.

Second, understanding the strength of genetic drift in island fox populations is important for knowing whether loss of genetic variation is a significant threat to their persistence. If extreme bottlenecks have substantially reduced genetic variation in island foxes, this could make these populations vulnerable to both inbreeding depression (Ralls *et al.* 1979; Ralls & Ballou 1983; Lacy 1997; Newman & Pilson 1997; Saccheri *et al.* 1998) and a reduced ability to adapt to future environmental change (Bürger & Lynch 1995), such as the predicted warming and drying of southern California in the coming century (Cayan *et al.* 2008; LaDochy & Witiw 2012; Cook *et al.* 2015).

Lastly, understanding divergent selection and adaptive differentiation among island fox populations is relevant to future consideration of genetic rescue as a management strategy (Tallmon *et al.* 2004; Whiteley *et al.* 2015). Outbreeding depression rather than genetic rescue could result if a source population is maladapted to the target population (Edmands 2007; Frankham *et al.* 2011). Thus, understanding patterns of adaptive differentiation among islands will inform predictions about the likelihood that genetic rescue would increase fitness and population sizes.

Our goal here was to use a population genomic approach with single-nucleotide polymorphism (SNP) data generated from Restriction-site Associated DNA (RAD) sequencing to investigate the roles of genetic drift and divergent selection in causing population divergence among island fox populations. Genomics greatly improves our ability to address this question compared to traditional population genetics approaches with small numbers of markers by increasing power to identify loci under selection, providing enough variability in small populations to estimate N_e and test for bottlenecks, and improving statistical power and precision. We had four specific aims. First, we characterize the genetic population structure of island foxes. Second, we test the hypothesis that genetic drift contributes to genetic differentiation among populations. Third, we test the hypothesis that divergent selection caused by environmental differences among islands contributes to genetic differentiation among populations. Fourth, we

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characterize patterns of population divergence at neutral vs. any detected adaptive loci. We conclude with a discussion of the conservation implications of our findings.

Methods

RAD sequencing and genotyping

We used Restriction-site Associated DNA (RAD) sequencing (Baird *et al.* 2008) to genotype 182 island foxes and 18 outgroup grey foxes (Goldstein *et al.* 1999; Hofman *et al.* 2015) collected between 2008 and 2011. DNA was extracted from blood samples or muscle tissue from road-killed foxes using DNeasy blood and tissue extraction kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We sequenced 18–46 individuals (median = 24 individuals) per island (or from the mainland, in the case of grey foxes; Fig. 1 and Table 1).

As there is currently no island fox reference genome, we used a two-step RAD sequencing approach. First, we assembled reference contigs using paired-end sequences from eight individuals at high coverage depth (~100 X) (Etter & Johnson 2012; Hohenlohe *et al.* 2013). DNA from these individuals was prepared in an individually barcoded RAD library following the method of Etter *et al.* (2011) using the restriction enzyme *SbfI*. Next, we selected fragments corresponding to insert sizes of 230–400 bp and sequenced this library in a single 150 bp paired-end Illumina HiSeq lane. After sequencing, we filtered for read quality and presence of a correct barcode and *SbfI* recognition site, identified and removed PCR duplicates, pooled the data from all individuals, identified loci using STACKS software (Catchen *et al.* 2011, 2013), and assembled consensus RAD contigs from the overlapping paired-end reads separately at each locus following methods and parameters outlined in Hohenlohe *et al.* (2013).

We next prepared individually barcoded RAD libraries for the remaining individuals (n = 192) as above and sequenced them on an Illumina HiSeq with single-end 100 bp reads at lower depth (~20 X) in a total of four lanes with 47–49 individuals per lane. After filtering reads as above, we aligned these "clean" reads against the reference RAD contigs, removing those loci that did not align uniquely, and called diploid genotypes along the 100 bp stretch using a maximum likelihood statistical method (Hohenlohe *et al.* 2010a; Catchen *et al.* 2013). The forward reads from the eight individuals used to assemble contigs were also aligned and genotyped.

This two-step procedure of first assembling reference contigs using paired-end sequencing, followed by aligning single-end reads to reference contigs provides multiple advantages over single-end sequencing alone. First, assembling reference contigs provides a high-confidence reference "genome" for the RAD loci. Longer paired-end reads and contig assembly better distinguish paralogous and duplicate loci and allow for a greater chance of finding functional information about high F_{ST} outliers with blasting than shorter, single-end contigs (Hohenlohe *et al.* 2013). Second, alignment of subsequent single-end reads to these reference contigs provides higher-confidence clustering of reads to the correct loci and an additional layer of filtering for the single-end read data (e.g., removing non-RAD sequence, quality filtering, etc.).

After calling SNPs, we performed several additional quality filters. First, we removed any loci for which more than half of the individuals had missing data (Hohenlohe *et al.* 2010a). Second, for those RAD tags with more than 1 SNP per contig, we only used the first (most 5′) SNP per contig, as the choice of which SNP to use per contig did not affect results. For example, there were no biases or statistically significant differences in genetic differentiation or within population genetic variation based on the first vs. latter SNPs. Third, we removed loci with minor allele frequencies <0.10, as low frequency alleles may represent PCR errors. Fourth, we removed any individuals with genotypes for <50% of loci. Finally, we removed loci with exceptionally high coverage (coverage greater than 2 SD above the mean), as these loci could be paralogs (Emerson *et al.* 2010).

Data analysis

Aim 1: characterize population structure. We used several analyses to characterize population structure among island fox and grey fox populations. First, we estimated two different indices of genetic differentiation among all island fox populations and mainland grey foxes: pairwise F_{ST} and Jost's D (Jost 2008; Verity & Nichols 2014). Pairwise F_{ST} estimates and their significance were calculated using 1000 permutations in ARLEQUIN 3.5 (Excoffier *et al.* 2005) and Jost's D estimates were calculated in GENALEX 6.5 (Peakall & Smouse 2006, 2012). In addition, we tested the sensitivity of F_{ST} estimates to our threshold for the allowed level of missing genotypes (<50%; see "RAD sequencing and genotyping" section of Methods above) by calculating F_{ST} again using a more stringent threshold (<20%) and then estimating the correlation coefficient between pairwise F_{ST} estimates calculated with these different thresholds. We also calculated the correlation coefficient between pairwise F_{ST} and Jost's D estimates.

Secondly, we inferred the number of island fox and grey fox populations using the Bayesian clustering

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algorithm implemented in program STRUCTURE 2.3.4 (Pritchard *et al.* 2000). STRUCTURE infers the best-supported number of clusters (K) in the sample and the proportion of each individual's genome assigned to each cluster (q_k). We ran STRUCTURE with an MCMC burn-in of 100 000 steps followed by 100 000 steps for inference of clustering (Willing *et al.* 2010) and used the admixture model with correlated allele frequencies. We tracked LnP(D), the probability of the data given K, over the course of the run to ensure that these values had stabilized at the end of the burn-in period. STRUCTURE was run for K = 1–10 with 10 replicates for each value of K. We inferred the best-supported value of K using a combination of mean LnP(K) and the ΔK method of Evanno *et al.* (2005) as implemented in STRUCTURE HARVESTER (Earl & Vonholdt 2012).

Third, we examined patterns of genetic divergence and similarity using two different analyses. Principal Components Analysis (PCA) was performed with the 'prcomp' package in program R (R Development Core Team 2010). Neighbour-net trees were then inferred using program SPLITSTREE4 (Bryant & Moulton 2004; Huson & Bryant 2006). For the Neighbour-net tree analysis, all heterozygous SNPs were coded according to International Union of Pure and Applied Chemistry (IUPAC), uncorrected_P distance was used as the metric, and ambiguous states were treated as average matches. These last two analyses were performed with and without grey foxes to test the sensitivity of patterns of genetic differentiation to inclusion of the outgroup.

Aim 2: test the contribution of genetic drift to genetic differentiation. Two main predictions stem from the hypothesis that genetic drift due to small effective population sizes (N_e), founder effects, and/or bottlenecks is a significant cause of high genetic differentiation among island fox populations: (i) island fox populations will have low genetic variation (indicating strong historical genetic drift), small N_e, and/or evidence of bottlenecks; and (ii) pairwise genetic differentiation between island fox populations and the mainland (grey foxes) will be negatively correlated with indices of historical genetic drift (estimates of within population genetic variation). In other words, following Jordan & Snell (2008) and Whiteley *et al.* (2010), we expected high F_{ST} values when within-population genetic variation was low, and vice versa.

To test the first prediction, we estimated several indices of within-population genetic variation, estimated N_e, and tested for bottlenecks. We used four measures to characterize within-population genetic variation for island foxes and grey foxes: observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_r) and nucleotide diversity (π). H_o, H_e and π were estimated directly from STACKS output and A_r was estimated using HP-RARE 1.0 (Kalinowski 2005). Importantly, estimates of H_o, H_e and π are relatively insensitive to sample size, especially when the number of loci used is large (Nei 1978); A_r uses a rarefaction approach to correct for variation in sample sizes.

We estimated effective population size (N_e) of island fox populations using the linkage disequilibrium (LD) method implemented in program NEESTIMATOR 2.01 (Do *et al.* 2014). This method is based on theory showing that the amount of linkage (i.e., gametic) disequilibrium at independent loci in randomly mating, isolated populations is purely a function of the magnitude of genetic drift and can therefore be used to estimate N_e (Hill 1981). The LD method provides a contemporary estimate of N_e in the previous generation, although it can also be affected by LD generated over several generations (Waples 2005). Because island foxes have overlapping generations, the LD method estimates the effective number of breeders (N_b) that produced the sampled cohort(s), which may or may not be the per-generation estimate of N_e. However, the relationship between N_e and N_b for single-sample estimates of N_e remains unclear (Waples 2010), so here, we refer to our estimates as N_e.

As this method assumes that markers are selectively neutral, we estimated N_e using only presumably neutral loci *not* identified as high F_{ST} outliers (which may be under divergent selection). For the purposes of this analysis, we identified high F_{ST} outliers as those loci with the top 5% of global F_{ST} values (Hohenlohe *et al.* 2010a) and then removed these outlier loci before running NEESTIMATOR, leaving 4615 loci for analysis. This simple nonmodel-based approach for identifying high F_{ST} outlier loci may incorrectly identify some loci as outliers that in reality are not under divergent selection (i.e., false positives) (Bierne *et al.* 2013). Thus, this is a conservative approach because it errs on the side of removing too many loci.

We tested for evidence of population bottlenecks in each of the six island fox subspecies and the mainland grey fox species using the program BOTTLENECK 1.2.02 (Cornuet & Luikart 1996; Piry *et al.* 1999). This analysis is based on the loss of rare alleles predicted in recently bottlenecked populations, resulting in heterozygosity excess. As this method assumes that markers are selectively neutral, we only used nonoutlier loci, identified using the "top 5% method" as explained above. We used the infinite alleles model (IAM) as the most appropriate evolutionary model for SNP loci. To test for significant heterozygosity excess compared to the level predicted under mutation-drift equilibrium, we used a one-tailed Wilcoxon signed rank test implemented in R v3.1.3. Stringent filters on minor allele frequency (MAF)

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could bias this analysis in favour of detecting bottlenecks because rare alleles are removed. Therefore, we carried out two sets of analyses, the first using all nonoutlier loci that met the standard MAF filter of >0.1, and the second using all nonoutlier loci that met a less stringent filter of MAF >0.02.

To test the second prediction that genetic differentiation between island fox populations and the mainland will be negatively correlated with the magnitude of genetic drift, we conducted linear regression analyses with pairwise estimates of F_{ST} between each island fox population vs. grey foxes as the response variable and H_o, H_e, A_r or π as the predictor variable (in four separate regression analyses). This analysis assumes that within island genetic variation is a reasonable index of the magnitude of historical genetic drift, which should be a valid assumption, due to minimal gene flow among island fox populations and grey foxes after initial colonization of the islands (Hofman *et al.* 2015). Therefore, gene flow would not be expected to contribute significantly to within-island genetic variation.

Aim 3: test the contribution of divergent selection to genetic differentiation. Divergent selection could contribute to genetic differentiation of a subset of loci or genome-wide. In the early stages of adaptive divergence, selection is predicted to target specific loci underlying traits involved in local adaptation, causing a relatively small subset of loci to be identified as high F_{ST} outliers with higher genetic differentiation than background, neutral levels (Beaumont & Nichols 1996; Beaumont & Balding 2004; Hohenlohe *et al.* 2010a,b). If adaptive divergence proceeds to the point of causing reproductive isolation in the process of ecological speciation, "isolation-by-adaption" can result whereby genome-wide genetic differentiation is correlated with environmental differences among populations (Nosil *et al.* 2008). We tested both of these possible outcomes of divergent selection in island foxes.

First, we identified high F_{ST} outliers among island fox populations (without grey foxes) using three outlier tests: a nonmodel-based method (loci with the highest 5% of F_{ST} values, described above); a maximum likelihood test implemented in FDIST2 (Beaumont & Nichols 1996; Beaumont & Balding 2004); and a Bayesian approach implemented in BAYESFST (Beaumont & Balding 2004). Both of these methods have been shown to be relatively robust to deviations from assumed population structure (Beaumont & Balding 2004). FDIST2 was run assuming either a simple island model or a hierarchical island model with islands nested in separate northern (SMI, SRI, SCI) and southern (SCA, SCL, SNI) groups. To standardize the false positive rate between FDIST2 and BAYESFST, we set the critical *P*-value to 0.01 for FDIST2 to compare with the Bayesian 10% level, as recommended by Beaumont & Balding (2004). With a critical *P*-value of 0.01, 1% of loci should be identified as high F_{ST} outliers by chance. Thus, following previous studies (Beaumont & Balding 2004; Willing *et al.* 2010), if >1% of loci were identified as high F_{ST} outliers, we interpreted this as evidence of true divergent selection and adaptive divergence. We also corrected for type I errors from multiple testing in FDIST2 using a false discovery rate (FDR) correction (Benjamini & Hochberg 1995). BAYESFST already addresses the problem of multiple testing through the prior distribution of the regression parameter for the locus effect (Beaumont & Balding 2004). We then ran a Gene Ontology (GO) term enrichment analysis (Mi *et al.* 2013) for those high F_{ST} outliers that blasted to genes in the dog (*Canis lupus familiaris*) genome (canFam3.1; Hoeppner *et al.* 2014). The GO analysis tested for overrepresentation of genes associated with specific biological processes relative to the full set of dog genes.

To complement our high F_{ST} outlier tests, we tested for loci associated with environmental variation with BAYESCENV (Villemereuil & Gaggiotti 2015). This method considers a model incorporating environmental data from each collection site, and compares that to both the null *F*-model and standard α-model to identify F_{ST} outlier loci that show variation associated with environmental differentiation. We considered five environmental variables hypothesized to be related to adaptive divergence among island fox populations: mean annual temperature and precipitation (Weigelt *et al.* 2013) and three dietary variables that reflect differences in prey availability among islands (proportion of insects, fruit or mice in the diet; Cypher *et al.* 2014).

BAYESCENV was run for each of the environmental variables with the parameter settings of: g (upper bound) = 10; α (mean prior) = −1.0; P = 0.50; and π = 0.10. After 20 pilot runs of 2000 iterations each and a burn-in of 50 000 iterations, 5000 MCMC samples were taken with 10 steps between each sample. Diagnostics of the log likelihoods and F_{ST} values for the 5000 sampled iterations were checked using the 'coda' package in R to confirm convergence and sample sizes of at least 2500.

Second, we tested for relationships between genome-wide genetic differentiation and environmental differences among populations using multiple regression on distance matrices (MRDM; Legendre *et al.* 1994; Balkenhol *et al.* 2009). MRDM regresses multiple predictor matrices against a response matrix of genetic distances, and uses permutation to assess statistical significance. The *MRM* function in the R package 'ecodist' (Goslee & Urban 2007) was run with 1000 permutations using the genetic distance matrices (F_{ST} or Jost's D) as the response variable. Each of three matrices representing climatic differences among the islands (mean

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temperature and precipitation; Weigelt *et al.* 2013) and dietary differences (Horn's similarity index; Cypher *et al.* 2014), along with the geographic distance matrix were used as the predictor variables. Prior to running MRDM, we used variance inflation factors (VIF) to assess multicollinearity among predictor variables. Geographic distance and temperature were highly correlated (>80%) but had VIF values <4; thus, both factors were retained for the subsequent analyses. To obtain a best-reduced model, stepwise regression with both forward and backward selection was implemented with the 'step' function in R. A full MRDM model that included all predictor variables was then run, as well as a model that considered geographic distance alone.

Aim 4: characterize population divergence at neutral vs. adaptive loci. Lastly, we used the results of the above outlier tests to partition our SNP data set into nonoutlier (presumably neutral) and high F_{ST} outlier (presumable adaptive) loci for examining patterns of genetic divergence and similarity using PCA and Neighbour-net trees (Funk *et al.* 2012). In addition, we used the previously described nonmodel-based method of identifying outliers, in which loci with the highest 5% of F_{ST} values were designated as outliers, as a third way to partition our data set.

Results

RAD sequencing and genotyping

After filtering for read quality and presence of a correct barcode and *SbfI* recognition site, a total of 93 314 044 "clean" read pairs were generated across the eight individuals included in the paired-end Illumina HiSeq lane. Of these, 69% were identified as PCR duplicates and were removed, leaving 29 357 186 read pairs from which we assembled a total of 126 264 unique consensus RAD contigs.

Single-end sequencing yielded 494 418 159 clean sequence reads across 192 individuals. These were added to 55 262 507 clean forward reads from the eight individuals included in the paired-end sequencing lane for a total of 549 680 666 clean reads from 200 individuals that were aligned to the above 126 264 RAD contigs. After all quality filters, a total of 4858 variable SNP loci were available for analysis when the grey fox outgroup was included and 5293 SNPs were available without grey foxes (Table 2). Mean coverage per locus (averaged across individuals) ranged from 5–40 (median = 20; Fig. S1, Supporting information) and the number of loci per individual (for the data set including grey foxes) ranged from 2381 to 4854 (median = 4419 [=91% of all variable SNPs]; Fig. S2, Supporting information).

Aim 1: characterize population structure

We found exceptionally high genetic differentiation among island fox populations. Pairwise F_{ST} values between most islands were extremely high, ranging from 0.463 to 0.963 (median = 0.749), and all values were statistically significant ($P < 0.00001$; Table 3). F_{ST} values were insensitive to the threshold used for the allowed level of missing genotypes, as revealed by a high correlation between pairwise F_{ST} values calculated using our standard threshold of <50% missing genotypes vs. a more stringent threshold of <20% missing genotypes ($r = 0.999$, $P < 0.00001$). Pairwise Jost's D values were also significantly correlated with pairwise F_{ST} values ($r = 0.711$, $P = 0.0003$), although SNI was more similar to SCL with Jost's D than with F_{ST} (Table 3).

The best-supported value of K in our STRUCTURE analysis was $K = 7$ based on mean LnP(K) and $K = 2$ based on the ΔK method. However, $K = 2$ was clearly an underestimate based on our F_{ST}, Jost's D, PCA, and Neighbour-net results. Interestingly, although $K = 7$ was best-supported based on mean LnP(K), no individuals had any measurable portion (to the thousandths place) of their genome assigned to the seventh cluster, meaning $K = 6$ effectively had the highest support. With $K = 7$, individuals were generally assigned to a single island (or to the mainland, in the case of grey foxes; Fig. 2). However, approximately 73% of the genomes of individuals from SRI were assigned to SMI and approximately 27% to SCI, indicating SRI has an intermediate genetic relationship to SMI and SCI. Several individuals on SCA also had a small proportion of their genomes (mean = 1.3%) assigned to SCI.

As expected, all island foxes grouped by island in the PCA (Fig. 3) and Neighbour-net tree (Fig. 4). Removal of grey foxes did not change this result (Figs 3b and 4b). Island fox populations grouped geographically,

Table 2 Counts of SNP loci after each step of filtering with or without grey foxes included

	With grey foxes included		Without grey foxes included	
Filtering step	Count	% of total	Count	% of total
(1) Total contigs	126 264	100.0	126 264	100.0
(2) SNPs w/genotypes for ≥50% individuals	50 135	39.7	20 153	16.0
(3) 1 SNP per contig	30 719	24.3	15 291	12.1
(4) Minor allele frequency (MAF) ≥0.10	4997	4.0	5404	4.3
(5) Coverage ≤ mean coverage + 2SD	4858	3.8	5293	4.2

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with two broad clusters representing northern island (SMI, SRI, and SCI) and southern island (SCA, SCL, SNI) populations.

Aim 2: test the contribution of genetic drift to genetic differentiation

Island fox populations had low within-population genetic variation compared to mainland grey foxes (Table 4). This pattern was evident for all four measures of genetic variation estimated (observed heterozygosity [H_o], expected heterozygosity [H_e], allelic richness [A_r], nucleotide diversity [π]), but was most pronounced for π, which is based on invariant sites as well as SNPs (whereas the other three measures are only based on SNPs). Effective population sizes (N_e) estimated using NEESTIMATOR were also generally small on islands, ranging from 2.1–89.7 (median = 19.4), relative to an N_e of 109.2 for our mainland grey fox population (Table 4). Finally, BOTTLENECK found overwhelming evidence for historical bottlenecks in all populations (island foxes and grey foxes), regardless of whether a MAF filter of >0.10 or >0.02 was used (Wilcoxon signed rank test, $P < 0.000001$).

Also as predicted, pairwise F_{ST} values between grey foxes vs. island fox populations were significantly predicted by within-island genetic variation (Fig. 5; H_o: $F_{1,4} = 28.35$, $P = 0.006$; H_e: $F_{1,4} = 28.44$, $P = 0.006$; A_r: $F_{1,4} = 29.56$, $P = 0.006$; π: $F_{1,4} = 42.45$, $P = 0.003$). For example, grey foxes were most genetically similar to island foxes on SCA, which had the highest within-population genetic variation, and were most genetically divergent from island foxes on SNI, which had the lowest genetic variation.

Table 3 Pairwise F_{ST} estimates below diagonal and Jost's D estimates above diagonal between all pairs of island fox and grey fox populations

	Grey foxes	SMI	SRI	SCI	SCA	SCL	SNI
Grey foxes	—	0.376	0.345	0.346	0.282	0.357	0.384
SMI	0.664	—	0.136	0.325	0.457	0.460	0.603
SRI	0.589	0.515	—	0.199	0.368	0.392	0.547
SCI	0.623	0.773	0.584	—	0.291	0.362	0.527
SCA	0.462	0.676	0.596	0.558	—	0.204	0.237
SCL	0.629	0.884	0.749	0.778	0.463	—	0.239
SNI	0.814	0.963	0.902	0.919	0.646	0.914	—

Pairwise F_{ST} and Jost's D estimates were calculated from 4858 SNP loci using ARLEQUIN 3.5 (Excoffier *et al.* 2005) and GENALEX 6.5 (Peakall & Smouse 2006, 2012), respectively. All pairwise F_{ST} estimates were statistically significant. See Fig. 1 for full names of islands abbreviated here.

Aim 3: test the contribution of divergent selection to genetic differentiation

Mean F_{ST} among all populations was 0.726, but many loci had F_{ST} values of one or close to one, suggesting they may be high F_{ST} outliers (Fig. 6). We used three different tests to identify high F_{ST} outlier loci with a signature of divergent selection and adaptive divergence among the six island fox populations: a non-model-based method (loci with the highest 5% of F_{ST} values); a likelihood model-based method (implemented in FDIST2); and a Bayesian model-based method (implemented in BAYESFST). As we set the critical P-value to 0.01 for the two model-based approaches, we expected 53 loci (1% of the total 5293 loci) to be identi-

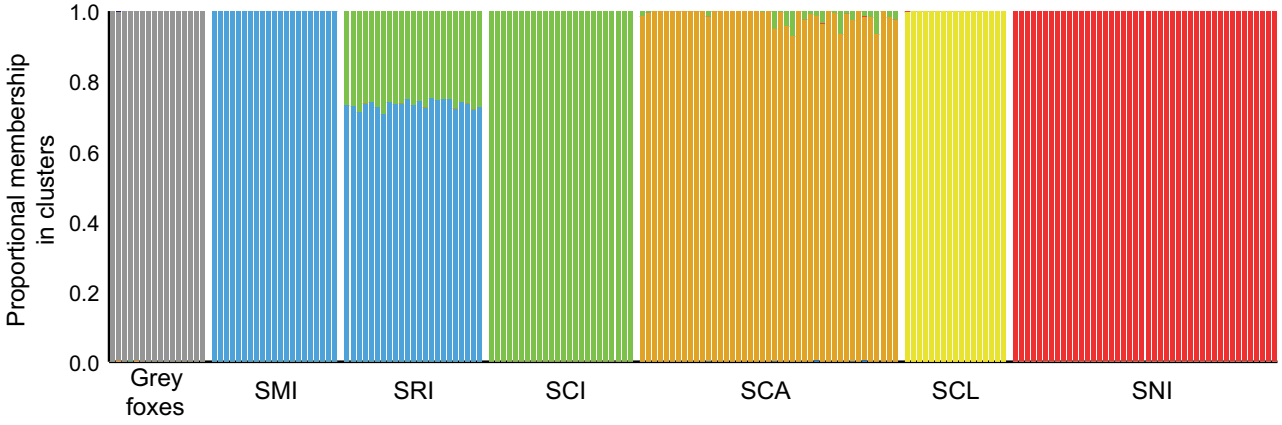


Fig. 2 Results from Bayesian individual clustering with STRUCTURE for $K = 7$. Each colour corresponds to a distinct genetic cluster and each bar corresponds to the proportion of an individual's genotype assigned to each cluster. Note that although $K = 7$ was the best-supported number of K, no individuals had any measurable portion (to the thousandths place) of their genome assigned to the seventh cluster, meaning $K = 6$ effectively had the highest support.

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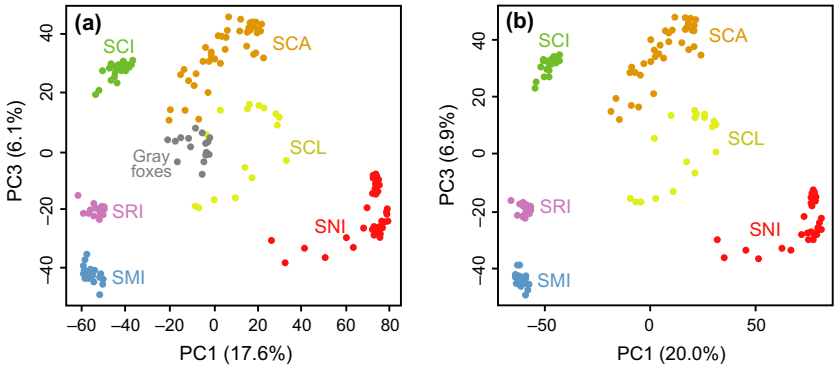


Fig. 3 Principal component analysis (PCA) to characterize genetic differentiation among island fox populations using SNP loci with (a) or without (b) the grey fox outgroup. As PC2 primarily reflected the amount of missing data, we used PC1 and PC3 to visualize genetic divergence among individuals. Colours and abbreviations correspond to different islands as shown in Fig. 1.

fied as significant high F_{ST} outliers by chance. However, 351 (6.6%) and 188 (3.6%) loci were actually identified as high F_{ST} outliers by FDIST2 and BAYESFST, respectively, suggesting that many of these loci (or linked loci) are under divergent selection. Moreover, the FDIST2 outlier test assuming a hierarchical island model (rather than the simple island model assumed above) still identified 325 (6.1%) loci as high F_{ST} outliers, indicating the results were insensitive to the model. Similarly, 297 (5.6%) loci were still identified by FDIST2 as high F_{ST} outliers even after the false discovery rate (FDR) correction.

In addition, 437 out of 439 loci identified as high F_{ST} outliers by at least one of the above three tests blasted to the dog genome (median E-value = 0; range = 0–1.41×10^{-24}) and 195 out of these 437 loci (44.6%) blasted to genes (Table S1, Supporting information), indicating many high F_{ST} outliers are functional. Gene Ontology (GO) term enrichment analysis of the outliers that blasted to dog genes uncovered three categories of gene function that were statistically overrepresented relative to the full set of dog genes. These included genes involved in regulation of catalytic activity (Bonferroni-corrected $P = 0.044$), cellular protein modification ($P = 0.046$), and regulation of molecular function ($P = 0.050$). However, no loci were significantly associated with the climatic or diet variables tested in BAYESCENV ($P > 0.05$).

We did not find any evidence of an effect of environmental differences among islands on genome-wide genetic distance. Matrix regression results (Table 5) using geometric distance as a predictor were not significant for either genetic distance measure (F_{ST}: $F_{1,13} = 0.236$, $r^2 = 0.018$, $P = 0.60$; Jost's D: $F_{1,13} = 0.021$, $r^2 = 0.002$, $P = 0.90$), nor was the full model that also incorporated all three habitat distance matrices as predictors (F_{ST}: $F_{5,9} = 0.406$, $r^2 = 0.140$, $P = 0.89$; Jost's D: $F_{5,9} = 0.285$, $r^2 = 0.102$, $P = 0.84$). In addition, model selection based on AIC did not identify any of the habitat distance matrices as significant predictors.

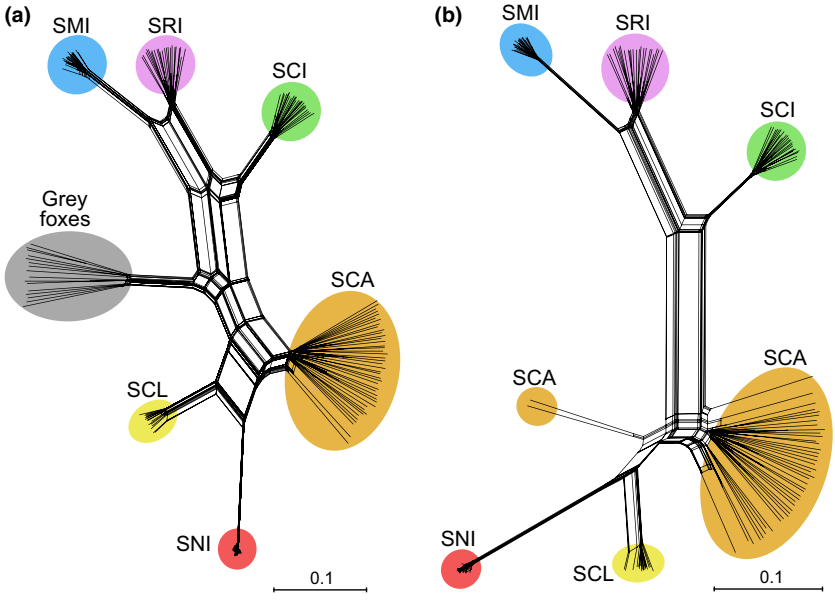


Fig. 4 Neighbour-net tree to characterize genetic differentiation among island fox populations using SNP loci with (a) or without (b) the grey fox outgroup. Colours and abbreviations correspond to different islands as shown in Fig. 1.

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Aim 4: characterize population divergence at neutral vs. adaptive loci

Patterns of population divergence based on high F_{ST} outlier (presumably adaptive) vs. nonoutlier (presumably neutral) loci differed in two different ways. The main consistent difference as seen with both PCA (Fig. 7; Figs S3 and S4, Supporting information) and Neighbour-net trees (Fig. 8; Figs S5 and S6, Supporting information) is that SCI is more similar to SCA when using outliers vs. nonoutliers. Moreover, SNI is very divergent from all other populations based on outlier loci identified using the top 5% method and FDIST2, but more similar to SCL based on outlier loci identified using BAYESFST.

Table 4 Observed heterozygosity (H_o), expected heterozygosity (H_e), allelic richness (A_r), nucleotide diversity (π), and effective population size estimates (N_e, with 95% confidence intervals) for mainland grey foxes and each island fox population

Site	H_o	H_e	A_r	π	N_e (95% confidence intervals)
Grey foxes	0.238	0.261	1.73	0.00296	109.2 (105.2–113.6)
SMI	0.060	0.059	1.16	0.00027	13.7 (13.2–14.1)
SRI	0.147	0.148	1.39	0.00054	13.6 (13.5–13.7)
SCI	0.114	0.112	1.30	0.00045	25.1 (24.6–25.5)
SCA	0.231	0.251	1.65	0.00082	47.0 (46.7–47.4)
SCL	0.065	0.064	1.17	0.00033	89.7 (77.1–107.0)
SNI	0.016	0.011	1.03	0.00012	2.1 (2.0–2.2)

Discussion

Our analysis of over 5000 SNPs revealed that genetic drift is the dominant evolutionary force driving genetic differentiation among island fox populations. Three lines of evidence support this conclusion. First, genetic variation, particularly nucleotide diversity (π), was much lower in island fox populations than in their sister species, the grey fox, or other species with published data on π. Second, most island fox populations have low (in some cases, extremely low) effective population sizes (N_e), and all have genetic signatures of historical bottlenecks. Third, the significant negative relationship between pairwise F_{ST} (between each island fox population and mainland grey foxes) and measures of within

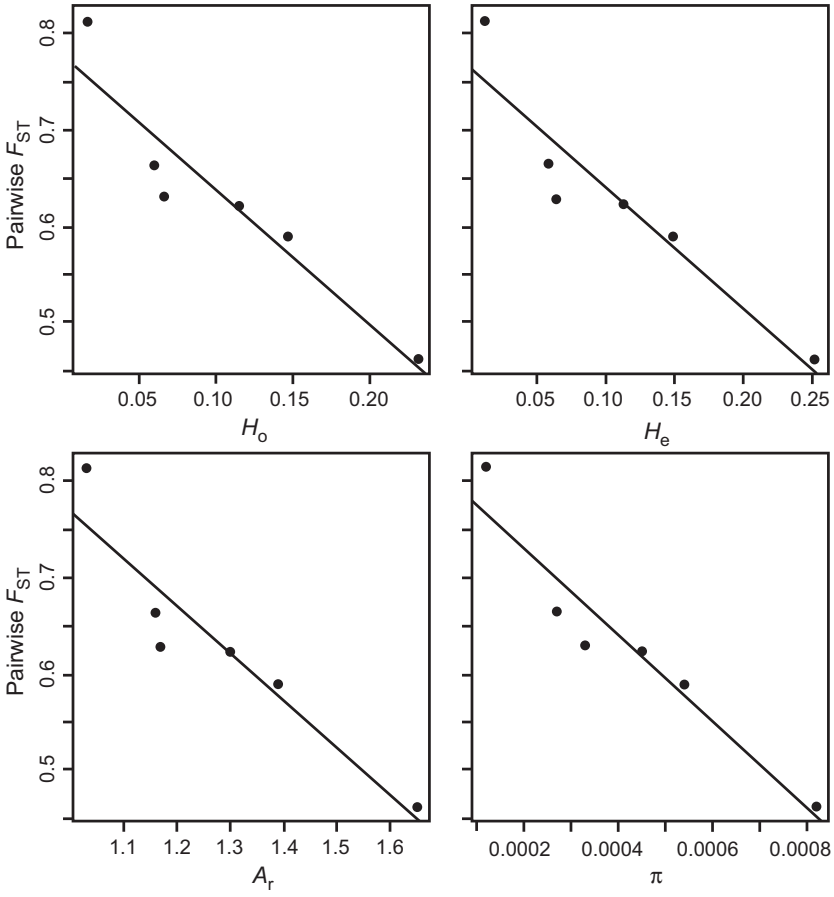


Fig. 5 Scatterplots of pairwise F_{ST} values between grey foxes and each island fox population vs. different measures of within population genetic variation (H_o, observed heterozygosity; H_e, expected heterozygosity; A_r, allelic richness; π, nucleotide diversity). All four relationships were statistically significant ($P < 0.05$; indicated by solid black regression lines).

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population genetic variation suggests that the strength of genetic drift determines the degree of divergence.

Nonetheless, we also uncovered evidence for adaptive divergence among island fox populations based on high F_{ST} outlier tests, indicating that divergent selection may have contributed to divergence despite strong genetic drift (McKay *et al.* 2001). No loci were associated with variation in climate or diet. However, patterns of population similarity at high F_{ST} outlier loci mirrored patterns of morphological similarity (discussed below), suggesting genetically based, adaptive differences exist among populations, supporting subspecies designation.

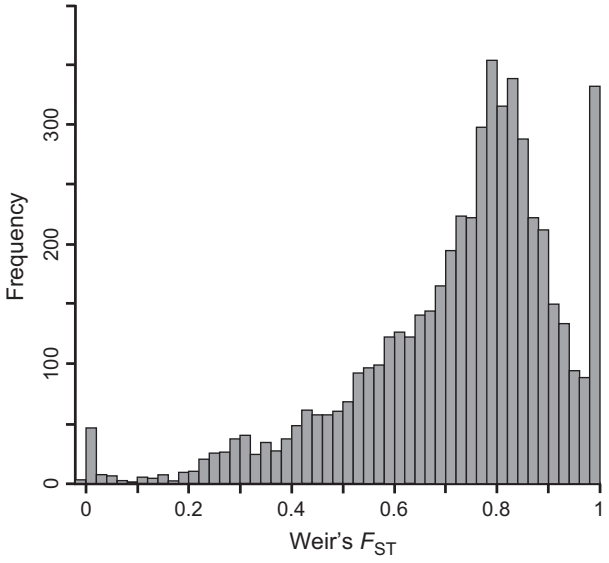


Fig. 6 Histogram of Weir's F_{ST} values among all island fox populations at 5293 SNP loci.

Alone, the finding of adaptive divergence among island fox populations suggests that they should continue to be managed separately. However, extremely low genetic variation and N_e found on some populations, particularly SNI, indicate that they are vulnerable to negative inbreeding effects and loss of genetic variation. These populations might therefore benefit from genetic rescue using source individuals from another island. These opposing management options—managing islands separately to maintain adaptive differences vs. supplementing small, declining populations with individuals from another island to boost fitness through genetic rescue—create a management conundrum. We argue that this uncertainty could best be resolved by research to determine the severity of inbreeding depression, if any, and the potential benefits/costs of genetic rescue. Below, we discuss these and other results in more detail.

High genetic differentiation

Our finding of high genetic differentiation among island fox populations using a large number of genome-wide markers is in agreement with the results of previous studies that used traditional molecular markers, including allozymes, minisatellites, mtDNA and microsatellites (Wayne *et al.* 1991; Goldstein *et al.* 1999). We found that in some cases, genetic differentiation was exceptionally high, particularly between SNI and other island populations, with pairwise F_{ST} values ranging from 0.646 to 0.963. Importantly, the measure of genetic differentiation we used did not affect our conclusion that island fox populations were highly divergent from each other. Genetic differentiation measured using F_{ST} and

Table 5 Results of multiple regression on distance matrices (MRDM)

Genetic distance	MRM model		Coef	*P*-value	r^2	*F*	*P*-value
F_{ST}	pF_{ST} ~ Geo	Int	0.547	0.70	0.018	0.236	0.60
		Geo	0.000	0.60			
	Full model	Int	0.447	0.77	0.140	0.406	0.89
		Geo	0.001	0.69			
		Diet	0.322	0.59			
		Temp	−0.112	0.66			
		Precip	0.000	0.90			
Jost's *D*	p*D* ~ Geo	Int	0.362	0.65	0.002	0.021	0.90
		Geo	0.000	0.90			
	Full model	Int	0.330	0.63	0.102	0.285	0.84
		Geo	0.000	0.74			
		Diet	0.210	0.56			
		Temp	−0.053	0.70			
		Precip	0.000	0.49			

Int = *y*-intercept; Geo = geographic distance; Diet = Horn's similarity index for diet; Temp = difference in mean annual temperature; Precip = difference in mean annual precipitation.

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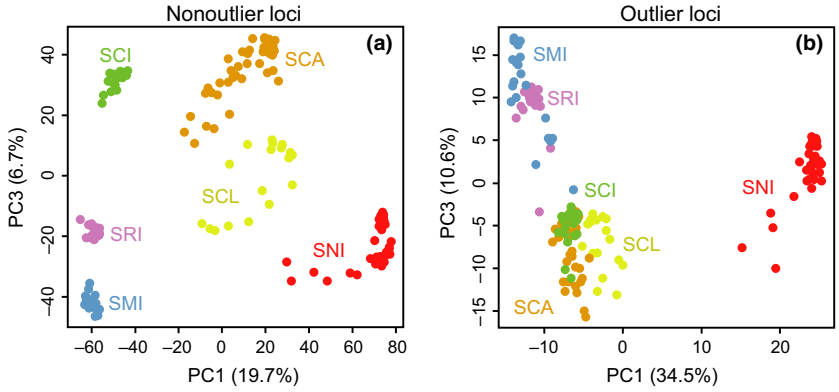


Fig. 7 Principle component analysis (PCA) to characterize genetic differentiation among island fox populations using high F_{ST} outlier SNPs or nonoutliers. (a) PCA based on 5028 presumably neutral SNPs not identified as high F_{ST} outliers or (b) 265 presumably adaptive SNPs identified as high F_{ST} outliers. Here, outlier loci were identified as the highest 5% of F_{ST} values. As PC2 primarily reflected the amount of missing data, we used PC1 and PC3 to visualize genetic divergence among individuals. Colours and abbreviations correspond to different islands as shown in Fig. 1. See Figs S3 and S4 (Supporting information) for PCA using four different methods for identifying outlier loci (highest 5% of F_{ST} values, FDIST2, FDIST2 with the false discovery rate correction, or BAYESFST) with (Fig. S3, Supporting information) or without (Fig. S4, Supporting information) grey foxes.

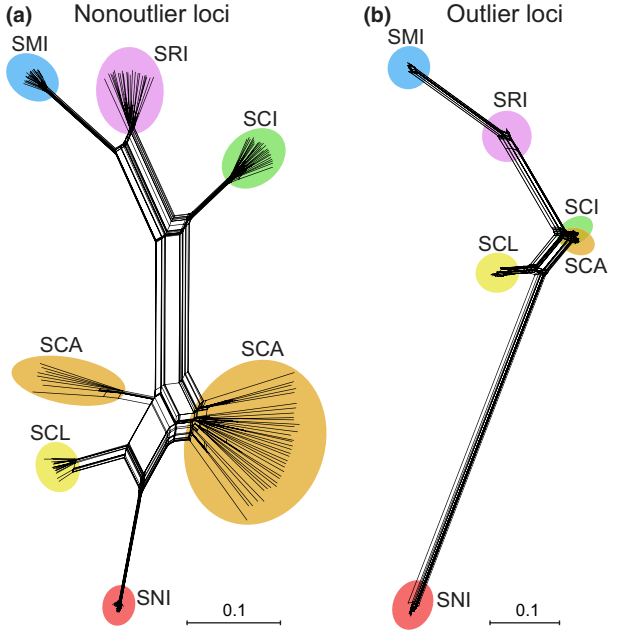


Fig. 8 Neighbour-net trees to characterize genetic differentiation among island fox populations using high F_{ST} outlier SNPs or nonoutliers. (a) Neighbour-net trees based on 5028 presumably neutral SNPs not identified as high F_{ST} outliers or (b) 265 presumably adaptive SNPs identified as high F_{ST} outliers. Here, outlier loci were identified as the highest 5% of F_{ST} values. Colours and abbreviations correspond to different islands as shown in Fig. 1. See Figs S5 and S6 (Supporting information) for Neighbour-net trees using four different methods for identifying outlier loci (highest 5% of F_{ST} values, FDIST2, FDIST2 with the false discovery rate correction, or BAYESFST) with (Fig. S5, Supporting information) or without (Fig. S6, Supporting information) grey foxes.

Jost's D were highly concordant ($r = 0.711$, $P < 0.00001$). The main exception to this concordance was that SNI was more similar to SCL using Jost's D than F_{ST}. Although some have recently argued that some measures of genetic differentiation are superior to others (Verity & Nichols 2014), our results were insensitive to the measure used.

As expected based on high F_{ST} values and previous work, STRUCTURE identified each island as a distinct genetic cluster, with the exception of SRI, in which each individual had an average of 73% of its genome assigned to SMI and 27% assigned to SCI, indicating a genetic composition intermediate to these two populations (Fig. 2). The intermediate position of SRI between SMI and SCI was also apparent in the PCA (Fig. 3) and Neighbour-net trees (Fig. 4). Interestingly, a small proportion of some individuals' genomes on SCA were assigned to SCI, which is in agreement with the finding of a SCI mtDNA haplotype in the SCA population, suggesting a recent human movement of island foxes from SCI to SCA (Hofman *et al.* 2015). This result is interesting in light of the potential use of genetic rescue in island foxes (see "Conservation implications" below); introduction of these individuals did not have any detectable, long-term negative consequences for the SCA population. SCA maintained its genetic distinctness despite anthropogenically-mediated gene flow.

The PCA and Neighbour-net analyses showed the same overall pattern of genetic relationships among island fox populations (Figs 3 and 4). Both clustered individuals by their island of origin, as expected based on high F_{ST} and Jost's D values among populations, and both grouped the northern islands (SMI, SRI, and

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SCI) together and the southern islands (SCA, SCL and SNI) together. These results, once again, are in general agreement with patterns uncovered from previous genetic studies (Wayne *et al.* 1991; Goldstein *et al.* 1999), suggesting that when genetic structure is pronounced, as in the case of island foxes, relatively small numbers of traditional markers may be sufficient for inferring population structure.

Contribution of genetic drift to genetic differentiation

We found overwhelming evidence for strong genetic drift in island fox populations. Genetic variation was much lower in island fox populations than in mainland grey foxes based on all four measures of genetic variation examined. In particular, nucleotide diversity (π), which is based on invariant sites in addition to SNPs (and therefore more comparable among populations and species), was 3.6–24.7 times higher in grey foxes than island fox populations. Similarly, π was approximately an order of magnitude higher in two bumble bee species (mean π = 0.0025–0.0041 for *Bombus impatiens* and 0.0027–0.0042 for *B. pensylvanicus*; Lozier 2014), sticklebacks (0.00203–0.00268 in *Gasterosteus aculeatus*; Hohenlohe *et al.* 2010a), and endangered European eels (0.00529 for *Anguilla anguilla*; Pujolar *et al.* 2013), indicating island foxes are among the most genetically depauperate populations of sexually reproducing animals analysed with SNPs to date.

Effective population sizes (N_e) were small in five of six island fox populations analysed (SMI, SRI, SCI, SCA and SNI), which had effective population sizes ranging from 2.1 to 47.0. In contrast, N_e was significantly higher (89.7) in the one population, SCL, which has not experienced any recent documented bottlenecks. These N_e estimates, which reflect N_e from the last one to several generations, generally mirror the severity of known bottlenecks (compare known bottlenecks in Table 1 to N_e estimates in Table 4). The N_e estimate of 2.1 from SNI is the lowest such value for any population of a sexually reproducing animal species of which we are aware.

We detected a genetic signal of population bottlenecks in all island fox populations, regardless of the minor allele frequency filter used. We were somewhat surprised by the discovery of a bottleneck in grey foxes on the mainland. However, in retrospect, this could reflect declines in this population, which is negatively affected by urbanization in southern California (Ordeñana *et al.* 2010).

Finally, the statistically significant negative relationship we found between pairwise F_{ST} (between each island fox population and the mainland grey fox population) and all measures of genetic variation strongly suggests that historical genetic drift has caused most variation in genetic differentiation among island fox populations. This same analysis has previously proven effective to test the effects of genetic drift and bottlenecks on population divergence (Jordan & Snell 2008; Whiteley *et al.* 2010).

Contribution of divergent selection to genetic differentiation

Although the multiple regression on distance matrices (MRDM) analysis failed to find evidence for a genome-wide association between genetic divergence and environmental factors (temperature, precipitation and diet), likelihood (FDIST2) and Bayesian (BAYESFST) outlier tests found evidence for high F_{ST} outlier loci that may be under divergent selection and involved in adaptive divergence. Many of these loci, as well as outliers identified using a nonmodel based approach (loci with the top 5% of F_{ST} values), blasted to genes in the dog genome, which were enriched for genes involved in regulation of catalytic activity, cellular protein modification and regulation of molecular function. However, no loci were associated with variation in the climatic or diet indices we used, suggesting that the high F_{ST} outlier loci identified must be involved in adaptation to other environmental factors.

Several recent studies have warned about the limitations of F_{ST} outlier tests for identifying loci under divergent selection, indicating that the results of these tests should be interpreted carefully. Other explanations for high F_{ST} outliers besides divergent selection include neutral factors, such as demographics (Lotterhos & Whitlock 2014), recombination rate heterogeneity (Roesti *et al.* 2012), or background selection within populations (Cruickshank & Hahn 2014). In addition, the assumptions of model-based outlier tests are rarely completely upheld. For example, like many outlier tests, FDIST2 and BAYESFST assume an island model in which migration is equally liked among all populations, an assumption that may not hold for island fox populations due to erratic and rare dispersal events among populations (for example, the human-mediated dispersal from SCI to SCA described above). Moreover, as these populations have become more and more divergent over time, even neutral loci may drift to fixation by chance, resulting in F_{ST} values equal to one. Nonetheless, the sensitivity of FDIST2, BAYESFST and other model-based outlier tests to their assumptions is poorly understood, so the high F_{ST} outliers identified may still be valid. This conclusion is supported by concordance between patterns of population similarity based on high F_{ST} outlier loci and patterns based on morphometric traits, discussed next.

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Population divergence at presumably neutral vs. adaptive loci

Overall, patterns of population divergence and similarity based on high F_{ST} outlier (presumably adaptive) vs. nonoutlier (presumably neutral) loci were similar, as seen in both PCA plots and Neighbour-net trees (Figs 7 and 8), but there were some notable exceptions. The principal consistent difference was the position of SCI. In particular, SCI was more similar to SCA based on outliers than nonoutliers, suggesting adaptive similarity between these populations. SNI was also very divergent from all other populations based on outliers identified using the top 5% method and FDIST2 (Figs 7 and 8). Given extremely low genetic variation and small N_e in SNI, this result could be an artefact of strong genetic drift causing fixation of alleles at many loci in SNI, such that these loci are identified as high F_{ST} outliers and SNI appears highly divergent from all other populations based on the outlier data set. In contrast, SNI was more similar to SCL based on outlier loci identified using BAYESFST (Figs S3–S6, Supporting information), suggesting SNI is most adaptively similar to SCL.

Interestingly, patterns of population similarity at high F_{ST} outlier loci were similar to patterns of similarity based on a suit of 29 morphometric (cranial and dentition) traits (Wayne *et al.* 1991). In particular, Wayne *et al.* (1991) found that, using these morphometric traits, SCI was most similar to SCA (the same pattern found here with high F_{ST} outliers) and that SNI was most similar to SCL (the same pattern found with high F_{ST} outliers identified using BAYESFST). The concordance between population similarity based on high F_{ST} outliers and morphology provides an independent line of evidence that high F_{ST} outliers or linked loci are under divergent selection and involved in adaptation. These high F_{ST} outlier loci or linked loci could underlie the actual morphological differences, or they could underlie other unmeasured, but correlated, traits also involved in adaptation to environmental heterogeneity among islands.

Conservation implications

Our results have several implications for island fox conservation and management. First, the high genetic differentiation we document, particularly at potentially adaptive, functional loci, coupled with previous studies documenting morphological differences among island fox populations (Grinnell *et al.* 1937; Wayne *et al.* 1991; Collins 1993), supports the current designation of each island fox population as a distinct subspecies. However, as we argue below, some circumstances might make it prudent for managers to consider supplementing a severely threatened subspecies with individuals from another subspecies, as has been done for Florida panthers (*Puma concolor coryi*; Hedrick 1995; Johnson *et al.* 2010).

Second, despite population rebounds on the northern islands and SCA following population crashes in the late 1990s, all island fox populations except SCL have very low genetic variation and small N_e, suggesting that they remain vulnerable to the increase in frequency and expression of deleterious recessive alleles and to the loss of additive genetic variation. Managers should therefore strive to maintain large populations (e.g., at or close to carrying capacity) and avoid future population crashes, which could exacerbate these negative genetic effects. In addition, low genetic variation and small N_e estimates documented here mean some populations may already be suffering from inbreeding depression (Ralls *et al.* 1979; Ralls & Ballou 1983; Lacy 1997; Newman & Pilson 1997; Saccheri *et al.* 1998). In particular, SNI has <300 adults (Table 1; Coonan 2015), a rapidly declining population ($\lambda = 0.77$), incredibly low genetic variation, and an extremely low N_e of 2.1; this population is highly vulnerable to extinction due to both demographic and genetic factors. We strongly recommend adjusting island fox monitoring programs to include tests for potential negative genetic effects in all subspecies. For example, genomic screening (e.g., Schwartz *et al.* 2007) would enable testing for variation in fitness related to inbreeding coefficients, average individual heterozygosity and genotypes at specific loci.

The adaptive differentiation among island fox populations that we document here and evidence that outbreeding depression is most likely in crosses between adaptively divergent populations (Edmands 2007; Frankham *et al.* 2011) might suggest that genetic rescue would have the unintended consequence of decreasing fitness, rather than the desired effect of reducing extinction risk. However, population genetic theory demonstrates that when N_e is small, the threat of swamping out locally adapted alleles is low because strong genetic drift precludes the maintenance of many of these alleles in the first place (Wright 1931, 1951). Selection would have to be very strong for an adaptive allele to be maintained by selection with N_e as small as observed in those island fox populations that would be the most likely candidates for genetic rescue. For example, on SNI with an N_e of 2.1, population genetic theory predicts that the selection coefficient would have to be $s > 1/(2N_e) = 0.24$ to maintain an adaptive allele (Conner & Hartl 2004), which is high relative to most empirical estimates of selection coefficients, at least for phenotypes (Conner 2001; Kingsolver *et al.* 2001). Moreover, if selection is this strong for a native, adaptive allele, then it is unlikely that a new, foreign, maladaptive allele will successfully "invade" and persist in the population.

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Thus, when N_e is extremely small, as observed in some island fox populations, concerns about inbreeding depression may be more important than concerns about outbreeding depression. If a population has an unacceptably high probability of going extinct, inbreeding depression significantly contributes to this high extinction risk, and genetic rescue is predicted to reduce inbreeding depression, then genetic rescue should be considered as a viable management option (Tallmon *et al.* 2004; Hedrick & Fredrickson 2010; Frankham 2015; Whiteley *et al.* 2015). Research is therefore critically needed to determine the viability of island fox populations, the magnitude of inbreeding depression, and potential fitness effects of interpopulation crosses and genetic rescue to determine if and when genetic rescue would be an effective management strategy. This research should be pursued as soon as possible so that these important management decisions can be made before population recovery is unlikely. Lessons from other systems—such as Isle Royale wolves (*Canis lupus*), Vancouver Island marmots (*Marmota vancouverensis*) and greater gliders (*Petauroides volans*)—highlight that waiting too long to make management decisions (and waiting too long to test the efficacy of management options) can cause imperiled populations to decline beyond the point of recovery (Lindenmayer *et al.* 2013; Marris 2015; Mlot 2015).

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Data accessibility

Raw sequence data, RAD contig sequences and genotype data: Dryad doi:10.5061/dryad.2kn1v.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Frequency histogram of mean coverage per locus averaged across individuals for the SNP data set including grey foxes.

Fig. S2 Frequency histogram of mean number of loci per individual for the SNP data set including grey foxes (total number of SNP loci was 4858).

Fig. S3 Principle component analysis (PCA) with grey foxes using (a, c, e, and g) presumably neutral SNPs not identified as high F_{ST} outliers or (b, d, f and h) presumably adaptive SNPs identified as high F_{ST} outliers.

Fig. S4 Principle component analysis (PCA) without grey foxes using (a, c, e and g) presumably neutral SNPs not identified as high F_{ST} outliers or (b, d, f and h) presumably adaptive SNPs identified as high F_{ST} outliers.

Fig. S5 Neighbour-net trees with grey foxes using (a, b, c and d) presumably neutral SNPs not identified as high F_{ST} outliers or (e, f, g, and h) presumably adaptive SNPs identified as high F_{ST} outliers.

Fig. S6 Neighbour-net trees without grey foxes using (a, b, c and d) presumably neutral SNPs not identified as high F_{ST} outliers or (e, f, g and h) presumably adaptive SNPs identified as high F_{ST} outliers. Outlier loci were identified using (a and e) the highest 5% of F_{ST} values, (b and f) FDIST2, (c and g) FDIST2 with a false discovery rate (FDR) correction, or (d and h) BAYESFST.

Table S1 High F_{ST} outlier loci that blasted to genes in the dog genome.

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