RESEARCH ARTICLE

Genetic integrity, diversity, and population structure of the Cascade red fox

Jocelyn R. Akins[1,2] · Keith B. Aubry[3] · Benjamin N. Sacks[2,4]

Abstract

The Cascade red fox (CRF) occurred historically throughout subalpine and alpine habitats in the Cascade Range of Washington and southernmost British Columbia, but now appears to be extremely rare. Causes for its apparent decline are unknown, as is the current distribution and connectivity of its populations. Additionally, the introduction of nonnative (fur-farm) red foxes to surrounding lowland areas during the past century raises concerns about their expansion to higher elevations and potential hybridization with the CRF. We conducted noninvasive genetic sampling and analyses of CRFs in a 5575 km^2 region in the southern portion of its range, which is thought to contain a significant proportion of the current population. We obtained 154 mitochondrial DNA sequences and microsatellite genotypes for 51 individuals to determine trends in genetic diversity, assess evidence for nonnative introgression, and describe population structure. Although heterozygosity ($H_e = 0.60$, SE = 0.03) was only slightly lower than an estimate obtained from samples collected during the 1980s ($H_e = 0.64$, SE = 0.05), genetic effective size of the current population based on a one-sample estimate was very small ($N_e = 16.0$, 95% CI 13.3–19.4), suggesting a loss of genetic diversity and the potential for inbreeding depression in future decades. Genetic connectivity was high and we found no evidence for hybridization with nonnative lowland red foxes. Thus, although a small effective population size indicates the possibility of inbreeding depression and loss of evolutionary potential, high connectivity and genetic integrity could mitigate this to some extent, indicating that the population could respond to conservation efforts. Ultimately, successful conservation of this species depends on a better understanding of the factors that originally contributed to its decline and that currently limit its growth.

Keywords Cascade red fox · Genetic diversity · Genetic effective size · Mountain biome · Montane red fox · Population structure · Range contraction · *Vulpes vulpes cascadensis*

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Introduction

In the American West, many carnivores suffered substantial declines in distribution and abundance, including widespread extirpations of predators, during the past century (Laliberte and Ripple 2004; Zielinski et al. 2005). Many historical threats, such as overharvest and predator-control programs, were eliminated, and some species have begun to recover and recolonize portions of their former range (McKelvey et al. 2014; Maletzke et al. 2016). However, habitat loss and fragmentation due to timber extraction, road building, and human development and recreation continue to impact carnivore populations (Bunnell et al. 2006; Nellemann et al. 2010). Additionally, carnivores face new threats associated with climate change (Parmesan 2006; Hansen et al. 2014) that in some cases could prevent their recovery.

Among those mesocarnivores most threatened by contemporary environmental changes are indigenous red foxes (*Vulpes vulpes*). Four native subspecies of red fox occur in the western United States, including three montane subspecies—Cascade (*V. v. cascadensis*), Rocky Mountain (*V. v. macroura*), and Sierra Nevada (*V. v. necator*) red foxes (Aubry et al. 2009)—and the lowland Sacramento Valley red fox (*V. v. patwin*; Sacks et al. 2010). These native subspecies represent remnant populations of a red fox lineage that was broadly distributed in open-forest refugia south of the ice sheets between 100,000 and 10,000 years ago during the last Pleistocene glaciation (Pielou 2008; Aubry et al. 2009; Statham et al. 2014). Today, their populations occur primarily in geographically restricted, high-elevation areas scattered throughout the major mountain ranges of the American West (Swanson et al. 2005; Perrine et al. 2007; Van Etten et al. 2007; Statham et al. 2012a, b; Volkmann et al. 2015; Green et al. 2017). In California, the Sierra Nevada red fox is considered critically endangered (USFWS 2015a, b).

The Cascade red fox (CRF) is endemic to Washington where it is considered a State Endangered Candidate Species, a United States Forest Service (USFS) Regional Forester's Sensitive Species, and a Species of Greatest Conservation Need in the Washington State Wildlife Action Plan (WDFW 2015). It once occurred at high elevations in the Cascade Range from southern British Columbia to the Columbia Gorge in southern Washington. However, it is now rarely detected in the North Cascades Ecosystem (NCE), despite numerous forest carnivore surveys conducted in recent years that should have detected them if they were present (K. Aubry, unpublished data). Little is known about the distribution, abundance, connectivity, genetic diversity, or genetic integrity of the CRF. Along with identification of factors that may have limited its distribution or abundance, understanding these basic population parameters is an essential step in its conservation.

Although proximate threats to Cascade and other montane red foxes are poorly understood, hybridization with nonnative red foxes has been identified as an immediate threat in the two other montane subspecies (Quinn and Sacks 2014; Green et al. 2017; Merson et al. 2017). In Washington, low-elevation red fox populations occur on either side of the Cascades where they are known (west of the Cascades) or presumed (east of the Cascades) to have derived from red foxes that were translocated for hound hunting or escaped from fur farms (Aubry 1984; Statham et al. 2012b). Ultimately, these nonnative red foxes are primarily of eastern Canadian and Alaskan ancestry (Statham et al. 2012b; Merson et al. 2017), but potentially reflect many generations of captive rearing and, therefore, selection for traits likely to be maladaptive in the habitats of native western red foxes (Sacks et al. 2016). Thus, another important question is whether nonnative lowland red foxes have come into contact

and hybridized with native CRFs. In this study, we used primarily noninvasively collected DNA samples from CRFs along with mtDNA sequencing and microsatellite genotyping to investigate genetic diversity, nonnative introgression, and population structure of the CRF throughout the southern portion of its range in the Washington Cascades.

Materials and methods

Study area

The study area comprised 5575 km^2 in the southern Washington Cascade Range, consisting of portions of the Cascades, and Eastern Cascades Slopes and Foothills ecoregions (US EPA 2003; Fig. 1). Specifically, we surveyed USFS and National Park Service (NPS) lands, including the Indian Heaven, Mount Adams, Goat Rocks, Norse Peak, and William O. Douglas Wilderness areas; surrounding areas on the Gifford Pinchot, Okanogan-Wenatchee, and Mt. Baker-Snoqualmie National Forest; and Mount Rainier National Park. We surveyed elevations ranging from 750 to 2250 m, encompassing lower elevation western hemlock (*Tsuga heterophylla*), Douglas-fir (*Pseudotsuga menziesii*), and grand fir (*Abies grandis*) forests; mid-elevation Pacific silver fir (*Abies amabilis*) forests; and upper-elevation subalpine fir (*Abies lasiocarpa*), mountain hemlock (*Tsuga mertensiana*), and whitebark pine (*Pinus albicaulis*) forests; as well as subalpine parklands and alpine grasslands (Lillybridge et al. 1995; Hall 1998; Crawford et al. 2009). Tree-line occurred from 1600 to 2000 m in elevation (Hemstrom and Franklin 1982). The climate consisted of annual temperatures averaging a low of 4.5 °C and a high of 18.6 °C, and a seasonal precipitation pattern characterized by summer drought and heavy winter snowfall (Hijmans et al. 2005). The study area received low to moderate human visitation during summer due to its remoteness, except for sites at Mount Rainier (Sunrise and Paradise) and Mount Adams (South Climb) where visitation was frequent, due to high-elevation road access. During winter, human visitation was extremely low, with the exception of one access point (Paradise) at Mount Rainier National Park, and another on the southern slopes of Mount Adams, where legal and illegal snowmobile activity occurred. There was no snowmobile activity in Mount Rainier National Park or in any other federally designated wilderness area.

Survey design

To obtain samples, we collected scats along trails, scats and urine along putative red fox snow tracks, and hair from hair-snagging devices at baited camera stations. We also received tissue samples from three individuals live-trapped

Fig. 1 Geographic extent of our study area in the southern Cascade Range in Washington (black polygon), and the localities where Cascade red fox samples were collected from 2009 to 2014, including Mount Rainier National Park (MR), and the Norse Peak (NP), William O. Douglas (WD), Goat Rocks and Hamilton Buttes (GR and HM), Mount Adams (MA), and Indian Heaven (IH) wilderness areas (yellow polygons). Ecoregions are shown in blue (Cascades), green (East Cascade Slopes and Foothills), and orange (North Cascades). (Color figure online)
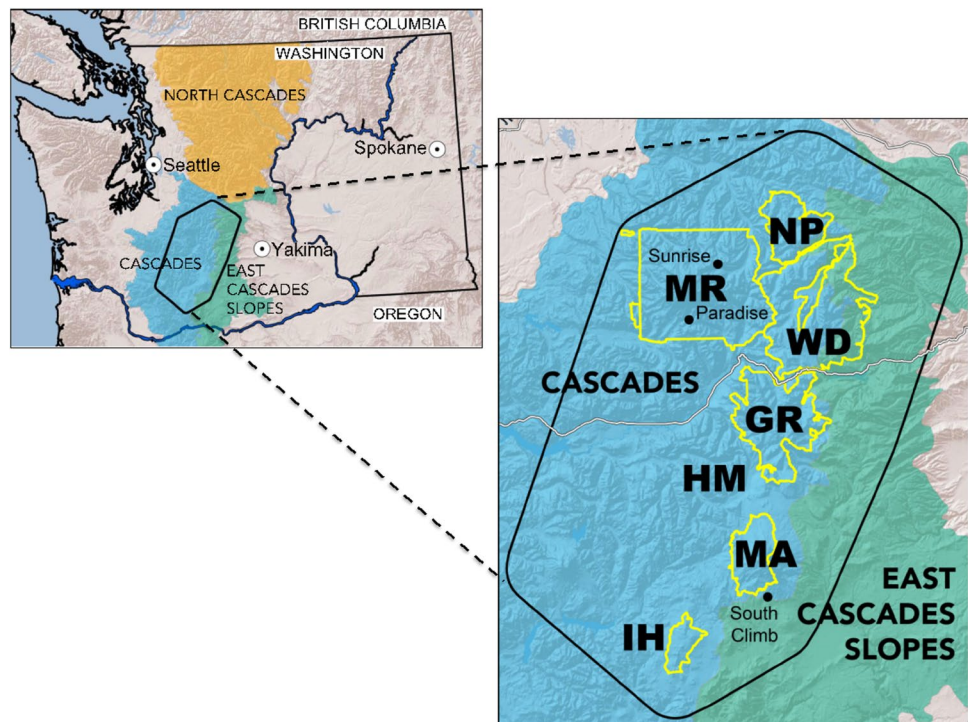
in Mount Rainier National Park for another study (Jenkins et al. 2014). We collected hair samples from hair-snagging devices deployed at noninvasive camera surveys during 6 years (2009–2014) using a spatially replicated design with 4-km² sampling units to approximate average CRF home range size based on limited data (Aubry 1983; Perrine 2005). We randomly selected a subset of 50 units from those that overlapped elevations ranging from 1500 to 2100 m, i.e., primarily containing subalpine parkland or upper montane forest. We surveyed a second adjacent unit to each randomly selected unit (i.e., 50 more units) to maximize survey efficiency. Lastly, we selected 24 additional units that were adjacent to our randomly selected units but that targeted lower elevations ranging from 525 to 1500 m to include mid-elevation forest (Fig. 1). To collect scats, we surveyed across a broad range of elevations, commencing at trailheads that were lower in elevation than we expected to find CRF samples. Finally, we followed fox tracks in snow that were encountered en route to camera stations and along set transects.

Sample collection and preservation

We collected DNA samples from hair, scat, urine, and tissue samples that were obtained from 2009 to 2014 using four noninvasive techniques: hair-snagging devices, hiking trails to collect scats, snow-tracking, and opportunistic sampling of road-killed carcasses. We received tissue samples (ear punches stored in 95–100% ethanol or desiccant) from three

individuals live-trapped during a previous study in Mount Rainier National Park (Jenkins et al. 2014). To estimate the lower elevation limit of their distribution, we surveyed a broad range of elevations, including relatively low-elevation areas where we did not expect to find CRF samples. We collected hair using hair-snagging devices deployed at baited camera stations. The device consisted of a webbing belt containing a row of six 0.30-caliber gun brushes, clipped around a tree with bait (deer, elk) mounted above. We selected hair samples for DNA analysis that were identified in the field provisionally as either red fox or Pacific marten (*Martes caurina*), based on length and color pattern. We collected hair samples in small paper envelopes and preserved them in desiccant in an airtight centrifuge tube. We only selected hair samples for which we detected a red fox at the corresponding camera station and analyzed hairs from each brush as a separate sample to minimize the chance of contamination among multiple individuals (which could be identified and discarded in subsequent analyses by > 2 alleles at any locus). We collected scats along trails and fox snow tracks, which we identified based on track size, stride, and straddle length. We collected ~ 1 ml of material from the ends and outer surface of each scat (to maximize retention of red fox epithelial cells), which we preserved in a 5-ml vial with ~ 4 ml of 95–100% ethanol. To determine the accuracy of field identifications, and prioritize DNA extractions given limited resources, we classified scats in the field as highly likely, probable, or possible red fox. We tested the validity of our provisional field identifications using mitochondrial

DNA (mtDNA) sequencing. We collected urine encountered along snow-tracks in a 25-ml vial including as little snow as possible, and kept samples in the freezer before transporting them to the laboratory in dry ice.

Laboratory analyses

We extracted, amplified, sequenced, and genotyped DNA samples at the Mammalian Ecology and Conservation Unit in the Veterinary Genetics Laboratory at the University of California, Davis. We extracted DNA from approximately 200 mg of each scat using a QIAmp Stool Mini Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol, except we eluted in 50 µl of buffer to obtain concentrated DNA (Miles et al. 2015). We thawed urine-soaked snow in the laboratory and pipetted 1.5 ml into a 2-ml microcentrifuge tube. We concentrated this volume to 600 µl using a vacuum centrifuge and extracted DNA using Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocol for blood, except that our final elution was in 50 µl of elution buffer. We extracted DNA from hair samples by first digesting the follicles (Pfeiffer et al. 2004) and then purifying the DNA with a phenol/chloroform method (Sambrook and Russell 2001). We extracted DNA from tissue samples using a Qiagen DNeasy Blood and Tissue kit according to the manufacturer's instructions.

We sequenced a 354-basepair (bp) portion of the mtDNA cytochrome *b* gene using primers RF14724 and RF15149 (Perrine et al. 2007) and a 342-bp portion of the D-loop control region using primers VVDL1 and VVDL6 (Aubry et al. 2009). We used the same PCR reaction for both mtDNA loci, consisting of 2 µl DNA, 1.1 µl PCR buffer (10×), 1.1 µl deoxynucleotide (dNTPs) solution (10 mM stock), 1.1 µl MgCl$_2$ (25 mM stock), 0.11 µl 1× bovine serum albumen (BSA), 1 µl forward primer (10 µM stock), 1 µl reverse primer (10 µM stock), 0.2 µl Taq polymerase (5 U/µl stock), and 4.84 µl de-ionized water (13 µl total volume). We allocated a negative control (all reagents except template DNA) for each extraction set (10–30 samples) to detect possible contamination. To amplify DNA, we used a PCR thermal profile that involved an initial 10-min denaturing step at 94 °C, followed by 40 cycles of a 30-s denaturing step at 94 °C, followed by a 30-s annealing step at 50 °C, and a 45-s extension step at 72 °C. These cycles were followed by a 10-min extension step at 72 °C. We purified the PCR products using exonuclease I and shrimp alkaline phosphatase (SAP), and sequenced the mtDNA in forward direction (cytochrome *b*) or in both directions (D loop) using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA). We purified the sequencing products with Sephadex and electrophoresed on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA).

We attempted to genotype red fox samples at 33 microsatellites and a sex marker for which primers, size range, original sequence sources, and multiplex reactions were described in detail by Moore et al. (2010): AHT121, AHT133, AHT137, AHT140, AHT142, AHT171, c01.424PET, CPH3, CPH11, CPH18, CXX-279, CXX-402, CXX-468, CXX-602, FH2004, FH2010, FH2088, FH2289, FH2328, FH2380, FH2848, REN105L03, REN162C04, REN169O18, REN247M23, REN54P11, RF08.618, RF2011Fam, RF2054, RF2457, RFCPH2, INU030, INU055; K9-AMELO. Forward primers were labeled fluorescently with 6-FAM, VIC, NED, or PET (Applied Biosystems) as described by Moore et al. (2010). We amplified nuclear markers using a Qiagen multiplex PCR kit with Q-solution and a 58 °C annealing temperature according to the manufacturer's instructions. For tissue-extracted DNA, we diluted samples 100-fold prior to PCR amplification. We genotyped each scat, hair, and urine sample twice with separate PCR reactions for each sample to detect and reduce allelic dropout errors, which were estimated previously for noninvasive DNA samples in the same laboratory (and using the same protocols) to be 2.3% (Sacks et al. 2011).

Data analysis

We aligned mtDNA sequences manually using Sequencher 4.8 (Gene Codes Corp 2007) and trimmed them to a 354-bp fragment for cytochrome *b* and a 342-bp fragment for the D-loop, consistent with homologous haplotypes from previous studies (e.g., Perrine et al. 2007; Aubry et al. 2009). We determined species identification for each sample using the Basic Local Alignment Search Tool (BLAST) in the Genbank nucleotide database, and then determined specific red fox haplotype sequences by comparing them to previously published homologous red fox reference sequences using Sequencher 5.3 (Gene Codes Corporation). Nomenclature for haplotypes follows Sacks et al. (2010), whereby cytochrome *b* fragments were indicated by a letter or letter/numeral combination preceding a dash and D-loop fragments were represented by a numeral following the dash (e.g., O-24 indicates cytochrome *b* haplotype O and D-loop haplotype 24).

We scored microsatellite genotypes manually using an internal size standard (Genescan 500 LIZ, Applied Biosystems) in the program STRand (Toonen and Hughes 2001) and combined the two replicate genotypes for each sample into a consensus genotype. We assigned samples to individual red foxes in accordance with the following microsatellite allele-sharing rule: we examined the frequency distribution of pairwise allele-sharing among all sample genotypes to select a cutoff value above which we assumed samples were from the same individual (e.g., Lounsberry et al. 2015). Based on the point where the frequency of shared alleles was

lowest between two modes in this distribution, we selected 85% allele sharing as the cutoff for identifying samples from the same individual. Once all samples were grouped according to their individual identity, we compared their consensus genotypes to identify and remove any remaining errors (false alleles or allelic dropout) where individual identity agreed otherwise. This produced a single 'best' genotype for each individual to use in subsequent analyses.

Microsatellite genetic diversity

We estimated observed and expected heterozygosity and the number of alleles with Microsatellite Toolkit (Park 2001), and rarefied allelic richness (average number of alleles per locus) and F_{IS} using FSTAT 2.9.3.2 (Goudet 1995). We tested for significant deviations from Hardy–Weinberg equilibrium (HWE) using FSTAT, correcting for multiple comparisons with the sequential Bonferroni correction (Rice 1989). We estimated pairwise relatedness (R) among individuals with ML-Relate (Kalinowski et al. 2006). We estimated genetic effective population size (N_e) based on a bias-corrected linkage disequilibrium method (Waples 2006) with LDNe software (Waples and Do 2008). We used the random-mating model, excluded all alleles with frequencies < 0.05, and reported jackknife-based 95% confidence intervals. To assess the sensitivity of our estimate of N_e to potential biases resulting from the oversampling of close relatives, we also estimated N_e using a subsample with the closest relatives ($R \geq 0.70$) removed and 30 individuals selected randomly from the remaining pool of individuals.

Population structure

To examine spatial patterns of population genetic structure, we employed model-based genetic admixture analyses using program Structure 2.3.4, which divides samples into preselected numbers of genetic clusters (K) based on genotypic frequencies (Pritchard et al. 2000). We investigated the assignment of individuals to K = 1–8 clusters with ten replicate runs for each K value. We conducted all Structure runs using 250,000 Markov Chain Monte Carlo (MCMC) cycles, following a burn-in period of 250,000 cycles for each run. We selected the admixture model with correlated allele frequencies (Falush et al. 2003) and no prior sampling information. To aid in evaluating the most likely number of genetic clusters, we plotted likelihood values in relation to K [LnP(K)] using Structure Harvester (Earl and von Holdt 2012).

Nonnative introgression

We investigated introgression by nonnative foxes based on both matrilineal (mtDNA) and biparentally inherited (microsatellite) ancestry. We tested for the presence of nonnative matrilines in our study populations using mtDNA cytochrome b and D-loop sequences. The only cytochrome b haplotypes known to be native to the Washington Cascades historically were A, O, and T (Aubry et al. 2009; Sacks et al. 2010). However, we could only evaluate native/nonnative maternal ancestry provisionally because the most common Cascade haplotype (O-24) has also been found in fur farms (Lounsberry et al. 2017), and both O-24 and O-26 were found in nonnative lowland populations in the western contiguous United States (West) that included fur-farm ancestry (Statham et al. 2011, 2012a, b; Sacks et al. 2016). Nevertheless, numerous nonnative haplotypes have been found in the West (Statham et al. 2011, 2012b; Sacks et al. 2016; Merson et al. 2017), and in the Washington lowlands, in particular (e.g., 80% of lowland red foxes in Washington were haplotype F or G; Statham et al. 2012b). Thus, although we could not rule out nonnative maternal haplotypes in our study area if we detected haplotypes O-24 or O-26, the presence of any other nonnative haplotype would provide conclusive evidence of their introgression into our study population.

Because males tend to be the primary mediators of gene flow among nonnative red foxes (e.g., Sacks et al. 2016; Merson et al. 2017), we also assessed nuclear gene flow from nonnative sources. For reference genotypes (i.e., knowns), we used previously published genotypes (including 14 of the loci used in this study) from 72 individuals sampled from both native red fox populations (i.e., historical museum specimens) and modern nonnative populations (Sacks et al. 2010) against which to compare our Cascade genotypes (treated as unknowns). Although these reference samples were analyzed in the same laboratory as the present study, 11 of them were reanalyzed to confirm consistency of genotypes (Merson et al. 2017). The genotypes matched for 96% of alleles and disagreements were due to random allelic dropout. We assigned prior (geographic) population information to each reference individual in Structure for our first analysis (Ancestry model = Use Population Information to test for migrants) by setting the USEPOPINFO flag to 1, keeping the USEPOPINFO flag set to 0 for samples collected during the present study. We then ran the dataset with no prior population information (Ancestry model = Use Admixture Model), effectively treating both reference and study genotypes as unknowns, which we ran for K = 1–8. We used the correlated allele frequencies model for all runs of both ancestry models (Falush et al. 2003), each with 250,000 Markov Chain Monte Carlo (MCMC) cycles following a 250,000 repetition burn-in period for each run.

Results

Field samples and species identification

We collected 886 putative red fox scat ($n = 711$), urine ($n = 87$), hair ($n = 84$), and tissue ($n = 4$) samples. We sequenced 665 (75.1%) of these samples (221 were unsuccessful; Table 1), of which 416 (63%) were red foxes, 174 (26%) coyotes (*Canis latrans*), 70 (11%) Pacific martens, and 5 (< 1%) bobcats (*Lynx rufus*). Among the 711 scats that we collected, 518 (73%) sequenced successfully, including 317 (61%) red foxes, 172 (33%) coyotes, 24 (5%) martens, and 5 (1%) bobcats. Our field identifications were correct for 271 (71%) of 382 scats that we considered highly likely to be red fox, 37 (45%) of 83 scats we considered probable red fox, and 9 (17%) of 53 scats we considered possible red fox. Most incorrectly assigned scats were from coyotes (Appendix S1; Supplementary Information). Among the urine samples, 73 (84%) were successfully sequenced, and 70 (96%) were red fox, 2 (3%) were coyote, and 1 (1%) was marten. Among the hair samples, 67 (80%) were successfully sequenced, and 22 (33%) were red fox and 45 (67%) were marten.

Microsatellite genetic diversity

We successfully genotyped 154 samples at ≥ 25 microsatellite loci collected during 2010–2013, from which we inferred the presence of 51 individuals. Samples were assigned to a unique individual based on sharing > 85% of alleles (Fig. S1; Supplementary Information). We assembled consensus genotypes only from samples with ≥ 25 loci to avoid erroneously identifying samples as unique individuals when they were actually multiple samples from the same individual due to false alleles and allelic dropout. Samples with fewer successful loci also likely had the highest allelic dropout rates. Based on the 51 individual genotypes, we estimated expected heterozygosity at 0.60 (SE = 0.03) and observed heterozygosity at 0.58 (SE = 0.01). The average number of alleles was 4.75 (SE = 0.28), and rarefied allelic richness was 4.20 (SE = 0.27). We found no statistically significant deviations from HWE for any locus and there was no significant heterozygote deficiency or excess in the population based on all loci ($F_{IS} = 0.03$, SE = 0.03). Genetic effective population size, N_e, was estimated at 11.4 (10.0–13.1) for the entire dataset ($n = 51$). Use of the reduced-relatedness subset of individuals ($n = 30$) resulted in a slightly larger estimate of $N_e = 16.0$ (13.3–19.4).

Population structure

Genetic clustering based on microsatellites resulted in two distinct groups (Fig. 2) as the greatest increase in posterior probability occurred at $K = 2$ (Fig. S2, S3; Supplementary Information). However, the pattern associated with these clusters (Fig. 3) was due to a large group of closely related individuals sampled at Mount Rainier National Park, that are likely multi-year descendants of a pair at a denning site on the south side of the mountain. Higher levels of K preserved this genetic cluster and revealed additional substructure with no spatial pattern throughout the study area. This additional structure likely corresponded to other family groups (Fig. 2; Fig. S4, Supplementary Information). Examination of the locations of pairs of close relatives throughout the study area showed that individuals dispersed across large portions of the landscape (Fig. S5; Supplementary Information), resulting in an overall weak spatial pattern of genetic structure.

Mitochondrial DNA characteristics

Based on the 51 individuals identified with microsatellites, we tallied the mtDNA haplotype frequencies (Table 2). Most haplotypes ($n = 38$) were O-24, although we also found O-28 in 4 individuals from Goat Rocks, Mount Rainier, and Indian Heaven. The cytochrome *b* fragment of one individual from Indian Heaven (also with a -28 D-loop fragment) exhibited both the O mutation and a heteroplasmic site with a novel, O2, substitution. We confirmed this heteroplasmic haplotype in five different samples from, or presumably from (if no genotype was available), this individual from the Indian Heaven Wilderness. Among the 251 samples that provided mtDNA haplotypes (excluding 11 that were incomplete) but for which no individual genotype was obtained, all but five cytochrome *b* haplotypes were O (the other five were the heteroplasmic O2 haplotype). We did not detect any other montane or nonnative red fox haplotypes in this study.

Table 1 Putative Cascade red fox samples collected from 2009 to 2014 in the southern Washington Cascade Range and verified with mitochondrial DNA sequencing at the cytochrome-*b* (354 bp) locus

Sample type	n	No. that sequenced	No. red fox	No. coyote	No. Pacific marten	No. bobcat
Scat	712	518	317	172	24	5
Urine	87	74	70	2	1	0
Hair	83	67	22	0	45	0
Tissue	4	3	3	0	0	0
Total	886	665	416	174	70	5
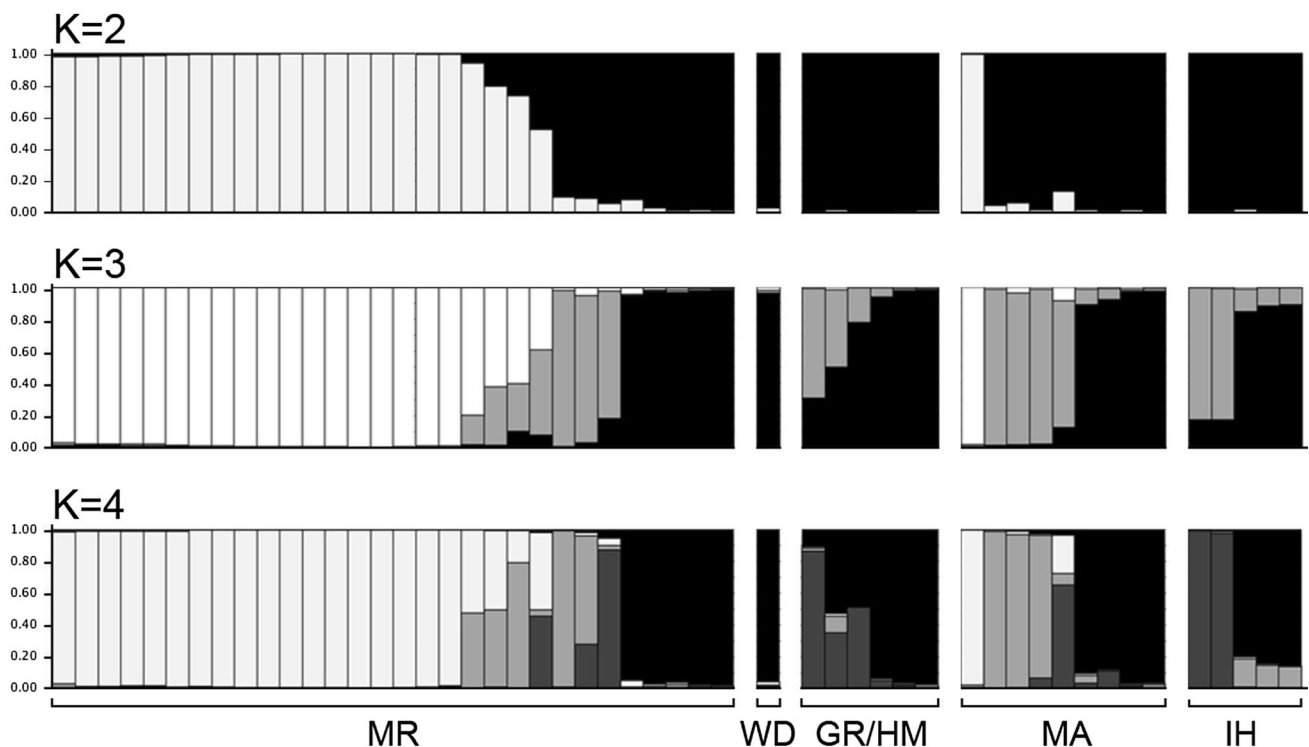
Fig. 2 Genetic assignment of 51 Cascade red foxes in program Structure based on the correlated allele frequencies model and 500,000 MCMC cycles following 250,000 burn-in cycles to $K=2$, $K=3$, and $K=4$ genetic clusters, southern Washington Cascade Range (2010–2013)

Nonnative introgression

Based on previously published genotypes for nonnative lowland red foxes from California and native montane populations from throughout the West as reference samples, we used Structure with prior information to assign samples from the present study as unknowns (no prior information) with respect to nonnative versus native ancestry. All samples assigned overwhelmingly as native (Fig. 4a). We then investigated genetic clustering of the reference and study samples together with no prior information specified regarding the population of origin using varying numbers of genetic clusters (K). The posterior probability [log Pr(D|K)] increased most from $K=1$ to $K=2$, but continued to increase with K up to $K=4$ (Fig. S2, S3; Supplementary Information). Individuals from this study (modern CRF samples, $n=51$) clustered together closely with CRF samples collected in the 1980s at $K=2$ ($n=8$; Fig. 4b). All other nonnative and native samples clustered together, indicating the strong distinctiveness of modern CRFs. The only difference at $K=3$ was that the nonnative red foxes clustered as a distinct population from all native populations. At $K=4$, the historical Sierra Nevada red fox from the southern portion of its range clustered out as distinct, but otherwise was the same. Thus, our study animals

were native and most similar genetically to CRFs from the same region that were sampled several decades earlier.

Discussion

There are multiple state and federal conservation listings for the CRF, suggesting that this subspecies has suffered significant population declines and is imperiled. However, the paucity of scientific research conducted on this subspecies to date has provided little data with which to evaluate the severity and relative significance of particular threats, or to gain a clearer understanding of the CRF's basic natural history, habitat requirements, or limiting factors. The present study represents the first attempt to obtain some of the fundamental information needed for proactive conservation of this subspecies. As the first comprehensive analysis of the genetic diversity and population structure of the CRF, our findings suggest that this southern population is small but functionally well connected.

Field scat identification and DNA verification

Because of significant size overlap between red fox and coyote scats, field identification can be challenging. We found
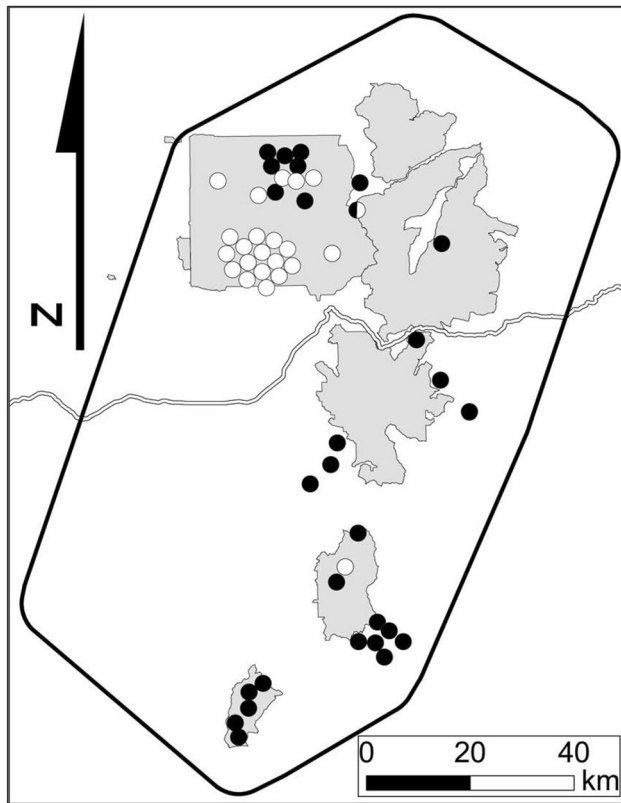
Fig. 3 Map of 51 Cascade red foxes (averaged location) sampled in southern Washington Cascade Range (2010–2013) based on their genetic assignment at $K = 2$ in program Structure (based on the correlated allele frequencies model and 500,000 MCMC cycles following 250,000 burn-in cycles). Dot color indicates assignment to one of two genetic clusters, or equal assignment to both clusters (bi-colored dot, see Fig. 2). White line is US Highway 12

that with adequate training and practice, a collector could achieve 84% accuracy in identifying red fox scats by using only those scats for which identification was most confident (highly likely; Akins 2017). Additionally, we were less

certain of correct field identification (i.e., probable or possible red fox) for 46 (15%) of the 317 red fox scats we collected, indicating that CRF scats would have been discarded had they not been identified genetically. Thus, it is important that species identification of scats be based on genetic analysis because similar-sized carnivores—especially coyotes—co-occur throughout the ranges of the CRF and other montane red foxes. In our study, we found the coyote to be most commonly misidentified as red fox, followed by Pacific marten.

Of additional note, we had a high success rate sequencing DNA from urine samples (84%) and correctly identifying them as red fox, which is likely due to the recent deposition of the sample and the high likelihood of identifying red fox snow tracks correctly with practice. Finally, the proportion of hair samples collected at camera stations that were confirmed to be red foxes was low ($n = 22$ of 84, 26%) of the total hair samples collected. Nonetheless, these noninvasively collected hair samples provide a potentially significant source of genetic material for CRFs.

Genetic diversity, population structure, and connectivity

The best overall metric of genetic diversity is the contemporary genetic effective population size, N_e, because this ultimately determines the rate of loss of genetic diversity (Lande and Barrowclough 1987; Hoehn et al. 2012) and, therefore, evolutionary potential and risks associated with inbreeding (Saccheri et al. 1998; Hedrick 2011). Our estimates of N_e in this population varied depending on inclusion of the closest relatives in our dataset; however, in all cases the upper 95% CI was < 20, which is considered to be very small (e.g., Funk et al. 1999; Sacks et al. 2010). The expectation based on an N_e of 20 is that the population will experience a loss in heterozygosity of approximately 25% over 10 generations (e.g., Waples 1989). Thus, assuming
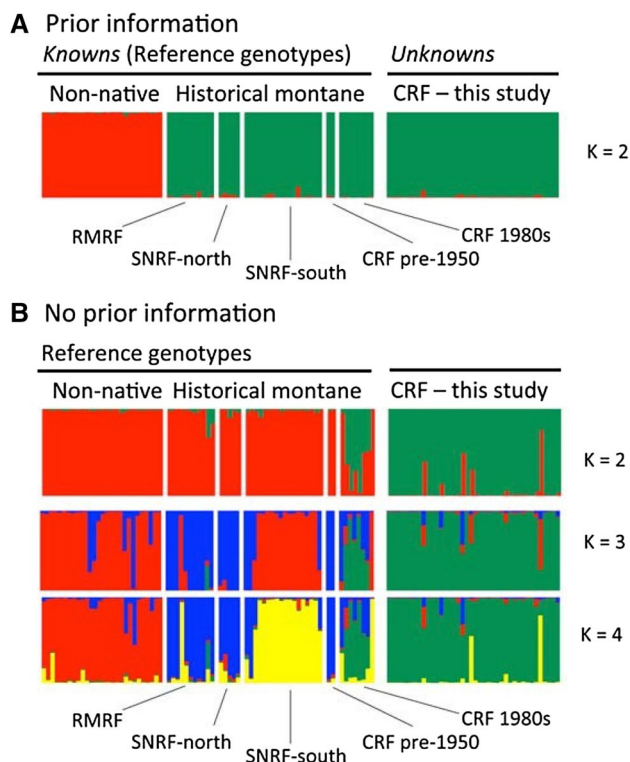
Table 2 Concatenated cytochrome-*b* and D loop haplotypes derived from mitochondrial sequencing of Cascade red foxes ($n = 51$) collected from 2009 to 2014 in the southern Washington Cascade Range including Mount Rainier National Park (MR), and the Norse Peak (NP), William O. Douglas (WD), Goat Rocks/Hamilton Buttes (GR and HM), Mount Adams (MA), and Indian Heaven (IH) wilderness areas

Haplotype	Wilderness area					
	IH	MA	GR/ HM	MR	WD	Total
O-24	2	7	4	25	0	38
O-28	2	0	1	1	0	4
O2-28	1	0	0	0	0	1
O-unknown	0	2	1	4	1	8
Total	5	9	6	30	1	51

Haplotype nomenclature based on Sacks et al. (2010): cytochrome *b* fragments indicated by letter or letter/numeral combination preceding the dash; D loop fragments indicated by numeral following the dash. The 8 haplotypes with cytochrome b component, O, for which the D-loop fragment was not successfully sequenced are indicated as "O-unknown."

A Prior information

B No prior information

Fig. 4 Admixture analyses based on 14 microsatellite loci run in program Structure using 72 reference samples from Sacks et al. (2010) and 40 Cascade red fox (CRF) samples from this study (we excluded 11 foxes that were missing data for 3 or more loci). **a** at $K=2$ genetic clusters with reference samples used as known nonnative or native montane foxes to assign samples from this study (treated as unknowns), and **b** at $K=2$, 3, and four genetic clusters using no prior information on population of origin. Only genotypes with 12 loci were included in this analysis. Reference samples included nonnative California red foxes ($n=28$), along with historical (pre-1950) native montane red foxes from the Rocky Mountains ($n=11$), Sierra Nevada red fox of the southern Cascades (SNRF-north, $n=5$) and the Sierra Nevada proper (SNRF-south, $n=18$), and CSR sampled before 1950 ($n=2$) and in the 1980s ($n=8$). **b** The logP(D|K) increased with increasing K up to K=4, values from K=1 to 6 as follows: -6225, -5748, -5556, -5402, -5347, -5339

a generation time of 2–5 years, heterozygosity is expected to decline to 0.45 over the next 20–50 years. In general, the longer a population remains small, the more of its genomic diversity will be lost, and the more likely the population will be to experience adverse effects from inbreeding (i.e., inbreeding depression).

Based on comparisons with a small sample of CRFs from the 1980s ($n=8$), it appears that the population has declined in abundance recently. Had the population been similarly small in the 1980s, we would have expected its heterozygosity to be substantially lower at present than we observed. However, heterozygosity estimated in this study for the current population ($H_e=0.60$, SE=0.03) was only slightly lower (and not significantly so) than that from

samples collected in the 1980s ($H_e=0.64$, SE=0.05; Sacks et al. 2010; Sacks, unpublished data). This suggests that the effective population size was larger in the 1980s than at present. If so, the population has probably not yet experienced the most significant impacts of small population size, such as inbreeding depression.

Connectivity within this population, and between this and other populations, also affects genetic effective population size and the rate of loss in genetic diversity. In the present population, closely related individuals were distributed throughout our study area (e.g., occurring on different Cascade volcanoes), with individuals dispersing across large distances. There was no broad-scale geographic pattern associated with population structure but rather with family groups dispersed across the study area. This suggests a strong level of connectivity, which is favorable to preserving genetic diversity if the population is able to increase in abundance through natural means.

Of additional note, in comparison to populations of other montane red foxes, the CRFs from this study, as well as those sampled in the 1980s (Sacks et al. 2010), clustered separately from Rocky Mountain and Sierra Nevada red foxes (Fig. 4b). By contrast, historical CRFs sampled earlier (1890s–1950s) clustered with these two other montane red fox subspecies, suggesting that the CRF has become increasingly isolated from other native montane red fox subspecies during the past century.

Nonnative introgression

We found no evidence of matrilineal or nuclear introgression by nonnative red foxes into our study population, suggesting that the native genetic integrity of this small, isolated population has been maintained. Mitochondrial sequences known to be native to the Washington Cascades historically were haplotypes A-19, A-25, A-29, O-28, O-24, and T-24 (Aubry et al. 2009; Sacks et al. 2010). In the present study, the population was characterized by only two of these matrilines, along with the heteroplasmic O2-28 haplotype, consistent with loss of diversity due to genetic drift. Although we cannot rule out that some of the native O-24 haplotypes may have resulted from introgression by nonnative foxes (Lounsberry et al. 2017), this seems unlikely. None of the unambiguous nonnative haplotypes (which comprised 80% of those in the adjacent nonnative population; e.g. Statham et al. 2012b) were found in this population.

Moreover, we found no evidence of nuclear gene flow from nonnative red fox populations into our study population based on microsatellite markers, yet male-mediated gene flow tends to be approximately four times greater than maternal gene flow (Sacks et al. 2016). Gene flow between native and nonnative red foxes in Washington was likely prevented by a wide, historical barrier of dense low- to

mid-elevation forest types separating them (Aubry 1984). Nevertheless, the current lack of nonnative gene flow does not indicate that such introgression will not occur in the future. Extensive timber extraction and road-building activities within these forests could eventually facilitate the movement of nonnative red foxes into subalpine habitats. In addition, the greater openness in lower elevation forests on the eastern slope of the Cascade Range may create better opportunities for nonnative introgression into CRF populations. The eastside mid-slopes consist of drier, open forests of Douglas-fir (*Pseudotsuga menziesii*), grand (*Abies grandis*) fir, lodgepole (*Pinus contorta*) and ponderosa (*P. ponderosa*) pines, and big sagebrush (*Artemisa tridentata*) that may provide more foraging opportunities for a cursorial predator such as the red fox (Aubry 1984). Nonnative introgression can occur rapidly, and has been documented in montane red fox populations elsewhere (Quinn and Sacks 2014; Merson et al. 2017).

Conservation implications and future research

Assessing the status of rare and little-studied species, and understanding the threats to their long-term persistence are fundamental steps in the development of an effective conservation plan. It is especially important to fill these knowledge gaps before species become functionally extinct. Our findings indicate that the CRF population in the southern Cascade Range exhibits a small genetic effective population size, and is therefore at risk of future losses in genetic diversity. We know even less about the CRF in the northern portion of its range, although the scarcity of recent verifiable reports, despite the implementation of forest carnivore surveys during the past several decades in many areas of the northern Cascades (K. Aubry, unpublished data), suggests that this population may be much smaller and, therefore, at greater risk than our study population. Understanding how the southern population is connected to individuals in the north will help to assess the prospects for their long-term persistence and identify key habitat needed for population connectivity. In addition, monitoring potential future introgression into this montane population will provide insights into which parts of the habitat are most susceptible to movements of lowland red foxes into the mountains.

We recommend the following research priorities to facilitate future CRF conservation efforts: (1) conduct occupancy surveys and create habitat models in the southern Cascades, (2) use resulting models to guide broad surveys in the northern Cascades, (3) continue genetic monitoring of the southern Cascades population to identify trends in genetic integrity, diversity, connectivity, and effective population size, and (4) implement new ecological studies in the southern Cascades to estimate demographic rates, characterize critical habitat, and identify the key factors that currently limit population size or growth rate.

References

Akins J (2017) Distribution, genetic structure, and conservation status of the Cascade red fox in southern Washington. Dissertation, University of California, Davis, CA, USA

Aubry KB (1983) The Cascade red fox: distribution morphology zoogeography and ecology. Dissertation, University of Washington, Seattle, WA, USA

Aubry KB (1984) The recent history and present distribution of the red fox in Washington. Northwest Sci 58:69–79

Aubry KB, Statham MJ, Sacks BN, Perrine JD, Wisely SM (2009) Phylogeography of the North American red fox: vicariance in Pleistocene forest refugia. Mol Ecol 18:2668–2686

Bunnell KD, Flinders JT, Wolfe ML (2006) Potential impacts of coyotes and snowmobiles on lynx conservation in the intermountain west. Wildl Soc B 34:828–838

Crawford RC, Chappell CB, Thompson CC, Rocchio FJ (2009) Vegetation classification of Mount Rainier, North Cascades, and Olympic National Parks. Natural Resource Technical Report NPS/NCCN/NRTR—2009/D-586. National Park Service, Fort Collins, CO, USA

Earl DA, von Holdt BM (2012) Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. Conserv Genet Res 4:359–361

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Funk WC, Tallmon DA, Allendorf FW (1999) Small effective population size in the long-toed salamander. Mol Ecol 8:1633–1640

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-Statistics. J Hered 86(6):485–486

Green GA, Sacks BN, Erickson LJ, Aubry KB (2017) Genetic characteristics of red foxes in northeastern Oregon. Northwest Nat 98:73–81

Hall (1998) Pacific Northwest ecoclass codes for seral and potential natural communities. Gen. In: Tech. Rep. PNW-GTR-418. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, OR. In cooperation with: Pacific Northwest Region

Hansen AJ, Piekielek N, Davis C, Haas J, Theobald DM, Gross JE, Monahan WB, Olliff T, Running SW (2014) Exposure of US National Parks to land use and climate change 1900–2100. Ecol Appl 24:484–502

Hedrick P (2011) The genetics of populations. Jones and Bartlett Publishers, Sudbury

Hemstrom MA, Franklin JF (1982) Fire and other disturbances of the forests in Mount Rainier National Park. Quat Res 18:32–51

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. Int J Climatol 25:1965–1978

Hoehn M, Gruber B, Sarre SD, Lange R, Henle K (2012) Can genetic estimators provide robust estimates of the effective number of breeders in small populations? PLoS ONE 7:e48464

Jenkins K, Quinn P, Reid M (2014) Indicators for habituated and food-conditioned Cascade red foxes in Mount Rainier National Park: preliminary assessment. U.S. Geological Survey administrative report to Mount Rainier National Park, U.S. National Park Service

Kalinowski ST, Wagner AP, Taper ML (2006) ML-Relate: a computer program for maximum likelihood estimation of relatedness and relationship. Mol Ecol Notes 6:576–579

Laliberte AS, Ripple WJ (2004) Range contractions of North American carnivores and ungulates. Bioscience 54:123–138

Lande R, Barrowclough GF (1987) Effective population size, genetic variation, and their use in population management. In: Soule ME (Ed) Viable populations for conservation. Cambridge University Press, Cambridge, MA, pp 88–90

Lillybridge TR, Kovalchik BL, Williams CK, Smith BG (1995) Field guide for forested plant associations of the Wenatchee National Forest. Gen. Tech. Rep. PNW-GTR-359. U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Portland, OR. In cooperation with Pacific Northwest Region, Wenatchee National Forest

Lounsberry ZT, Forrester TD, Olegario MT, Brazeal JL, Wittmer HU, Sacks BN (2015) Estimating sex-specific abundance in fawning areas of a high-density Columbian black tailed deer population using fecal DNA. J Wildlife Manage 79:39–49

Lounsberry ZT, Quinn CB, Angulo C, Kalani T, Tiller E, Sacks BN (2017) Investigating genetic introgression from farmed red foxes into the wild population in Newfoundland, Canada. Conserv Genet 18:383–392

Maletzke BT, Wielgus RB, Pierce DJ, Martorello DA, Stinson DW (2016) A meta-population model to predict occurrence and recovery of wolves. J Wildlife Manage 80:368–376

McKelvey KS, Aubry KB, Anderson NJ, Clevenger AP, Copeland JP, Heinemeyer KS, Inman RM, Squires JR, Waller JS, Pilgrim KL, Schwartz MK (2014) Recovery of wolverines in the western United States: recent extirpation and recolonization or range retraction and expansion? J Wildl Manag 78:325–334

Merson C, Statham MJ, Janecka JE, Lopez RR, Silvy NJ, Sacks BN (2017) Distribution of native and nonnative ancestry in red foxes along an elevational gradient in central Colorado. J Mammal 98:365–377

Miles KA, Holtz MN, Lounsberry ZT, Sacks BN (2015) A paired comparison of scat-collecting versus scat-swabbing methods for noninvasive recovery of mesocarnivore DNA from an arid environment. Wildl Soc B 39:797–803

Moore MS, Brown SK, Sacks BN (2010) Thirty-one short red fox (*Vulpes vulpes*) microsatellite markers. Mol Ecol Res 10:404–408

Nellemann C, Vistnes I, Jordhøy P, Støen OG, Kaltenborn BP, Hanssen F, Helgesen R (2010) Effects of recreational cabins, trails and their removal for restoration of reindeer winter ranges. Restor Ecol 18:873–881

Park S (2001) Microsatellite toolkit. Department of Genetics, Trinity College, Dublin

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. Annu Rev Ecol Evol Syst 37:637–669

Perrine JD (2005) Ecology of red fox (*Vulpes vulpes*) in the Lassen Peak region of California, USA. Dissertation. University of California Berkeley California, USA

Perrine JD, Pollinger JP, Sacks BN, Barrett RH, Wayne RK (2007) Genetic evidence for the persistence of the critically endangered Sierra Nevada Red fox in California. Conserv Genet 8:1083–1095

Pfeiffer I, Volkel I, Taubert H, Brenig B (2004) Forensic DNA-typing of dog hair: DNA extraction and PCR amplification. Forensic Sci Int 141:149–151

Pielou EC (2008) After the ice age: the return of life to glaciated North America. University of Chicago Press, Chicago, IL

Pritchard PK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Quinn CB, Sacks BN (2014) Ecology, distribution, and genetics of Sierra Nevada red fox. Draft final report to CDFW: Agreement No. P1080019

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Saccheri I, Kuussaari M, Kankare M, Vikman P (1998) Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494

Sacks BN, Statham MJ, Perrine JD, Wisely SM, Aubry KB (2010) North American montane red foxes: expansion, fragmentation, and the origin of the Sacramento Valley red fox. Conserv Genet 11:1523–1539

Sacks BN, Moore M, Statham MJ, Wittmer HU (2011) A restricted hybrid zone between native and introduced red fox (*Vulpes vulpes*) populations suggests reproductive barriers. Mol Ecol 20:326–341

Sacks BN, Brazeal JL, Lewis JC (2016) Landscape genetics of the non-native red fox of California. Ecol Evol 6:4775–4791

Sambrook J, Russell DW (2001) Molecular cloning—a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Statham MJ, Trut LN, Sacks BN, Kharlamova AV, Oskina IN, Gulevich RG, Johnson JL, Temnykh SV, Acland GM, Kukekova AV (2011) On the origin of a domesticated species: identifying the parent population of Russian silver foxes (*Vulpes vulpes*). Biol J Linn Soc 103:168–175

Statham MJ, Rich AC, Lisius SK, Sacks BN (2012a) Discovery of a remnant population of Sierra Nevada red fox (*Vulpes vulpes necator*). Northwest Sci 86:122–132

Statham MJ, Sacks BN, Aubry KB, Perrine JD, Wisely SM (2012b) The origin of recently established red fox populations in the contiguous United States: translocations or natural range expansions? J Mammal 93:52–65

Statham MJ, Murdoch J, Janecka J, Aubry KB, Edwards CJ, Soulsbury CD, Berry O, Wang Z, Harrison D, Pearch M, Tomsett L, Chupasko J, Sacks BN (2014) Range-wide multilocus phylogeography of the red fox reveals ancient continental divergence, minimal genomic exchange, and distinct demographic histories. Mol Ecol 23:4813–4830

Swanson BJ, Fuhrmann RT, Crabtree RL (2005) Elevational isolation of red fox populations in the Greater Yellowstone Ecosystem. Conserv Genet 6:123–131

Toonen R, Hughes S (2001) Increased throughput for fragment analysis on an ABI Prism 377 Automated Sequencer using a membrane comb and STRand software. Biotechniques 31:1320–1324

U.S. Environmental Protection Agency (2003) Level III ecoregions of the continental United States (revision of OMERNIK, 1987): Corvallis, Oregon, U.S. Environmental Protection Agency-National Health and Environmental Effects Research Laboratory, map scale 1:7,500,000. https://www.epa.gov/eco-research/level-iii-and-iv-ecoregions-continental-united-states

U.S. Fish and Wildlife Service (2015a) Species report: Sierra Nevada red fox (*Vulpes vulpes necator*). https://www.fws.gov/sacramento/outreach/2015/10-07/docs/20150814_SNRF_SpeciesReport.pdf. Accessed 14 Aug 2015

U.S. Fish and Wildlife Service (2015b) Endangered and threatened wildlife and plants; 12-month finding on a petition to list Sierra Nevada red fox as an endangered or threatened species; proposed rule. Fed Reg 50:60990–61028

Van Etten KW, Wilson KR, Crabtree RL (2007) Habitat use of red foxes in Yellowstone National Park based on snow tracking and telemetry. J Mammal 88:1498–1507

Volkmann LA, Statham MJ, Mooers A, Sacks BN (2015) Genetic distinctiveness of red foxes in the Intermountain West as revealed through expanded mitochondrial sequencing. J Mammal 96:297–307

Waples RS (1989) A generalized approach for estimating effective population size from temporal changes in allele frequency. Genetics 121:379–391

Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conserv Genet 7:167–184

Waples RS, Do CH (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Mol Ecol Res 8:753–756

Washington Department of Fish and Wildlife (2015) Washington's State Wildlife Action Plan. Washington Department of Fish and Wildlife, Olympia, Washington

Zielinski WJ, Truex RL, Schlexer FV, Campbell LA, Carroll C (2005) Historical and contemporary distributions of carnivores in forests of the Sierra Nevada, California, USA. J Biogeog 32:1385–1407