An efficient noninvasive method for simultaneous species, individual, and sex identification of sympatric Mojave Desert canids via in-solution SNP capture

Running title: Using SNP capture to genotype canids from scat

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Abstract

Understanding predator population dynamics is important for conservation management because of the critical roles predators play within ecosystems. Noninvasive genetic sampling methods are useful for the study of predators like canids that can be difficult to capture or directly observe. Here, we introduce the FAECES* method (Fast and Accurate Enrichment of Canid Excrement for Species *and other analyses) which expands the toolbox for canid researchers and conservationists by using in-solution hybridization capture to produce SNP genotypes for multiple canid species from scat-derived DNA using a single enrichment. We designed a set of hybridization probes to genotype both coyotes (*Canis latrans*) and kit foxes (*Vulpes macrotis*) at hundreds of polymorphic single nucleotide polymorphism (SNP) loci. We tested the probes on both tissue and field-collected scat samples. We enriched and genotyped 52 coyote and 70 kit fox scats that we collected in and around a Mojave Desert conservation easement near Boulder City, Nevada. We demonstrate that the FAECES* method produces genotypes capable of differentiating coyotes and kit foxes, identifying individuals and their sex, and estimating genetic diversity and effective population sizes, even using highly degraded, low-quantity DNA extracted from scat. We found that the study area harbors a large and diverse population of kit foxes and a relatively smaller population of coyotes. Future studies can replicate our methods to monitor canid populations and assess the impacts of management decisions. This is important for conservationists because both coyotes and kit foxes are known predators of the federally threatened Mojave desert tortoise (*Gopherus agassizii*) in the study area.

Keywords: noninvasive genetic sampling**,** conservation genetics**,** hybridization capture, canid, coyote (*Canis latrans*)**,** kit fox (*Vulpes macrotis*)

Introduction

Studying the population dynamics of predators, including canids, is critical for conservation and ecosystem management because of the direct and indirect impacts predators have on prey species and the status of predators as sentinels, i.e. they are sensitive to changes in the environment and can serve as indicators of ecosystem health (Sergio et al., 2008). Both coyotes (*Canis latrans*) and kit foxes (*Vulpes macrotis*, Figure 1) are known to consume the federally threatened Mojave desert tortoise (MDT, *Gopherus agasizzii*; Cypher et al., 2018). Coyotes are the primary cause of predation on adult tortoises (Esque et al., 2010), while kit foxes are the primary predators of tortoise nests (Bjurlin & Bissonette, 2004). It is therefore important for managers to monitor these canids in desert areas where the tortoise occurs.

Because canids tend to be elusive and difficult to study, noninvasive genetic sampling methods have been utilized extensively, for example, to estimate abundance and genetic diversity of coyotes (e.g. Morin et al., 2016; Prugh et al., 2005; Woodruff et al., 2021) and kit foxes (e.g. Lonsinger et al., 2018; Wilbert et al., 2015; Wilbert et al., 2019). Scat, the most commonly collected material in noninvasive genetic studies (Waits & Paetkau, 2005), is particularly easy to identify in the field because canids defecate to mark territories (Morin et al., 2016). In areas where visual detection is difficult, scat searching dogs (*Canis lupus familiaris*) have proven effective at locating scats (Ralls et al., 2003; Smith et al., 2001). In addition, scats both enable high-resolution analysis of host genetics and allow researchers to study predation by identifying DNA from consumed species (Banks et al., 2003).

Noninvasive genetic sampling

Historically, most noninvasive studies targeted mitochondrial DNA for host species identification (e.g. Bozarth et al., 2010; Dalén et al., 2004; Paxinos et al., 1997) and nuclear microsatellites to reliably identify individuals and estimate population genetic structure (Lampa et al., 2007; Schwartz et al., 2006; Smith et al., 2006). However, single nucleotide polymorphism (SNP) loci are being used more often as new genotyping methods have made it efficient and cost-effective to simultaneously genotype hundreds of individuals at hundreds to thousands of SNPs (Carroll et al., 2018). To date, the majority of noninvasive studies that have used SNP markers generated genotypes by using polymerase chain reaction (PCR) to amplify loci followed by measurement of fluorescence (e.g. with the Fluidigm platform; von Thaden et al., 2017) or direct amplicon sequencing (Natesh et al., 2019). However, the use of multiplex PCRs in noninvasive applications presents some challenges, including the need for species-specific references to design primer pairs, the potential for PCR inhibition due to co-extracted inhibitors in scat-derived DNA, complex optimization of the multiplex reaction to avoid interactions between primers, and the requirement for DNA extracts >1ng/uL (von Thaden et al., 2020).

In-solution DNA hybridization capture ("capture" hereafter) is an alternative to multiplex PCR amplification that is well-suited to noninvasive DNA applications. First, unlike the primers used in PCR amplification, hybridization probes can be as much as 39% divergent from the target sequences (Li et al., 2013) precluding the need to have a reference genome for the target species. Second, capture methods are compatible with highly degraded, low-quantity DNA extracts with a high proportion of exogenous DNA such as those derived from scat, which is why they are often employed in ancient DNA studies (Ávila-Arcos et al., 2011). Finally, bait design is highly flexible, allowing one to minimize allelic dropout by probe tiling of the target region

(Cruz-Dávalos et al., 2017) and including probes matching alternate alleles. It allows one to target as many regions as desired including whole nuclear genomes or sequences from multiple taxa in a single assay (Campana et al., 2016a).

Despite the similar challenges posed by ancient and noninvasive DNA, capture methods have been widely adopted in ancient DNA studies but have rarely been used for noninvasive applications (Carroll et al., 2018). This may be in part due to the complexity of bioinformatics involved in probe design, the high cost of generating probes (Meek & Larson, 2019), or the incidence of off-target capture (Jensen et al., 2020; von Thaden et al., 2020). Although several authors have demonstrated that it is possible to enrich and sequence primate DNA from scat samples (e.g. Hernandez-Rodriguez et al., 2018; Perry et al., 2010; Snyder-Mackler et al., 2016; White et al., 2019), to our knowledge these methods have not been applied to any taxonomic group outside of primates, have not been used to enrich for SNP loci, and have not targeted multiple species in a single enrichment.

Study objectives

We aimed to address these gaps by demonstrating that, by using capture methods, it is possible to generate SNP genotypes for individuals of two different canid species using a single assay, and that these SNP data are capable of differentiating individuals, assigning sex, and estimating kinship, genetic structure, and genetic diversity. Accordingly, our first objective was to characterize and validate a set of informative SNP markers capable of identifying individual coyotes and two kit foxes subspecies: desert kit foxes (*V. m. arsipus*, DKF) and San Joaquin kit foxes (*V. m. mutica,* SJKF).

Our second objective was to assess coyote and kit fox populations in the Boulder County Conservation Easement Area (BCCEA) in the Mojave Desert (Nevada, USA) by using capture methods to enrich field-collected scat samples for our newly developed SNPs. We aimed to estimate genetic diversity, genetic population structure and effective population sizes for both species. The methods described here can be replicated to enable the use of scats for both applied and basic research on canids. Researchers can utilize these methods to monitor predator population dynamics to evaluate the effects of management actions including minimizing anthropogenic subsidies such as refuse and water, which can result in elevated predation on federally threatened MDTs (Esque et al., 2010).

Materials and methods

Study site and sample collection

We collected 340 scat samples at randomly selected points within the Boulder City Conservation Easement Area (BCCEA) and adjacent areas between September 2015 and April 2018 (Figure 2). Based on visual inspection, the scats were rarely fresh and some may have been several weeks old. The BCCEA is a 34,800-hectare area of public land in the northeastern Mojave Desert within the Eldorado Valley in southern Nevada, USA, that was established in 1995 for the conservation of the MDT and other desert wildlife.

We used stratified random sampling (Ratti & Garton, 1994) to select locations for scat collection. Using ArcGIS, we selected 84 random points that were at least 1 km from any other sampling point. We included multiple sampling locations within three areas that we defined based on dominant land-use: city, BCCEA, and desert reference. City consisted of urbanized

areas within Boulder City boundaries, the BCCEA zone included areas within and close to the BCCEA boundaries, and the desert reference area consisted of lands managed by the Bureau of Land Management south of the BCCEA, not including the highland mountains. Additional samples were collected opportunistically at power towers and fences. At each point, circles of 10-meter diameter were searched for scats, and all scats within this perimeter were collected. Collectors visually identified the species that produced the scat and recorded this information as well as the date and collection point using a Garmin Montana 650 GPS device. Scats were bagged individually unless they were found as part of a latrine (Ralls and Smith, 2004), in which case multiple scats were placed in a single bag. Bags were marked with the date and location.

Samples were stored dry in sealable plastic bags and shipped to the Center for Conservation Genomics (CCG), Smithsonian Institution, Washington, D.C. Subsamples of approximately 1.5 cm^3 were taken and stored dry in 15 mL conical tubes until DNA extraction. The remaining scat was saved for a separate study on morphological dietary analysis. In cases where multiple scats were collected per locality during the same sampling session (i.e. multiple samples were taken from a latrine), we attempted to take one sample per putative individual based on visual inspection.

FAECES* probe design

We designed a set of probes to enrich scat-derived DNA samples for a subset of SNP loci that are polymorphic in both coyotes and kit foxes and a subset that are fixed (or with a low minor allele frequency) in one species but polymorphic in the other to confirm species identification, identify individuals of each species, and calculate population genetics parameters.

We also designed and included probes targeting the zinc finger-Y and -X genes (*ZFX*/*ZFY*) for sex identification (Figure 3). We refer to this method of using capture to genotype multiple canid species using DNA extracted from scat as FAECES*, or Fast and Accurate Enrichment of Canid Excrement for Species *and other analyses.

First, we generated a reference dataset from which to select SNPs by enriching and genotyping coyote and kit fox tissue-derived DNA samples for a set of ~20,000 SNP loci that we previously found to be polymorphic in African wild dogs (*Lycaon pictus*, Campana et al., 2016b; Supporting Information: *Lycaon pictus* probe design, Probes available in Figshare, 10.25573/data.14633298). We extracted DNA from eight western coyote tissue samples obtained from the Museum of Southwestern Biology (Accession numbers in Table S1) and one eastern coyote obtained from the tissue collection at the CCG, using a DNeasy Blood and Tissue Kit (Qiagen, Valencia CA) following the manufacturer's protocol for tissue. We also obtained nine DNA samples that were previously extracted from San Joaquin kit fox (SJKF) tissues collected in Bakersfield, CA (Wilbert et al., 2015). We quantified DNA samples with a Qubit® 4 fluorometer (Life Technologies) using a 1× dsDNA HS assay. We sheared DNA to an average length of 250 base pairs (bp) using a Bioruptor® Pico sonicator (Diagenode Inc., Denville, NJ). The number of cycles required for adequate shearing varied by sample and ranged from 45 – 60; each cycle was 30 seconds on followed by 30 seconds off. We visualized the sheared DNA with a TapeStation 4200 System (Agilent Technologies, Santa Clara, CA) using High Sensitivity D1000 reagents. We then prepared dual-indexed libraries using the 'BEST' single-tube method described in Carøe et al. (2017) with revisions as described in Mak et al. (2017). We quantified libraries after index PCR with the Qubit® fluorometer and enriched samples in single-plex

reactions following the myBaits Manual v3 standard protocol. We quantified the enriched libraries using Qubit® and visualized them on the TapeStation as above. Finally, we pooled samples equimolarly and sequenced with paired-end 150 bp reads on an Illumina MiSeq at the CCG (Illumina, Inc., San Diego, CA).

We downloaded demultiplexed sequence data from the BaseSpace Server (Illumina) and used the program FastQC v0.11.8 (Andrews, 2010) to check for sequence quality and adapter content. We removed adapter sequences using TrimGalore v0.6.4 (Krueger, 2019). We aligned reads to the domestic dog (*Canis familiaris*) reference genome (Hoeppner et al., 2014; GenBank: CanFam3.1) using the 'mem' algorithm in BWA v0.7.17 (Li, 2013). We used SAMTools v1.3.1 (Li et al., 2009) to sort BAM files and convert to the SAM format. Following the SAMtools variant calling workflow v1.0 (http://www.htslib.org/workflow/#mapping_to_variant), we then marked duplicates with Picard Tools v 2.20.6 (Picard Toolkit, 2019, https://github.com/broadinstitute/picard) and realigned reads around indels (insertions/deletions) using the GenomeAnalysisToolKit (GATK v3.7, McKenna et al., 2010). We identified sequence variants (minimum quality 20) using the SAMtools 'mpileup' command (-C50 option) and the BCFtools v1.4.1 'call' command (Li et al., 2009). We removed indels, any site with more than 10% missing data, and sites with average coverage <10 reads using VCFtools v0.1.15 (Danecek et al., 2011). We then used the "vcf2baits" command in BaitsTools v1.2.0 (Campana, 2018) to generate probe sequences. We utilized three options to improve the likelihood of enrichment success: 1) generating short (80 bps) probes (option -L 80), 2) designing probes with alternate alleles represented (option -a), 3) tiling probes to cover each variant site with ~4× average depth with an offset of 20 bp between probes (option -O 20). Because the domestic dog reference

195 genome is more similar to the coyote than to the kit fox genome, we initially recovered more
196 coyote variants. We then incorporated an option to balance the number of probes by designated
197 taxon, setting a maximum of 500 variants for each, to include some loci that are fixed within
198 species in our samples but that vary between, as well as loci that are variable within each species
199 (using options 'taxacount' and 'popcategories').

200 To test the ability of the selected variants to differentiate species and individuals, we ran
201 PCAs and pairwise relatedness analyses with the SNPRelate v1.8.0 package (Zheng et al., 2012)
202 in R v4.0.3 (R Core Team 2020, applies to all uses of R). First, we filtered the multi-sample VCF
203 file to restrict loci to only those selected by BaitsTools. We then ran three PCA analyses: one
204 with coyotes only, one with kit foxes only, and one with both species. We calculated pairwise
205 kinship values by maximum likelihood estimation (MLE) with minor allele frequency >= 0.1. To
206 simulate the effect of locus dropout, we randomly removed 20% of loci from each dataset and
207 ran all analyses again to ensure that the patterns were consistent.

208 Finally, we downloaded reference sequences for *ZFX* and *ZFY* genes for coyotes
209 (Williams et al., 2003, GenBank AY145847 and AY145848, respectively) and kit foxes (Ortega
210 et al., 2004, GenBank AY310919 and AY310920, respectively) and aligned them using the
211 *MAFFT* v7.450 plugin (Katoh, 2005) in Geneious v9.1.2 (Biomatters Ltd., Auckland, New
212 Zealand). We exported the alignment in FASTA format and generated probes using the
213 "aln2baits" command in BaitsTools with the same options as above (80 bp probes with 20 bp
214 offset and ca. 4× tiling). The final set of probe sequences, targeting both autosomal and sex-
215 linked loci, was further filtered by Arbor Biosciences (Daicel Arbor Biosciences, Ann Arbor,
216 MI) using their standard pipeline to remove loci that overlapped >25% with repeat-masked

regions in the dog genome. Finally, we purchased myBaits® probes (myBaits-1 kit) from Arbor Biosciences (Probes available in FigShare, 10.25573/data.14633298).

Scat samples

Laboratory methods: DNA extraction

We extracted DNA from scat samples using a Mag-Bind® Stool DNA kit (Omega Bio-Tek, Norcross, CA) following the manufacturer's "Standard Protocol" with several modifications. First, bead bashing was omitted and replaced with overnight digestion with 30 µl DS buffer, 20 µl proteinase k (>600mAU/mL), and enough SLX-Mlus Buffer to completely cover the sample. Samples were incubated at 56°C with agitation at 40 RPM. After digestion, samples were centrifuged for 2 minutes at 3,000 × g, and approximately 700 µl supernatant was transferred to a 2.2 mL 96-deepwell plate. The plate was then centrifuged again at 4,000 × g for 10 minutes, and 600 µl supernatant was transferred to a new 2.2 mL 96-deepwell plate, taking care not to disturb the pellet. We then added 1.2 mL Mag-Bind® particles in XP2 buffer to each sample and mixed by pipetting. After a 5-minute incubation, the plate was centrifuged again for two minutes at 3,000 × g and then placed on a magnetic separation device. After elution with 100 µl of water, DNA extracts were quantified using a Qubit® fluorometer with a 1× dsDNA HS assay.

Library preparation and enrichment using FAECES probes*

Before making genomic libraries from the scat-derived DNA samples, we performed a PCR-based species identification assay following Bozarth et al. (2010). Briefly, we amplified a small fragment of the mitochondrial control region (CR) that is a different length in each canid

239 species in the study area. We included both positive and negative controls in each reaction.
240 Fragment length was determined by running PCR products on an ABI 3130xl at CCG. We then
241 proceeded with library preparation on samples that were identified as either coyote or kit fox.
242 To validate our SNP genotyping and sexing methodology, we included 5 of the tissue-
243 derived coyote DNA samples used for probe design above and 21 additional SJKF DNA samples
244 collected in LoKern, CA from previous studies: 7 tissue-derived samples and, for each
245 individual, two additional fecal-derived samples that were previously identified genotype
246 matches (Ralls, Maldonado, & Smith, unpublished data) at 6 microsatellite loci shown to be
247 sufficient to identify individuals (Smith et al., 2006). The kit fox fecal and tissue samples were
248 previously sexed following PCR-based protocols developed by Ortega et al. (2004). Finally, we
249 included previously extracted DNA from hair samples of 5 SJKF individuals from Bakersfield,
250 CA (Bremner-Harrison et al., 2006) to test whether our methodology would result in SNP
251 genotypes capable of discriminating SJKF from different populations.
252 We sheared all DNA samples to an average length of 250 bp using a Q800R sonicator
253 (QSonica, Newtown, CT). We then prepared dual-indexed libraries using the single-tube method
254 as above (Carøe et al., 2017; Mak et al., 2017). We quantified libraries after index PCR with a
255 Qubit® fluorometer and pooled three samples equimolarly into each capture reaction. We diluted
256 the probes three-fold and performed target enrichment using the standard protocol in the myBaits
257 Manual v4. After post-capture PCR, we quantified enriched library pools using a Qubit®
258 fluorometer and visualized them on a 4200 TapeStation System (Agilent Technologies) with
259 High Sensitivity D1000 reagents. Finally, we pooled captured libraries equimolarly and
260 sequenced with paired-end 150 bp reads on an Illumina MiSeq (CCG) and an Illumina NovaSeq

6000 (Vincent J. Coates Genomics Sequencing Laboratory at the University of California Berkeley).

Data analysis

SNP calling

We trimmed raw reads for adapter content and quality, aligned reads to the dog reference genome (using BWA v0.7.17), and identified variants following the SAMtools workflow as above. We removed indels and filtered sites to include only autosomal variants in the 140 bp regions targeted by the baits using VCFtools. We then filtered the all-sample VCF file for sites with a minimum depth of 5 reads and no more than 80% missing data, and then used the option 'thin -500000' to select only one variant per baited region. We then performed a PCA analysis with the SNPRelate package in R as above to confirm species identification. For subsequent analyses, we retained only samples which could be identified as coyote or kit fox according to separation by PC1.

We then separated samples according to species and called variants for coyotes and kit foxes separately using SAMtools 'mpileup' and BCFtools v1.9 'call' as above. Using VCFtools, we filtered the all-sample VCF files to include one variant per autosomal baited region and only sites with a minimum depth of 5 reads, minor allele count >=2, and no more than 25% missing data. Finally, we removed all sites that were out of Hardy-Weinberg equilibrium after Bonferroni correction ($\alpha = 0.05$).

Identification of individuals and recaptures

283 Using the SNPRelate package in R, we performed PCA on both the kit fox and coyote
284 variant datasets. For kit foxes, we performed analyses with both DKFs and SJKFs included and
285 also on each subspecies separately. We then calculated identity-by-state (IBS) and identity-by-
286 descent (IBD) with the maximum likelihood estimation, which has been shown to be more
287 accurate than the method of moments procedure (Blue et al., 2016). We characterized recaptures
288 and resampling events as pairs of samples with IBD >0.4 (IBS>0.95) and first-order relatives
289 (parent-offspring or full-siblings) with IBD >=0.2 and <=0.4 (Milligan, 2003). We defined
290 recapture events as sampling the same individual on different dates and/or in different locations.
291 We classified resampling events as occasions when the same individual was identified in
292 multiple samples collected at the same site on a given date (i.e. multiple scats from a single
293 individual in a latrine), because we cannot rule out the possibility that multiple samples from the
294 same individual were deposited on the same day. Using GenAlEx v6.503 (Peakall & Smouse,
295 2012), we calculated the probability of identity assuming siblings are present in the data (P_{IDsibs},
296 Waits et al., 2001).
297 We used VCFtools to calculate observed (H_o) and expected heterozygosity (H_e) and the
298 inbreeding coefficient (F_{IS}) for all unique individuals and all 'unrelated individuals', i.e., with
299 first degree relatives removed. For kit foxes, we calculated diversity metrics for SJKFs and
300 DKFs separately. To determine if our SNPs are capable of discriminating between kit fox
301 subspecies and between SJKF populations, we ran STRUCTURE v2.3.4 (Pritchard et al., 2000)
302 on the dataset including all unique individuals and for SJKFs and DKFs separately. For each
303 STRUCTURE run, we used a burn-in of 250,000 steps followed by 1,000,000 recorded steps.
304 We used the admixture model, no location priors, and assumed correlated allele frequencies

(Falush et al., 2003). We performed simulations with K 1–8 with 5 replicates each, and identified meaningful K values using the ΔK method (Evanno et al., 2005) implemented in STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt, 2012). We combined replicate runs using CLUMPP v1.1.2 (Jakobsson & Rosenberg, 2007). We quantified the differentiation between kit fox groups by running an analysis of molecular variance (AMOVA) and estimating pairwise F_{ST} in GenAlEx. We assessed statistical significance with a permutation test of 10,000 replicates.

We estimated the effective population sizes (N_e) of coyotes and kit foxes in the BCCEA using the linkage disequilibrium model with random mating (Waples and Do, 2008) in NeEstimator v2.1 (Do et al., 2014). We report N_e values using minor allele frequency $P_{crit} = 0$, because our variants were already filtered for frequency, and 95% confidence intervals generated by the 'Parametric method' (Waples and Do, 2008). For coyotes, we then estimated the census population size based on the number of recaptures of different frequencies using the maximum likelihood program CAPWIRE v1.1.4 in R (Pennell et al., 2013). We used the likelihood ratio test to select between the null even capturability model (ECM) and the two innate rates model (TIRM) with default parameters.

For kit foxes, population turnover is generally high - previous studies have reported mean annual survival to be 0.42 (Cypher et al., 2000). Because our total sampling period spans multiple years, leading to a violation of the assumption of population closure, we estimated the census population size of kit foxes by using the estimated N_e/N_c ratio as 0.55 (Wilbert et al., 2019).

Finally, we investigated local spatial structure in both species. Using a Mantel test implemented in GenAlEx, we tested for a correlation between pairwise genotypic distance and

Euclidean geographic distance, with 9,999 permutations to generate the null distribution. Also using GenAlEx, we generated a Mantel correlogram to test for spatial correlation between pairs of individuals at different distance classes. We used the Sturges rule (Sturges, 1926) to determine the number of classes, and defined each class to ensure a sufficient number of comparisons within each class. We selected the 'Spatial' option and performed 9,999 permutations.

Sex identification

For each sample positively identified as either a kit fox or coyote by PCA, we aligned trimmed reads to the appropriate species reference for *ZFX* and *ZFY* separately. We followed the steps outlined above for alignment and variant calling except for the 'mpileup' command, during which we omitted the -C50 option. This option downgrades map quality for reads with excessive mismatches. Although omission of the -C50 option increases the likelihood of spurious alignments, we disabled it because the multiple SNPs separating the *ZFX* and *ZFY* gene copies in the short reference region produced strong bias towards the reference gene copy (e.g., inclusion of the -C50 option yielded 99.25% kit fox Y alleles when aligned against *ZFY* but only 7.62% Y alleles when mapped against *ZFX*).

Preliminary sex designations were automatically assigned using a voting algorithm (script available: https://github.com/campanam/FAECES). For the automated sexing assignments, we discarded the *ZFY* alignments because mapping against the coyote *ZFY* yielded 100% Y alleles after removing the -C50 option, indicating significant asymmetric alignment bias. We catalogued SNP alleles that separate canid *ZFY* (male) alleles from their *ZFX* (female) homologs. At each sex-specific site, each read 'voted' for the 'male' or 'female' allele. A minimum of 10 allele

copies (across all sex-specific sites) were required to call sex. Since our data were unphased, we assumed that each allele and every site was independent. We recognize that alleles are linked on individual reads within the short region examined, which could potentially bias these preliminary assignments (e.g., through drop-out of multiple Y SNPs if a single male DNA sequence is not retained). For each sample, we determined false negative (Y drop-out) and false positive (Y drop-in/sequence misalignment) male determinations using the binomial probability of the deviation of the Y allele vote distributions from the expected Y allele frequency. We used an uncorrected $\alpha = 0.05$ for these statistical tests. Hybridization capture bias and reference bias can produce strong deviations from the expected 0.5 Y allele frequencies. Therefore, we empirically estimated the expected Y allele frequencies including these biases for each species using samples of known sex. These known samples were derived from tissues, where we can expect a minimum of allelic drop-out and empirical frequencies closer to the expected 0.5. This penalizes male sex estimation in the scat samples, where drop-out is more likely and statistical artifacts due to low DNA concentrations are much more likely to generate strong deviations from expected allele frequencies. To maximize sensitivity for males, we used the minimum observed Y allele frequency for each species in the known datasets as the expected Y allele frequency for the unknown samples. While we also experimented using the mean Y allele frequency and hard cut-offs at set *z*-scores, we found that the minimum Y frequency produced the results most consistent with the known samples (data not shown).

After initial automated sex estimation, we manually checked all results against known individuals and between sample replicates. We also checked all samples preliminarily identified as "female" where a significant number of Y alleles were detected (>10) as these likely represent

Y drop-out events, skewing the Y allele distribution from that expected from the known tissue samples. First, we imported BAM files into Geneious and generated consensus sequences with options 'Assign Quality Total', call 'N' if coverage <2 reads, and call heterozygotes >30%. We then aligned *ZFX* and *ZFY* consensus sequences to the appropriate reference *ZFX*/*ZFY* alignment and determined sex by visual inspection.

Results

FAECES probe design and test*

The nine coyote and nine SJKF tissue samples that were enriched for ~20,000 SNP loci using the African wild dog probe set (see Supplemental Information) were sequenced with a mean of approximately one million reads per sample (range of 65,3640 – 1,254,195; PRA project ID). Our final probe set targeted 835 autosomal SNP loci and the *ZFY*/*ZFX* genes for both coyotes and kit foxes and comprised 4,132 unique baits (FigShare 10.25573/data.14633298). Of the 835 autosomal SNPs, 382 were polymorphic in coyotes and 364 were polymorphic in SJKFs. The PCA including all samples showed separation of kit foxes and coyotes, and eastern coyote from western coyote (Figure 4). Results of PCA and kinship analyses on coyote and kit fox datasets were similar after simulating the effect of dropout by randomly removing 20% of loci. The kinship analysis including all SNP loci with a minor allele frequency >=0.1 included 249 SNPs, and after simulating 20% dropout, 89 SNPs; the coefficient of determination (R^2) between kinship estimates was 0.80.

DNA sequencing

After excluding samples that failed to amplify in the species identification PCR, mixed-species samples, or multiple samples that were collected at a latrine and stored together, 275 scat samples from the BCCEA remained. Of these, 84 were identified as coyote and 191 kit fox by the species identification PCR (Bozarth et al., 2010). Including the known individual SJKF and coyote tissue samples (n = 31), we sequenced a total of 306 samples with a mean of 1.5 million reads per sample (376 – 11.3 million; SD = 1,045,501). Across all samples, a mean of 75% of reads (0.19% – 99%; SD = 0.21%) mapped to the dog reference genome. For scat samples, a mean of 73% (SD = 19%) of reads mapped; for tissues, a mean of 99% (SD = 0.2%) of reads mapped.

Species identification of canid scat samples

After filtering variants from the joint species all-sample VCF, 668 sites remained. Based on the ability of the PCA to discriminate species (PC1, 57% of variation in the data), we filtered out samples with fewer than 30 SNPs, leaving 70 kit fox samples and 52 coyote samples. The species identification based on PCA matched the known species for all tissue samples and matched the identification based on our PCR assay in all but two of the 122 scat samples. These two scats were classified as kit foxes by the species ID assay, but clustered with coyotes in the PCA. For these two samples, we assembled reads that did not map to the dog reference genome to both a coyote and a red fox mitochondrial genome (GenBank Accessions NC_008093 and AM181037, respectively) using the Geneious algorithm (Medium-low sensitivity and up to 5 iterations). We generated consensus sequences (options 'Total' quality), assigning ambiguity codes if at least 40% of reads disagreed at a given site, and aligned consensus sequences and

415 references using the *MAFFT* v7.450 (Katoh, 2005) plugin. Visual inspection of alignments at the
416 *12S rRNA* gene revealed bases matching both the coyote and kit fox reference. These two mixed
417 samples were either the result of contamination, perhaps due to a mixed-species latrine (Ralls
418 and Smith, 2004) or coyote predation of kit fox (Ralls and White, 1995) and were removed from
419 further analyses.
420
421 *Individual identification, genetic diversity, and estimates of coyote population size*
422 For samples confirmed as coyotes, 301 polymorphic sites remained after filtering. The
423 PCA showed no apparent pattern of clustering among samples (Figure S1). Among the 47 coyote
424 samples from the BCCEA, we identified five individuals that were recaptured in different
425 locations during multiple sampling sessions (Figure 5), and five that were resampled in a single
426 session (i.e. pairwise kinship >0.40 or identity-by-state >95%), leaving 30 unique individuals.
427 We identified 11 individuals in 13 samples in 2015, four individuals in five samples in 2016, and
428 22 individuals in 23 observations in 2017; three individuals were recaptured in multiple years.
429 The maximum distance observed between recaptured individuals was 25.5 km, between two
430 samples collected in September 2015 and March 2017; the shortest distance was 1.1 km between
431 two samples collected in January 2016 and May 2017.
432 For subsequent estimation of genetic diversity, we used only unique individuals, selecting
433 the sample with the least missing data for each. We also identified several first-order relatives
434 (parent-offspring or full sibling pairs, kinship >0.2) – after removing the individual with the most
435 missing data in each related pair, 22 individuals remained. Including first-order relatives, the
436 average observed heterozygosity across variable sites was 0.24 (SD 0.04) and the inbreeding

coefficient, F_{IS} was 0.05 (SD 0.18); excluding these individuals, average observed heterozygosity was 0.25 (SD 0.04) and F_{IS} was 0.05 (SD 0.14, Table 1). Bartlett's test revealed that the observed and expected heterozygosity were not significantly different in either dataset ($p > 0.1$). The probability of two individuals having identical genotypes, (P_{ID}), was 1.30×10^{-65}; assuming siblings are present in the data, the probability (P_{IDsibs}) was 1.1×10^{-33}. The number of SNPs at which the P_{IDsibs} was <0.0001 was 38.

Including first order relatives ($n = 30$), the NeEstimator estimated effective population size (N_e) of coyotes in the BCCEA is 37.6 individuals (95% CI = 33.8 – 42.1). Inclusion of close relatives can result in artificially lower estimates of N_e, so we also ran the analysis on a dataset with first order relatives removed ($n = 22$). As expected, the estimated N_e was higher when using this dataset: $N_e = 64.9$ (95% CI = 53.5 – 81.8). We found no evidence of isolation-by-distance (IBD, $R_{xy} = 0.14$, $p = 0.169$), or spatial autocorrelation among individuals.

Because of the small number of recaptures, we pooled recapture data across years for the CAPWIRE analysis. We were not able to reject the ECM ($p = 0.1$); based on this model, the estimated census population size in the BCCEA was 83 (95% CI = 48 – 210).

Individual identification, genetic diversity and structure, and estimates of kit fox population size

After filtering, 136 polymorphic SNPs remained in our kit fox dataset. These loci were able to differentiate between SJKFs and DKFs, as well as between SJKF from two localities in California. Including all 70 samples, PCA separated the two subspecies (PC1, accounting for 14.8% of variation, Figure 6). Analyzing only the 13 SJKF samples, the PCA separated the SJKFs from the two localities, LoKern and Bakersfield (PC1, accounting for 16.4% of variation,

Figure S2). Including all unique kit foxes (n = 62), the results of the STRUCTURE analysis indicated the most likely number of population clusters is two, with individuals separated by subspecies (Table S2). Analyzing only SJKF (n = 13), the most likely number of population clusters was one when using no location priors, and two with location priors. Individuals were divided between those from LoKern and Bakersfield (Table S3). Pairwise F_{ST} between the two kit fox subspecies was 0.16 (p = 0.0001); the results of the AMOVA showed that between-subspecies variation accounts for 15% of total variation in the dataset. Between the two SJKF localities, F_{ST} = 0.094 (p = 0.005). The average observed heterozygosity among SJKFs is 0.32 (SD 0.04), and Bartlett's test revealed no significant difference in variances between observed and expected heterozygosity (p > 0.1). The average F_{IS} was 0.04 (SD 0.13).

Of the seven sets of matching LoKern SJKF scat (two each) and tissue pairs, only one scat sample produced enough sequencing reads for individual identification. The estimated pairwise kinship between the tissue and its putative corresponding scat sample was 0.27, indicating probable first-degree relatives. Based on P_{IDsibs}, our SNP dataset provides more statistical power than the microsatellite data previously used to distinguish between individual recaptures and first-degree relatives (1.2×10^{-18} and 7.95×10^{-3}, Smith et al., 2006, respectively).

Among the 57 BBCEA desert kit fox samples, we identified 49 unique individuals, 4 recaptured individuals (Figure 5), and 3 individuals that were resampled. Of the 4 recaptured individuals, two were captured in multiple years (2015 and 2017). The maximum geographical distance between observations of individuals was 4.4 km, sampled on October 2015 and January 2018. We identified 27 first-degree relative pairs; after removing the individual with the most

481 missing data in each pair, 36 'unrelated' individuals remained. Average observed heterozygosity
482 among the 49 individuals was 0.30 (SD 0.06), and average F_{IS} was -0.01 (SD 0.20). Excluding
483 putative first-degree relatives, average observed heterozygosity was 0.29 (SD 0.05) and F_{IS} was -
484 0.002 (SD 0.15, Table 2). Bartlett's test revealed that the variances in observed and expected
485 heterozygosity were not significantly different in either dataset ($p > 0.1$). P_{ID} was 1.30×10^{-34}
486 and P_{IDsibs} was 1.2×10^{-18}. The number of SNPs at which the P_{IDsibs} was <0.0001 was 34. Using
487 genotypes from unrelated individuals captured between January and April 2017 ($n = 34$), the
488 NeEstimator estimated effective population size was 179 (95% CI = 92 – 1644). Assuming an
489 N_e/N_c ratio of 0.55, N_c = 325 (166 – 2989), or 0.4 kit foxes/km^2 (0.2 – 3.7 foxes/km^2). The
490 Mantel test revealed no significant correlation between genetic distance and genotypic distance
491 (R_{xy} = -0.054, p = 0.250), and the Mantel correlogram showed no evidence of spatial
492 autocorrelation of individuals at any distance class.
493
494 *Sex identification*
495 The estimated expected Y allele frequencies using samples of known sex were 0.8315 for
496 kit foxes and 0.4382 for coyotes. The minimum observed Y allele frequencies in the known
497 dataset for each species, which we used for the expected Y allele frequency for unknown
498 samples, were 0.7994 for kit fox and 0.4036 for coyotes. Of the 30 individual coyotes identified
499 in our surveys of the BCCEA, 16 were male, 12 were female, and 2 were undetermined. The
500 estimated sex across all samples representing resamples of individuals ($n = 5$ individuals, $n = 6$
501 resample events) matched; all recaptures ($n = 4$ individuals, $n = 6$ recapture events) also

matched, except for one recapture event, where the sex of one sample could not be determined. Our sex assignments matched the known sex for all tissue references.

Of the 49 individual kit foxes from the BCCEA, 22 were female, 26 male, and one undetermined. The assigned sex matched across all samples for recaptured individuals (n = 4 individuals, n = 5 events) and resampled individuals (n = 3 individuals, n = 3 events). For all SJKF tissue samples, the sex estimated from the data matched the known sex of each individual (n = 12). Regarding the matching LoKern SJKF scat and tissue pairs, for which scat sexing was previously conducted using a PCR method (Ortega et al., 2004), only one scat produced enough sequencing reads for sex identification. As discussed above, based on pairwise kinship, these two samples most likely represent first-order relatives. Based on the high mean sequence depth, it is unlikely that this scat sample represents dropout of *ZFY* alleles; mean sequencing depth was 711 reads (0 – 1618) with 2577 reads mapped to the *ZFX* reference.

Discussion

Use of the FAECES method to genotype scat samples*

We showed that by using the FAECES* method, employing in-solution hybridization capture, it is possible to generate SNP genotypes capable of identifying individual canids and their sex using scat samples from multiple canid species in a single assay. We enriched for 382 and 364 polymorphic SNPs in coyotes and kit foxes, respectively, and successfully genotyped individuals of both species using even very low quantity scat-derived DNA extracts (<1ng/uL). The average amount of starting DNA (ng) that went into library preparation was significantly higher (Wilcoxon two-sample t-test, p = 0.003) for samples that successfully produced genotypes

524 of at least 35 SNP loci than for those that did not (199.9 ng and 113.5 ng, respectively); however,
525 we generated successful genotypes from scats of both species with starting DNA concentrations
526 as low as 0.1ng/uL (~ 3ng total). Our method worked reliably for DNA extracted from tissue
527 samples - all tissue-derived samples resulted in full genotypes with no missing data, even using
528 low quantity DNA that was extracted more than 20 years ago (LoKern kit fox DNA, 1.34 – 5.5
529 ng/uL). The high concentration scat DNA extracts that failed to generate genotypes likely had
530 low percentages of endogenous canid DNA content (i.e., they had a high percentage of prey or
531 microbial DNA), which we did not quantify (Cruz-Dávalos et al., 2017).
532 Our final datasets consisted of 136 polymorphic kit fox SNPs and 301 coyote SNPs.
533 Fifty-eight percent of coyote samples and 33% percent of kit fox samples were successfully
534 genotyped. Given that we used the dog reference genome for the probe sequences, the lower
535 success rate and smaller number of SNPs recovered from kit fox samples is most likely due to
536 divergence between probe and target DNA sequences, as has been documented in previous
537 studies (van der Valk et al., 2017). Dogs and kit foxes are separated by approximately 9 – 10
538 million years of evolution (Lindblad-Toh et al., 2005), while dogs and coyotes only diverged ~1
539 million years ago (vonHoldt et al., 2011). However, our success rates for both species fall within
540 the range of success previously reported in microsatellite studies on coyotes and kit foxes using
541 scat (27.5% - 91.4%, Eriksson et al., 2020; Lonsinger et al., 2018). Although our success rates
542 were lower than previous studies that genotyped amplicons using the Fluidigm platform (80 –
543 97%, von Thaden et al., 2017), by using the FAECES* method we were able to include a larger
544 number of loci than the Fluidigm platform in a single enrichment (i.e. > 96). In addition, the kit

545 fox SNPs recovered were sufficient to differentiate between subspecies and populations of
546 SJKFs, and to identify DKF individuals with high probability ($P_{ID} = 1.3 \times 10^{-34}$)

547 Given that the aim of this study was to test the use of capture methods to generate SNP
548 genotypes from field-collected scat samples which vary greatly in quality and quantity, we did
549 not selectively collect fresh scat or pre-screen DNA extracts for quality. Future studies could
550 improve the FAECES* method success rate by 1) preferentially collecting fresh scat samples in
551 the winter when DNA degrades at a slower rate due to lower temperatures and less UV radiation
552 (Lonsinger et al., 2018), 2) pre-screening samples for endogenous content through qPCR assays
553 or amplification of microsatellite loci (Fontsere et al., 2021), and/or 3) performing multiple DNA
554 extractions and/or library preparations on each sample and pooling prior to enrichment (Fontsere
555 et al., 2021; Hernandez-Rodriguez et al., 2018; Perry et al., 2010).

556 Capture methods provide greater flexibility for SNP genotyping than methods based on
557 the generation of amplicons and subsequent genotyping by sequencing or fluorescence because
558 of the ability for probes to hybridize with sequences up to 39% divergent, therefore precluding
559 the need for a species-specific reference as well as the need to optimize PCR conditions for large
560 multiplexes. Capture recovers flanking and target sequence data (Faircloth et al., 2012) which
561 could be used to study genes that may be under selection. In addition, we implemented several
562 cost-saving measures that increased the economic feasibility of our capture methodology,
563 including probe dilution, multiplexing three samples per capture reaction (Hernandez-Rodriguez
564 et al., 2018), and using a single-tube library preparation method (Carøe et al., 2017). Including
565 the cost of probes, our estimated per-sample cost for library preparation, capture, and sequencing
566 was ~33 USD (prices for reagents purchased in 2018, Table S4), approximately half of the

567 estimated per-sample cost to genotype 96 samples for 96 SNPs using the Fluidigm platform (~70
568 USD, Carroll et al., 2018). Designing enrichment probes and generating SNP genotypes using
569 sequence capture data requires some bioinformatics skills (Meek & Larson, 2019) which can be
570 a barrier to the use of capture methods. However, the use of BaitsTools (Campana, 2018), a fast
571 and user-friendly software, automates and facilitates probe design.
572
573 *Canid populations in the BCCEA and implications for the conservation of MDTs*
574 Given the proportion of scats that were identified as coyotes (31%) and estimated
575 effective and census population sizes, our data suggest that the population of coyotes in the
576 BCCEA is smaller than that of kit foxes and on the low end of estimates reported in previous
577 studies. From our scat DNA analyses, we estimated the census population size (N_c) of the
578 coyotes in this study area to be 83. Assuming that the total suitable habitat in the sampled region
579 is 800 km^2, i.e. the total area sampled not including the mountainous habitat in the southeast, the
580 density of coyotes in the area is 0.10 individuals/km^2 (95% CI = 48 – 210 or 0.06 – 0.26
581 coyotes/km^2). Previous studies of western coyotes have reported values including 0.053 – 0.112
582 coyote/km^2 (Woodruff et al., 2021), 0.14 coyotes/km^2 (Ralls and White, 1995), and 0.07 – 0.08
583 coyotes/km^2 (Lonsinger et al., 2018). In general, coyote densities tend to be lower in desert areas
584 where they are sympatric with kit foxes because coyotes have higher water needs than kit foxes
585 which are better adapted to arid environments (Lonsinger et al., 2018). In undisturbed
586 landscapes, coyote densities are expected to be lower than those of kit foxes because of their
587 larger size (4 – 5×) and higher energetic requirements (Golightly & Ohmart, 1984). However,
588 coyote densities can be higher than those of kit fox in landscapes with anthropogenic

589 disturbances including invasive plant species and artificial water sources, both of which decrease
590 available kit fox habitat and prey species while increasing the number of coyotes, the primary
591 competitors (and occasional predators; Ralls and White, 1995) of kit foxes (Arjo et al., 2007).
592 Because we pooled recapture data across several years, we likely violated the assumption
593 of population closure; that is, it is probable that there were immigration, emigration, birth, and/or
594 death events during the total sampling period. However, it is unlikely that we underestimated the
595 coyote population size because violating the closure assumption decreases the likelihood of
596 recapture and increases the estimated population size.
597 Conversely, we estimated that the population of kit foxes is relatively large. Sixty-nine
598 percent of scats collected were identified as kit foxes; of 54 genotyped DKF scats, we identified
599 49 individuals. The estimated mean N_e of the DKF population is more than 2× that of coyotes
600 (179 compared to 64.9). The estimated population density (0.4/km^2) overlaps with a previous
601 study of SJKF based on mark-recapture at the Naval Petroleum Reserves in southern San Joaquin
602 Valley, California (0.2 – 1.7/km^2, Cypher et al., 2000) but is higher than the density reported for
603 SJKF in the Ciervo-Panoche Natural Area (CPNA, 0.12 – 0.24 foxes/km^2, Wilbert et al., 2019),
604 and greatly exceeds contemporary estimates of DKF in UT (0.02 foxes/km^2, Lonsinger et al.,
605 2018).
606 While we found no evidence of isolation-by-distance or spatial autocorrelation among
607 DKF individuals, Wilbert et al. (2019) reported that SJKF individuals in the CPNA found within
608 6 km have significantly higher relatedness than expected by chance. These authors also reported
609 a strong signature of IBD and population structure likely caused by bisection of the landscape by
610 a major highway and the complex, heterogenous habitat. This suggests that by contrast, the

611 landscape around the BCCEA provides adequate kit fox habitat capable of supporting high and
612 unimpeded gene flow.
613 Given the large DKF population and its potential impact on threatened Mojave desert
614 tortoises as nest predators (Bjurlin & Bissonette, 2004), land managers should consider
615 addressing factors that can support increased canid populations during times of low prey
616 availability, for example, supplemental, anthropogenic sources of food including garbage
617 (Cypher et al., 2018). Both coyotes and kit foxes are known to consume anthropogenetic food
618 sources during times of low prey availability, such as during winter and after periods of low
619 precipitation when rodent and lagomorph populations decline (Kelly et al., 2019). Anthropogenic
620 subsidization could sustain canid abundance through these times of natural food shortages, which
621 may in turn increase predation pressure on species including the MDT (i.e. hyperpredation,
622 Esque et al., 2010). By replicating the methodology we used here, managers in the BCCEA
623 could monitor canid populations over time to assess the effects of actions to control
624 anthropogenic landscape alteration. Future studies could also estimate the frequency of canid
625 predation on MDTs by developing efficient molecular methods to detect MDT DNA in scats and
626 thus evaluate the impact that canids are having on this protected species.
627
628 **Conclusion**
629 We described and validated the FAECES* method, utilizing in-solution DNA
630 hybridization capture of SNPs to genotype canids from noninvasively collected scat samples.
631 Using this method, we showed that the landscape in and surrounding the BCCEA in the Mojave
632 Desert harbors a relatively large and genetically diverse population of desert kit foxes and a

633 smaller population of coyotes. The FAECES* method can be replicated in the future to enable
634 noninvasive population genetic studies, including capture-recapture, in multiple sympatric
635 species using a single capture assay, thus expanding the toolbox available to researchers and
636 conservation practitioners studying rare or elusive taxa like canids. Because of the flexibility of
637 capture methods, additional markers of interest can easily be incorporated into the probe set we
638 designed (Figure 3). Alternatively, our methods can be replicated for any species of interest by
639 performing reduced representation sequencing to identify SNP loci for probe design (e.g. by
640 using RADcap, Hoffberg et al., 2016).
641

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Data accessibility

DNA sequences: Submission to SRA in progress (raw fastq files for sequences from tissues and scat, with sample metadata including sampling localities)
Probe sequences: Will be made available on FigShare (DOI: 10.25573/data.14633298)
Sexing script: https://github.com/campanam/FAECES

Author contributions

WIB conceived the project and WIB, RCF, JEM secured funding; LDP, MGC, TRW, KR, RCF, WIB, JEM designed the study; WIB, RB, conducted the field work and collected samples; LDP, JDQ, IR carried out lab work; LDP analyzed and archived the data and wrote the manuscript with input from all of authors; MGC participated in data analysis and wrote the script for sample sexing; LDP, WIB and RB drafted figures and maps; all authors participated in revisions and acceptance of the submitted version of the manuscript.

Table 1: Mean observed and expected heterozygosity (H_o and H_e), inbreeding coefficients (F_{IS}), and effective population sizes (N_e) for coyotes. Standard deviations are in parentheses (95% confidence intervals for the N_e estimates).

	H_o	H_e	F_{IS}	N_e (95% CI)
All coyotes (n = 52)	0.24(0.04)	0.25(0.009)	0.037(0.15)	N/A
Unique individuals (n = 30) in the BCCEA	0.24(0.04)	0.25(0.01)	0.049(0.18)	37.6(33.8-42.1)
Unrelated individuals (n = 22) in the BCCEA	0.25(0.04)	0.26(0.007)	0.05(0.14)	64.9(53.5-81.8)

Table 2. Mean observed and expected heterozygosity (H_o and H_e) and inbreeding coefficients (F_{IS}) for San Joaquin kit foxes (SJFK) and desert kit foxes (DKF) in the BCCEA. Standard deviations are in parentheses.

	H_o	H_e	F_{IS}
All (n = 70)	0.27(0.06)	0.29(0.008)	0.078(0.2)
SJKF (n = 13)	0.32(0.04)	0.33(0.002)	0.04(0.13)
DKF (n = 57)	0.29(0.06)	0.29(0.008)	-0.0071(0.2)
Unique DKF individuals (n = 49)	0.30(0.06)	0.29(0.008)	-0.016(0.19)
Unrelated DKF individuals (n = 36)	0.29(0.05)	0.29(0.006)	-0.017(0.15)

Figure 1. Trail camera photographs of a coyote (left) and kit fox (right) in the BCCEA, Boulder County, NV. Animals are shown next to MDT models.

Figure 2. Map of sample collection localities. (Data organized and map generated using Esri ArcMap 10.4.1. Base map source: Esri © OpenStreetMap contributors 2021)

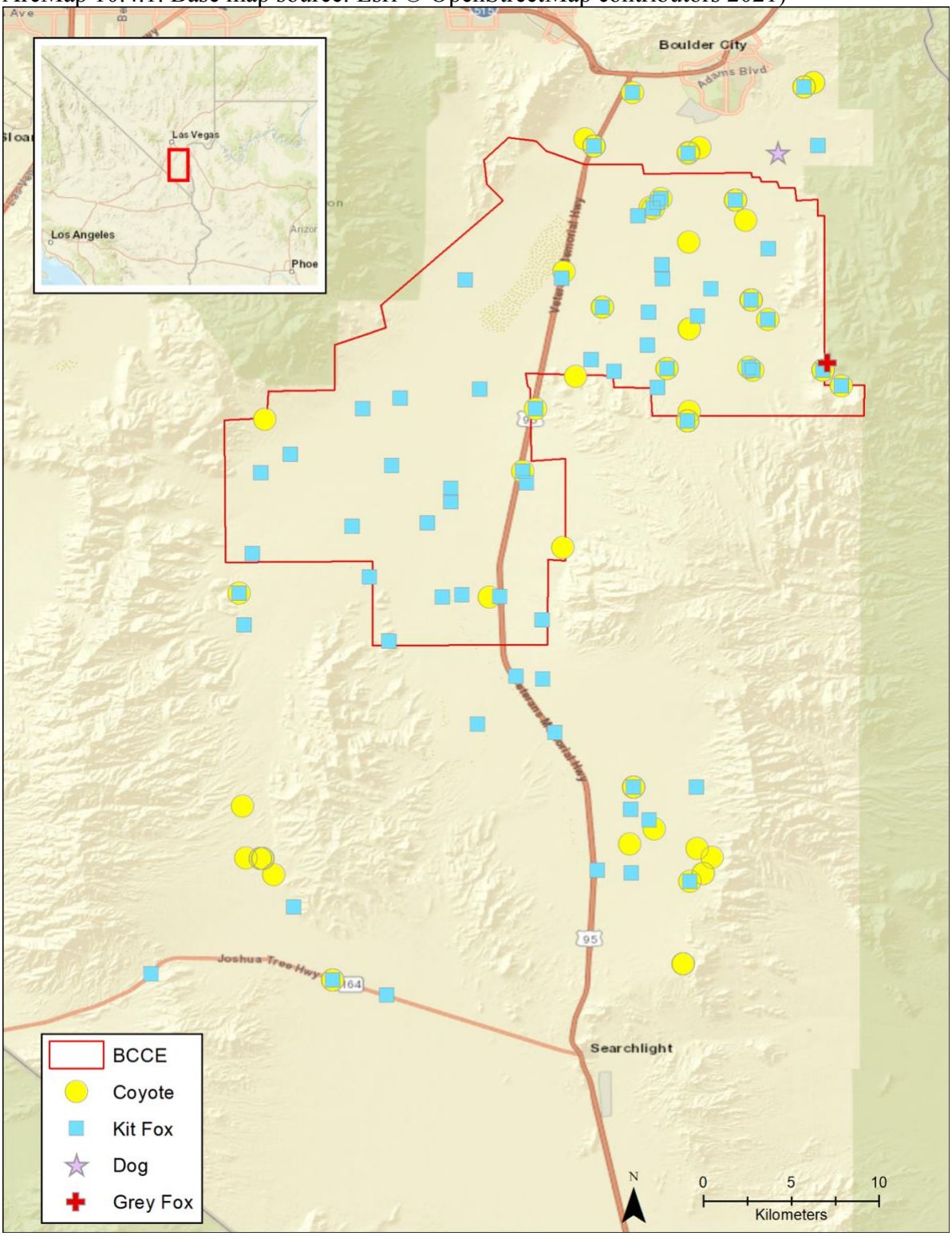


Figure 3. Workflow for FAECES* probe design, testing, and implementation.

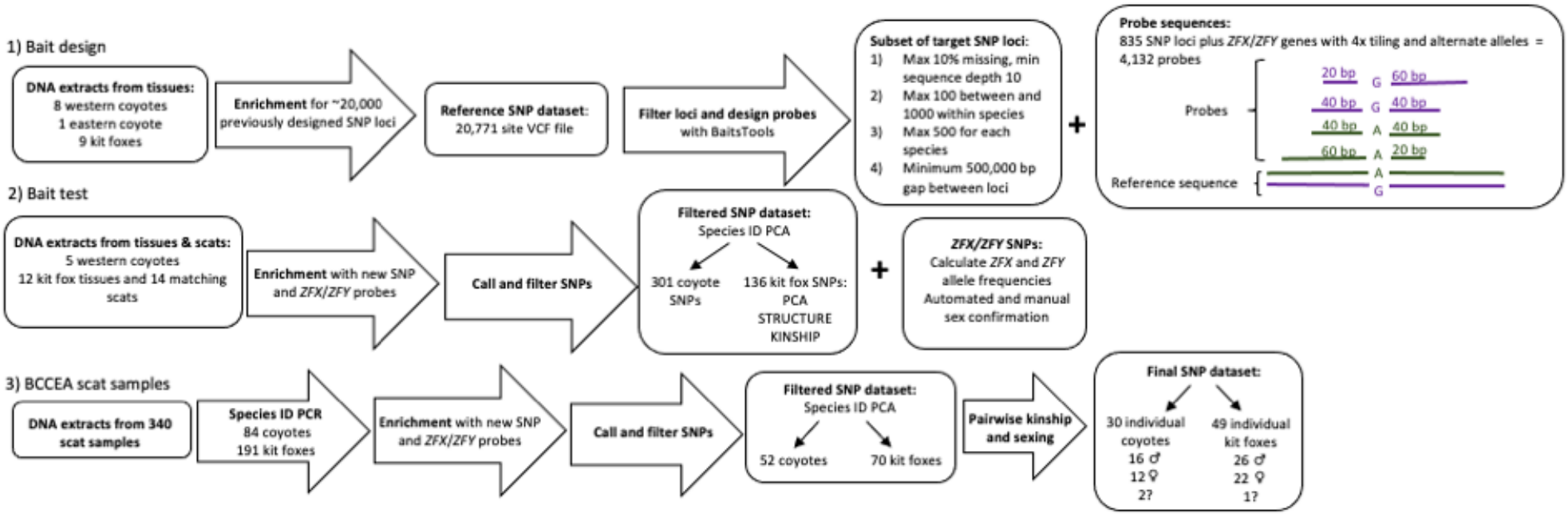


Figure 4. PCA of SNPs derived from coyote (*n* = 9) and San Joaquin kit fox (*n* = 9) tissues. PC1 accounts for 6.3% of variance; PC2 accounts for 4.1%.

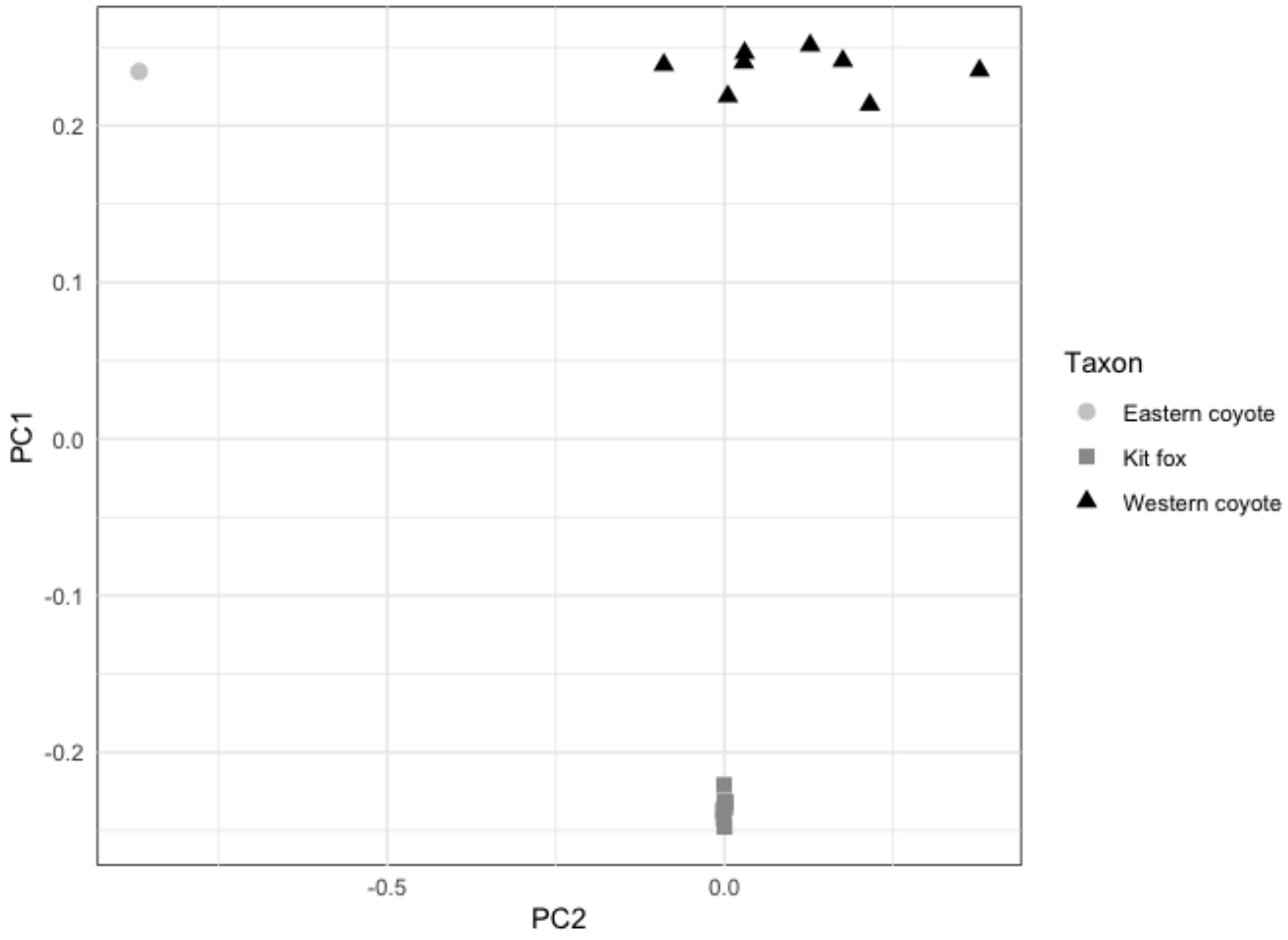


Figure 5. Map of coyote and kit fox recaptures colored by individual. Triangles represent DKFs and circles coyotes. (Data organized and map generated using Esri ArcMap 10.4.1. Base map source: Esri © OpenStreetMap contributors 2021)

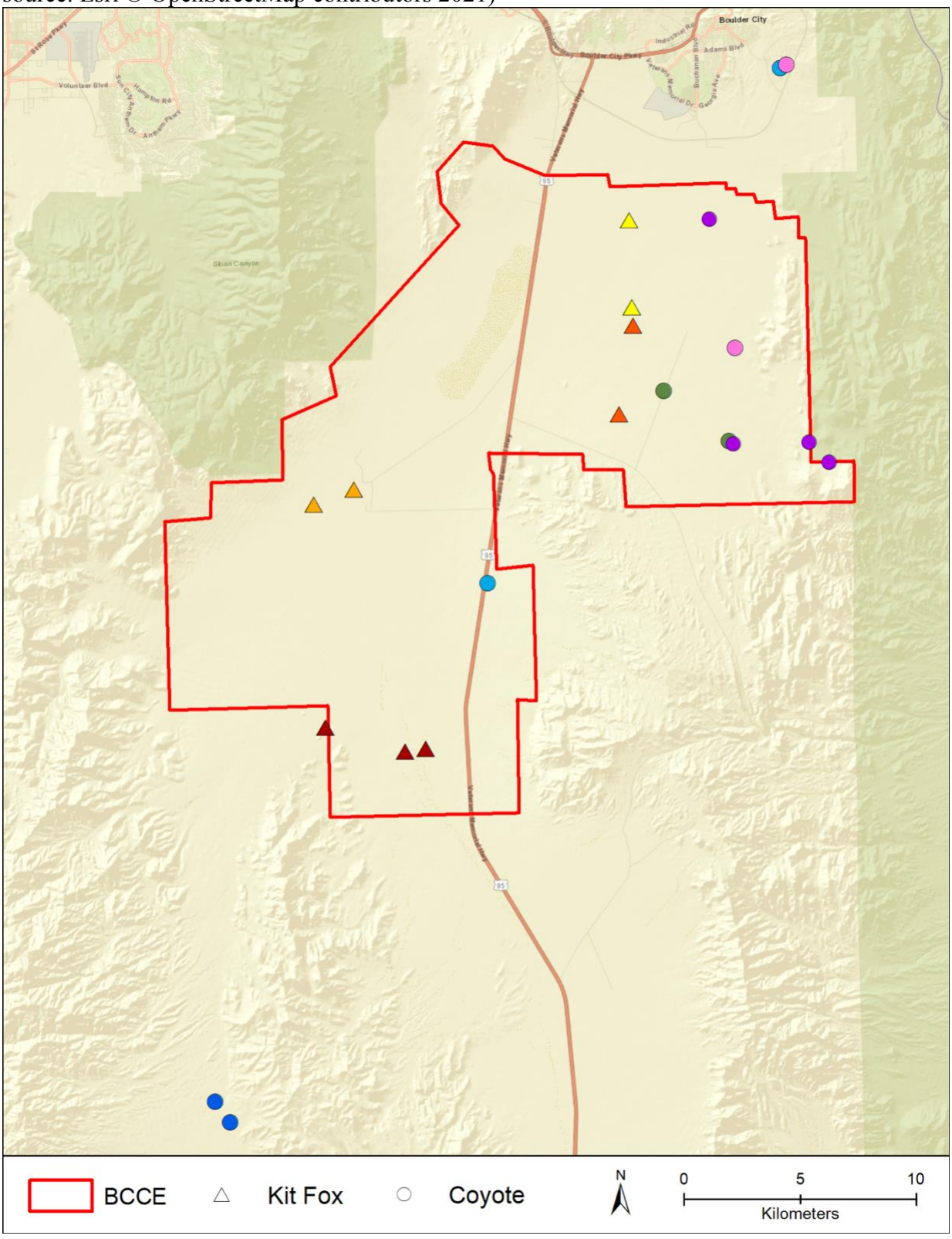
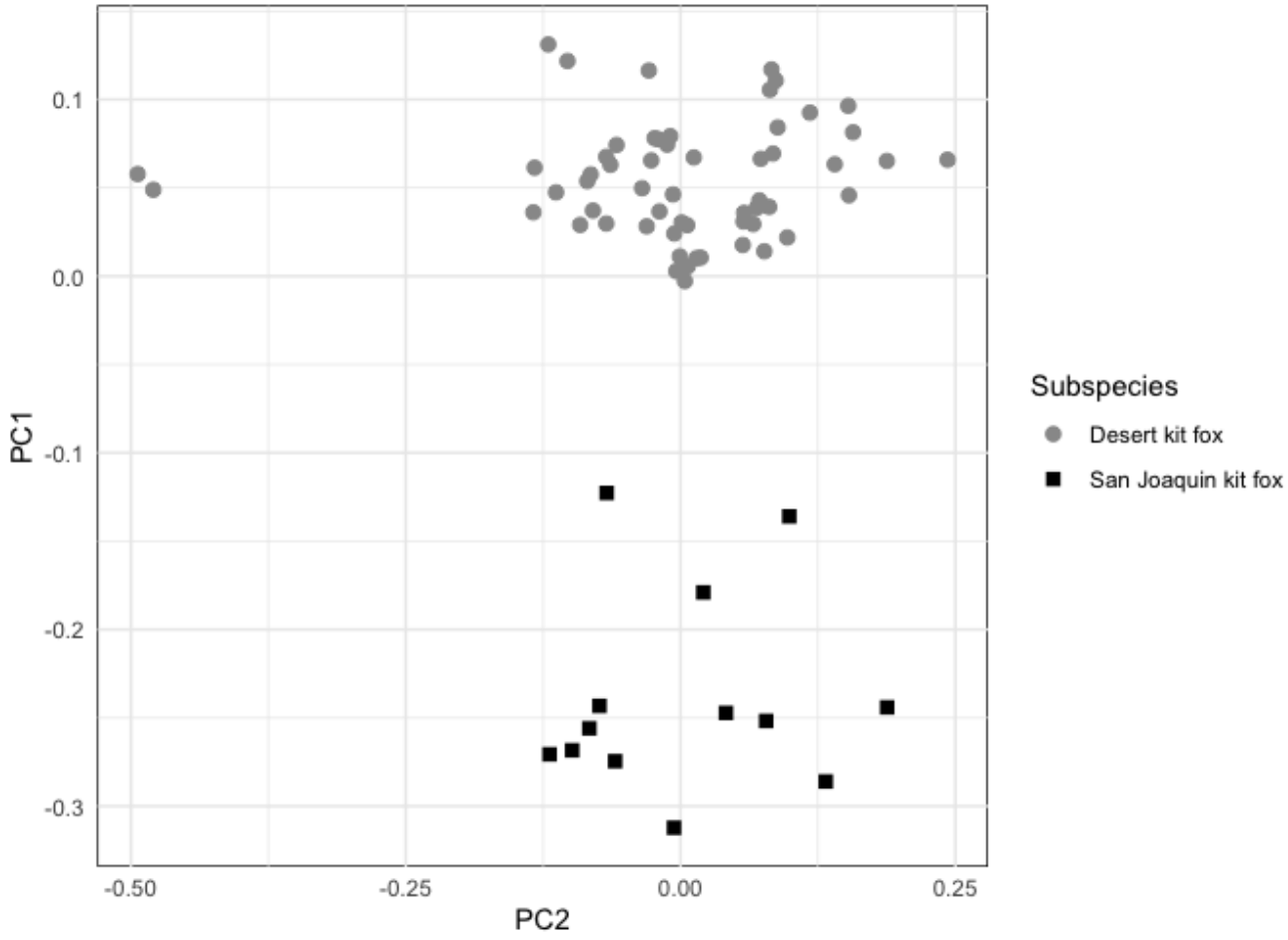


Figure 6. PCA of all kit fox samples (*n* = 70); PC1 accounts for 14.8% of variation; PC2 5.1%.



Supporting Information

Lycaon pictus probe design

Using two previously reported African wild dog (*Lycaon pictus*) genomes (Campana et al. 2016), we designed a 20,000-probe set to capture 19,729 nuclear SNPs that were polymorphic between the two genomes, along with the complete *Lycaon* mitochondrial genome (271 probes total). Paired raw reads from the two genomes were trimmed using Trimmomatic 0.33 (Bolger et al. 2014) with the parameters ILLUMINACLIP:NexteraPE-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:28 MINLEN:36. Trimmed reads were merged using FLASH 1.2.11 (Magoc and Salzberg 2011). Merged and remaining unmerged/unpaired reads were then concatenated and treated as single-end reads for downstream processing. Processed reads were then aligned to the CanFam 3.1 contigs (Hoeppner et al. 2014) using BWA-MEM 0.7.12 (Li 2013). Alignments were converted to BAM format, co-ordinate sorted, and PCR duplicates were removed using SAMtools 1.2 (Li et al. 2009) with the commands 'view', 'sort', and 'rmdup', respectively. Variants were called using the SAMtools 1.2 'mpileup' (options -u) and BCFtools 1.2 'call' (options -m -v) commands. We used the BCFtools 'filter' command to retain SNPs with a genotype quality > 50, alternate allele frequency < 1, a mapping quality > 30, a minimum sequencing depth of 10, and a maximum sequencing depth of 40. From the remaining 936,265 SNPs, we selected 30,000 SNPs using a preliminary version (select_snps-0.1) of BaitsTools (Campana 2018). To minimize SNP linkage, selected SNPs were required to be separated by a minimum distance of 10,000 bp and no more than two SNPs were selected per CanFam3.1 contig. Using the CanFam3.1 reference sequence, we then generated 120 bp probes with the selected SNPs at position 61 of the probe sequence. We discarded 92 truncated probes (e.g., SNPs located too close to the end of contigs to generate the full-length probe), leaving 29,908 candidate nuclear SNP probes for further filtration using the myBaits pipeline (MYcroarray, now Arbor BioSciences). After myBaits filtration, 19,729 of the filtered nuclear probes were randomly selected for the 20,000 probe set.

To generate the remaining 271 mitochondrial probes, we aligned the previously processed *Lycaon* reads against the circularized dog mitogenome reference sequence (NC_002008.4) using Geneious 8.1.4 (Biomatters, Ltd) using low sensitivity mode with 10 refinement iterations and a minimum mapping quality of 30. We submitted the consensus

mitogenomes to MYcroarray to generate 120 bp baits to cover each of the two mitogenomes at 1× tiling density each (135 and 136 probes for the two individuals).

Table S1. Samples used in this study.

Sample ID	Species ID (mtDNA PCR assay)	Species ID (SNP PCA)	Sample type	Collection locality (GPS) Lat	Collection locality (GPS) Lon	Collection date (year.month.day)	Sex (Known)	Sex (SNPs)	Recap ID	Number of sequence reads (post-QC)	Percent reads mapped to dog genome
109_K_Coyote	Coyote	Coyote	Scat	35.83701434	-114.86411	161022	n/a	F	1C	1180807	0.89
301_C_Coyote	Coyote	Coyote	Scat	35.81716374	-114.83363	170326	n/a	F	1C	2613797	0.81
53_C_Coyote	Coyote	Coyote	Scat	35.83701434	-114.86411	161022	n/a	F	1C	2708973	0.91
128_Unk_Coyote	Coyote	Coyote	Scat	35.84810796	-114.90878	150930	n/a	?	2C	1435436	0.72
23_C_b_Coyote	Coyote	Coyote	Scat	35.85176499	-114.83259	170106	n/a	M	2C	1414286	0.95
23_C_d_Coyote	Coyote	Coyote	Scat	35.85176499	-114.83259	170106	n/a	M	2C	1324598	1.00
24-c_Coyote	Coyote	Coyote	Scat	35.85176499	-114.83259	170106	n/a	M	2C	1868371	1.00
292_K_Coyote	Coyote	Coyote	Scat	35.81663995	-114.87571	170326	n/a	M	3C	918198	0.98
299_K_Coyote	Coyote	Coyote	Scat	35.81663995	-114.87571	170326	n/a	M	3C	2562033	0.98
110_K-C_a_Coyote	Coyote	Coyote	Scat	35.81590402	-114.79558	150927	n/a	M	4C	2262003	0.78
110_K-C_c_Coyote	Coyote	Coyote	Scat	35.81590402	-114.79558	150927	n/a	M	4C	2402830	1.00
112_Unk_Coyote	Coyote	Coyote	Scat	35.81588684	-114.83152	170105	n/a	M	4C	747322	0.92
305_C_Coyote	Coyote	Coyote	Scat	35.8078988	-114.7863	170326	n/a	M	4C	1823139	0.88
78_K_Coyote	Coyote	Coyote	Scat	35.90299401	-114.84045	150929	n/a	M	4C	1975902	0.94
217_C_a_Coyote	Coyote	Coyote	Scat	35.5115686	-114.86738	170326	n/a	F	5C	2338693	0.95
217_C_b_Coyote	Coyote	Coyote	Scat	35.5115686	-114.86738	170326	n/a	F	5C	2846973	0.87
16-C_Coyote	Coyote	Coyote	Scat	35.56564604	-115.08405	160130	n/a	M	6C	1284213	0.91
18-C_Coyote	Coyote	Coyote	Scat	35.55750401	-115.07711	170525	n/a	M	6C	761030	0.79
176_C_Coyote	Coyote	Coyote	Scat	35.76416899	-114.94961	170326	n/a	M	7C	2310774	0.97
85_K_Coyote	Coyote	Coyote	Scat	35.96089401	-114.80523	150927	n/a	M	7C	1666378	0.75
113_Unk_Coyote	Coyote	Coyote	Scat	35.96089401	-114.80523	150927	n/a	?	8C	740142	0.61
23_C_c_Coyote	Coyote	Coyote	Scat	35.85176499	-114.83259	170106	n/a	F	8C	1066228	0.90
1_C_Coyote	Coyote	Coyote	Scat	35.81251396	-114.92231	170406	n/a	F		3350729	0.99

117_Unk_Coyote	Coyote	Coyote	Scat	35.59260098	-115.09305	150930	n/a	M		4036773	0.58
2-C_Coyote	Coyote	Coyote	Scat	35.55786696	-114.85694	170406	n/a	M		970567	0.95
208_C_a_Coyote	Coyote	Coyote	Scat	35.93066552	-114.91305	170326	n/a	F		2018671	0.99
209_C_a_Coyote	Coyote	Coyote	Scat	35.93066552	-114.91305	170326	n/a	F		1524175	0.88
209_C_b_Coyote	Coyote	Coyote	Scat	35.93066552	-114.91305	170326	n/a	F		1931048	0.91
21_C_Coyote	Coyote	Coyote	Scat	35.84810796	-114.90878	150930	n/a	F		2960679	0.73
210_K_Kit_fox	Kit Fox	Coyote	Scat	35.93066552	-114.91305	170326	n/a	?		1505836	1.00
218_C_Coyote	Coyote	Coyote	Scat	35.5115686	-114.86738	170326	n/a	F		603981	0.72
23_C_a_Coyote	Coyote	Coyote	Scat	35.85176499	-114.83259	170106	n/a	F		1595907	0.95
27_C_b_Coyote	Coyote	Coyote	Scat	35.79607763	-114.9429	170105	n/a	F		1777144	0.74
273_K_Coyote	Coyote	Coyote	Scat	35.96304238	-114.80065	170326	n/a	F		1239969	0.82
28_C_Coyote	Coyote	Coyote	Scat	35.90299401	-114.84045	150929	n/a	M		5375319	0.84
29_C_a_Coyote	Coyote	Coyote	Scat	35.90299401	-114.84045	150929	n/a	M		2332870	0.67
3_C_Coyote	Coyote	Coyote	Scat	35.55786696	-114.85694	170406	n/a	M		1920808	0.93
30_C_Kit_fox	Kit Fox	Coyote	Scat	35.90299401	-114.84045	150929	n/a	F		2737120	0.77
33_C_Coyote	Coyote	Coyote	Scat	35.72502298	-114.92893	170105	n/a	M		448736	0.82
35_C_Coyote	Coyote	Coyote	Scat	35.95796722	-114.89324	170106	n/a	M		818817	0.92
37_C_Coyote	Coyote	Coyote	Scat	35.92728401	-114.86447	150927	n/a	?		1443087	0.46
4-C_Coyote	Coyote	Coyote	Scat	36.4110916	-114.86587	170406	n/a	M		1048031	0.74
42_C_Coyote	Coyote	Coyote	Scat	35.60226096	-114.89272	170524	n/a	M		2523076	0.76
44_C_Coyote	Coyote	Coyote	Scat	35.9343253	-114.91764	160524	n/a	?		1217943	0.70
45-c_Coyote	Coyote	Coyote	Scat	35.50334503	-115.04699	150930	n/a	M		639540	0.81
47_C_Coyote	Coyote	Coyote	Scat	35.78967797	-114.86504	150927	n/a	M		774303	0.80
5-C_Coyote	Coyote	Coyote	Scat	35.69970704	-114.96661	170405	n/a	F		1537652	0.91
51_C_Coyote	Coyote	Coyote	Scat	35.79460803	-114.86429	160402	n/a	M		1281573	0.69
68_K_c_Coyote	Coyote	Coyote	Scat	35.790604	-115.08176	150928	n/a	F		2578170	0.86
MSB-127530	Coyote	Coyote	Tissue	40.4436	-109.3175		F	F		566926	1.00

MSB-230633	Coyote	Coyote	Tissue	32.97388333	-109.15702		M	M		1170486	1.00
MSB-231176	Coyote	Coyote	Tissue	35.0442049	-106.67087		M	M		997126	1.00
MSB-231470	Coyote	Coyote	Tissue	36.596814	-108.00889		M	M		1014985	1.00
MSB-284096	Coyote	Coyote	Tissue	34.5970415	-111.31348		F	F		1060324	1.00
231_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.700783	-114.98062	180116	n/a	F	DKF1	4839155	1.00
287_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.69971131	-114.99061	170326	n/a	F	DKF1	1161904	0.98
91_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.70970598	-115.02814	151001	n/a	F	DKF1	2691189	1.00
303_C_Kit_fox	Kit Fox	Kit Fox	Scat	35.80148606	-115.0124	170326	n/a	F	DKF2	731762	0.90
81_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.79615064	-115.03143	170105	n/a	F	DKF2	1060325	0.92
115_Unk_Kit_fox	Kit Fox	Kit Fox	Scat	35.90360203	-114.87883	170405	n/a	F	DKF3	851241	0.83
63_K_b_Kit_fox	Kit Fox	Kit Fox	Scat	35.86985875	-114.87825	170105	n/a	M	DKF3	2260445	0.82
157_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.86265325	-114.87792	170326	n/a	F	DKF4	1241057	0.94
77_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.82857897	-114.88564	150929	n/a	F	DKF4	2549978	0.88
73-k_Kit_fox	Kit Fox	Kit Fox	Scat	35.76317104	-115.08369	150928	n/a	F	DKF5	224895	0.87
75_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.76317104	-115.08369	150928	n/a	F	DKF5	1481669	1.00
200_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.67701949	-115.01811	170326	n/a	M	DKF6	2245484	0.93
201_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.67701949	-115.01811	170326	n/a	M	DKF6	11309105	0.73
70_K_a_Kit_fox	Kit Fox	Kit Fox	Scat	35.815325	-114.90284	150929	n/a	M	DKF7	3136670	0.85
70_K_c_Kit_fox	Kit Fox	Kit Fox	Scat	35.815325	-114.90284	150929	n/a	M	DKF7	3211437	0.91
102_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.55974602	-114.9112	170105	n/a	M		3400842	0.72
105_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.590922	-114.89427	150930	n/a	F		1643655	1.00
10R_6155_5_Tissue	Kit Fox	Kit Fox	Tissue				M	M		1195750	1.00
118_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.9016493	-114.88058	160612	n/a	M		1015163	0.92
119_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.85753098	-114.85308	151212	n/a	M		734339	0.71
12_C_a_Kit_fox	Kit Fox	Kit Fox	Scat	35.55408396	-114.86395	151212	n/a	M		1436178	0.81
122_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.76707155	-115.01663	150927	n/a	M		1558196	0.82
13R_5447_8_Tissue	Kit Fox	Kit Fox	Tissue				F	F		2542496	1.00

16R_6154_9_Tissue	Kit Fox	Kit Fox	Tissue				M	M		1861676	1.00
173_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.76416899	-114.94961	170326	n/a	F		2337423	0.95
18R_1424_9_Scat	Kit Fox	Kit Fox	Scat				M	M		4067176	0.97
196_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.80148606	-115.0124	170326	n/a	M		1409397	0.78
198_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.6879059	-114.93952	170326	n/a	M		1502016	0.82
199_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.6879059	-114.93952	170326	n/a	F		2021692	0.91
19R_5443_10_Tissue	Kit Fox	Kit Fox	Tissue				M	M		880915	1.00
1R_5448_1_Tissue	Kit Fox	Kit Fox	Tissue				F	F		1168736	1.00
207_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.93066552	-114.91305	170326	n/a	F		1300112	1.00
213_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.93066552	-114.91305	170326	n/a	M		2295945	0.81
227_K_b_Kit_fox	Kit Fox	Kit Fox	Scat	35.73580837	-115.03693	170724	n/a	F		1385637	0.62
230_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.87802172	-114.82356	170416	n/a	M		2876960	0.83
236_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.87802172	-114.82356	171229	n/a	M		1511705	0.97
241_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.84541016	-114.88494	170326	n/a	F		841597	0.96
242_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.8416549	-114.82386	170326	n/a	F		1112439	0.86
25_C_Kit_fox	Kit Fox	Kit Fox	Scat	35.85176499	-114.83259	170106	n/a	M		2245314	0.91
254_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.8078988	-114.7863	170326	n/a	M		334852	0.76
267_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.81663995	-114.87571	170326	n/a	F		1061221	1.00
27_C_a_Kit_fox	Kit Fox	Kit Fox	Scat	35.79607763	-114.9429	170105	n/a	M		2079081	0.76
271_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.80148606	-115.0124	170326	n/a	F		1182086	1.00
272_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.9307397	-114.79811	170326	n/a	F		687955	0.96
281_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.65897811	-114.9531	170326	n/a	F		436050	0.75
284_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.87802172	-114.82356	180226	n/a	M		2459528	1.00
290_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.77277611	-115.06862	170326	n/a	M		466199	1.00
302_C_Kit_fox	Kit Fox	Kit Fox	Scat	35.73768586	-114.99849	170326	n/a	M		1227146	0.72
334_U_Kit_fox	Kit Fox	Kit Fox	Scat	35.80690118	-114.88044	170326	n/a	M		2119460	0.92
34_C_Kit_fox	Kit Fox	Kit Fox	Scat	35.80614304	-114.97167	150928	n/a	F		1142477	0.96

345_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.75514667	-114.98635	180430	n/a	?		1499561	0.72
4R_5450_2_Tissue	Kit Fox	Kit Fox	Tissue				F	F		1703586	1.00
62_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.86985875	-114.87825	170105	n/a	M		1090023	0.94
63_K_a_Kit_fox	Kit Fox	Kit Fox	Scat	35.86985875	-114.87825	170105	n/a	F		2040880	0.97
65_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.74834479	-114.9865	170105	n/a	F		2390904	0.96
67_K_a_Kit_fox	Kit Fox	Kit Fox	Scat	35.81588684	-114.83152	170105	n/a	M		2661200	0.97
67_K_b_Kit_fox	Kit Fox	Kit Fox	Scat	35.81588684	-114.83152	170105	n/a	M		1490788	0.92
71_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.79607763	-114.9429	170105	n/a	F		984813	0.70
7R_6142_3_Tissue	Kit Fox	Kit Fox	Tissue				F	F		892578	1.00
83_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.80614304	-114.97167	150928	n/a	F		1973258	0.52
89_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.60226096	-114.89272	170524	n/a	F		1798995	0.89
90_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.54094101	-115.06681	160331	n/a	F		1544611	1.00
93_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.60233598	-114.86037	151001	n/a	M		1156721	0.99
94_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.63445333	-114.97268	151001	n/a	M		508649	0.81
96_K_Kit_fox	Kit Fox	Kit Fox	Scat	35.63445333	-114.97268	151001	n/a	F		1199425	0.81
JEM_6121	Kit Fox	Kit Fox	Tissue				F	F		866520	1.00
JEM_6193	Kit Fox	Kit Fox	Tissue				M	M		1130607	1.00
JEM_6203	Kit Fox	Kit Fox	Tissue				F	F		850460	1.00
JEM_6253	Kit Fox	Kit Fox	Tissue				M	M		1312215	1.00
JEM_6449	Kit Fox	Kit Fox	Tissue				M	M		1212105	1.00

Table S2. Evanno table for STRUCTURE run including all kit fox samples.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln"(K)|	Delta K
1	5	-6526.54	0.1342	NA	NA	NA
2	**5**	**-5985.4**	**0.3808**	**541.14**	**603.34**	**1584.4484**
3	5	-6047.6	30.9788	-62.2	21.24	0.685631
4	5	-6131.04	45.7583	-83.44	40.98	0.895575
5	5	-6255.46	33.7364	-124.42	80.08	2.373694
6	5	-6459.96	35.0972	-204.5	345.28	9.837824
7	5	-6319.18	62.3156	140.78	127.42	2.044753
8	5	-6305.82	43.147	13.36	NA	NA

Table S3. Evanno table for STRUCTURE run with only SJKFs (n = 13), using location priors in the model.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln"(K)|	Delta K
1	5	-1159.4	0.886	NA	NA	NA
2	**5**	**-1145.52**	**2.1406**	**13.88**	**15.56**	**7.269124**
3	5	-1147.2	4.0908	-1.68	0.32	0.078223
4	5	-1148.56	2.6586	-1.36	2.68	1.00806
5	5	-1147.24	3.7099	1.32	10.24	2.760218
6	5	-1156.16	5.6168	-8.92	14.12	2.513905
7	5	-1150.96	8.6526	5.2	23.28	2.690512
8	5	-1169.04	30.8542	-18.08	NA	NA

Table S4. Approximate cost of reagents to process 400 samples, including library preparation, enrichment, and sequencing.

Company	Item	Catalog #	Price per unit (2018)	Quantity	Total Price (USD)
New England Biotech	T4 Polynucleotide Kinase	M0201L	224	2	448
New England Biotech	T4 DNA Polymerase	M0203L	268	1	268
New England Biotech	T4 DNA Ligase	M0202L	256	2	512
New England Biotech	Bst 2.0 Warmstart polymerase	M0538L	300	1	300
New England Biotech	T4 DNA ligase buffer	B0202S	22	1	22
New England Biotech	BSA	B9000S	25	10	250
Sigma-aldrich	PEG-4000	95904-250G-F	41	1	41
Kapa/Roche	HiFi Hotstart Readymix	KK2602	420	2	840
Agilent	TapeStation HS DS1000 reagents	5067-5584, 5585	500	1	500
Arbor Biosciences	myBaits-1 48 reactions	300148	5391	1	5391
Arbor Biosciences	extra reagents	300048	973	1	973
Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley	NovaSeq SP - 1 lane		2812	1	2812
ThermoFisher Scientific	Dynabeads MyOne Streptavidin C1	65001	568	1	568
ThermoFisher Scientific	Qubit HS dsDNA assay	Q32854	320	1	320
				TOTAL	13,245

Figure S1. PCA of coyote samples (*n* = 52). Coyote tissues shown in black squares; scat samples from the BBCEA in grey circles.

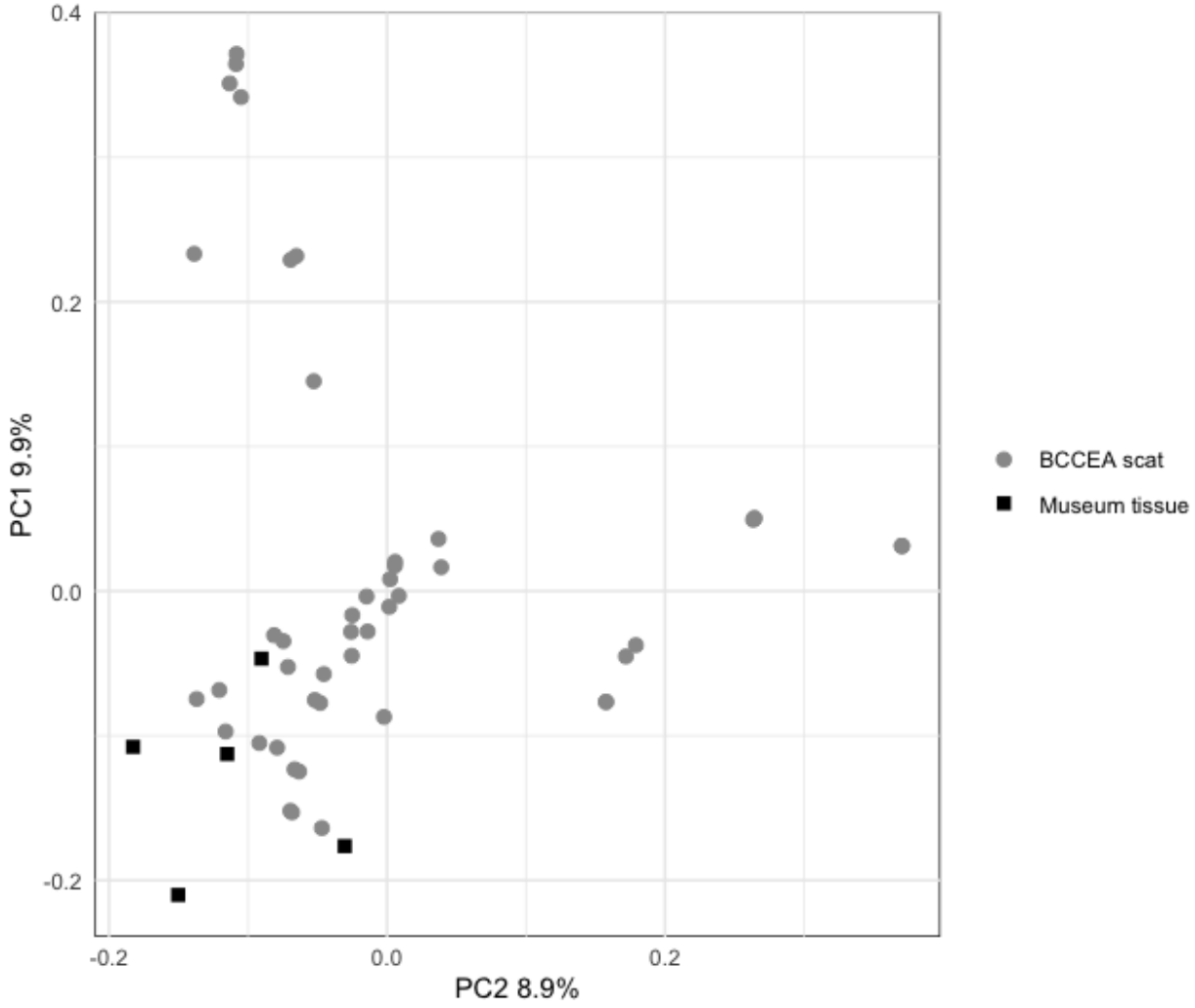
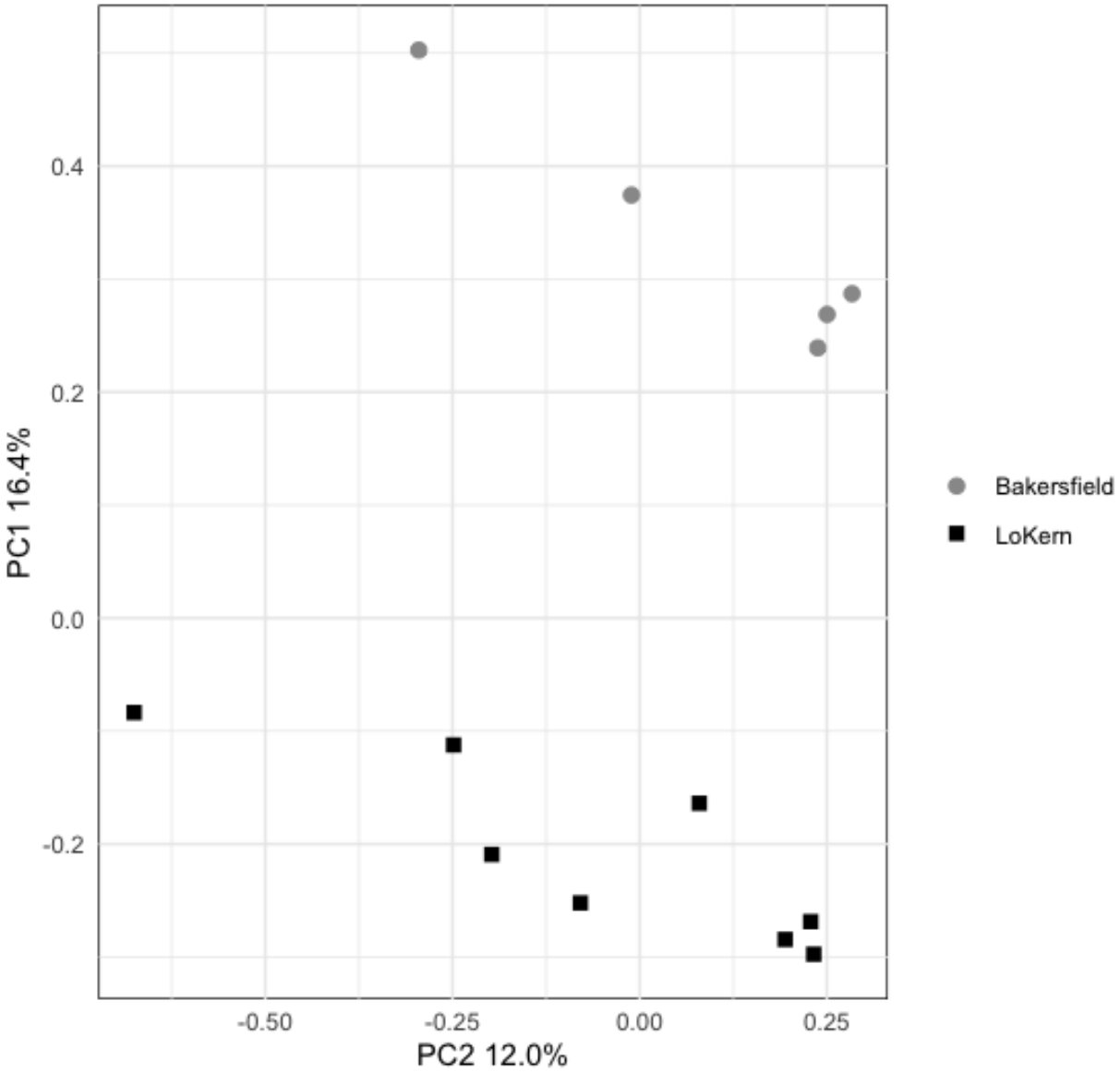


Figure S2. PCA of SJKF samples (*n* = 13). Individuals from Bakersfield, CA are shown in grey circles and foxes from LoKern, CA in black squares



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