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Conservation implications of the evolutionary history and genetic diversity hotspots of the snowshoe hare

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Abstract

With climate warming, the ranges of many boreal species are expected to shift northward and to fragment in southern peripheral ranges. To understand the conservation implications of losing southern populations, we examined range-wide genetic diversity of the snowshoe hare (*Lepus americanus*), an important prey species that drives boreal ecosystem dynamics. We analysed microsatellite (8 loci) and mitochondrial DNA sequence (cytochrome b and control region) variation in almost 1000 snowshoe hares. A hierarchical structure analysis of the microsatellite data suggests initial subdivision in two groups, Boreal and southwestern. The southwestern group further splits into Greater Pacific Northwest and U.S. Rockies. The genealogical information retrieved from mtDNA is congruent with the three highly differentiated and divergent groups of snowshoe hares. These groups can correspond with evolutionarily significant units that might have evolved in separate refugia south and east of the Pleistocene ice sheets. Genetic diversity was highest at mid-latitudes of the species' range, and genetic uniqueness was greatest in southern populations, consistent with substructuring inferred from both mtDNA and microsatellite analyses at finer levels of analysis. Surprisingly, snowshoe hares in the Greater Pacific Northwest mtDNA lineage were more closely related to black-tailed jackrabbits (*Lepus californicus*) than to other snowshoe hares, which may result from secondary introgression or shared ancestral polymorphism. Given the genetic distinctiveness of southern populations and minimal gene flow with their northern neighbours, fragmentation and loss of southern boreal habitats could mean loss of many unique alleles and reduced evolutionary potential.

Keywords: climate change, core-periphery, evolutionarily significant units, landscape genetics, *Lepus americanus*, phylogeography

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Introduction

Over the next century, North America's southern boreal forests are predicted to undergo rapid fragmentation and loss due to climate change and human activities (IPCC 2007). Understanding the conservation implications of southern habitat loss for boreal species requires evaluating range-wide genetic structure of individual species and assessing the generality of these patterns across taxa. Specifically, insights into large-scale genetic diversity and population differentiation would clarify the relative importance of southern boreal populations as hotspots of diversity and evolutionarily significant

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units (ESUs; Moritz 1994), with implications for adaptive potential.

Distribution of genetic diversity and structure across a species' range reflects historical range contraction and recolonization from glacial refugia as well as current habitat fragmentation and dispersal. During the Quaternary ice ages (approximately 2.6 mya–present), the alternation of glacial and interglacial periods caused repeated changes in species' distributions. A frequently invoked 'southern refugia' model in phylogeography suggests that when ice sheets advanced, many boreal species persisted primarily in refugia in southern latitudes (Hewitt 1996). Interglacial periods enabled northward range expansion, with leading-edge populations carrying a subset of the genetic diversity of refugial populations. Simultaneously, range contraction to higher elevations in southern populations may have reduced connectivity and increased local diversification (Moritz *et al.* 2008). For some species in North America and Europe, a pattern of decreasing genetic diversity with increasing latitude ('southern richness, northern purity') may reflect a dominant influence of historical southern refugia on patterns of diversity (Pielou 1991; Hewitt 1996; Soltis *et al.* 1997).

The generality of the southern refugia model has been challenged for species with high dispersal and large contemporary ranges (Hewitt 2000; Provan & Bennett 2008). For many North American species, fossil evidence and phylogeographic studies have identified additional glacial refugia in eastern Beringia, the Canadian Arctic, coastal British Columbia, the Maritimes, and other northern locations (Soltis *et al.* 2006; Provan & Bennett 2008; Godbout *et al.* 2010). When expanding populations from separate refugia met in zones of secondary contact, they often created hotspots of genetic diversity (Provan & Bennett 2008). Geographic and genetic subdivision within major refugia further complicate genetic patterns (Gomez & Lunt 2007).

Contemporary gene flow also impacts genetic structure and diversity. Many boreal species have populations occupying peninsular habitat extensions into montane forests of the USA (Shugart *et al.* 2005). In addition to natural habitat fragmentation, these southern boreal forests are heavily impacted by logging and habitat conversion (Hansen *et al.* 2010; Powers *et al.* 2012), which could lead to differentiation in remnant habitats. The core-periphery hypothesis suggests that connected populations in the boreal range core should have higher genetic diversity than the fragmented populations of the southern periphery (Eckert *et al.* 2008). But while low gene flow and chronic genetic drift may reduce genetic diversity in peripheral populations, these processes may simultaneously facilitate genetic differentiation and preserve unique alleles (Eckert *et al.* 2008).

Given the complex interplay of forces that shape intraspecific distribution of genetic diversity, what are the consequences of losing southern boreal populations? In this study we examined range-wide genetic diversity of the snowshoe hare (*Lepus americanus*) to address this question.

Snowshoe hares are important prey for most boreal carnivores, structuring food web dynamics as strong interactors (Krebs *et al.* 2001). Fossil evidence suggests the persistence of snowshoe hares in extensive refugia south of the ice sheets and in the northern refugium of Beringia during the Last Glacial Maximum (LGM; FAUNMAP Working Group 1994). Snowshoe hare populations near historical Beringia and in Montana harbour high genetic diversity and are genetically differentiated from each other (Burton *et al.* 2002). Morphological differences among snowshoe hare populations in and around the Pacific Northwest suggest genetically differentiated populations (Dalquest 1942).

We analysed mtDNA and microsatellite data throughout the contemporary range of snowshoe hares to test the hypotheses that: (i) extant snowshoe hare populations derive from Beringia and southern refugia; and (ii) snowshoe hare populations near the core of the range exhibit higher genetic diversity than populations near the periphery. We predict highest genetic diversity and uniqueness in the species' southern range and near the Alaska-Yukon border, that is, in likely refugia, with reduced diversity in range-edge populations outside of these areas.

We then discuss how anticipated loss of southern boreal habitats might affect snowshoe hare genetic diversity. First, we determine whether multiple snowshoe hare ESUs (sensu Moritz 1994) are warranted. Second, we examine genetic diversity and uniqueness across a latitudinal gradient, with particular focus on populations below 49°N, the approximate southernmost extent of the LGM. Many scenarios of climate change predict the climate envelope for North America's boreal ecosystem will shift north of 49°N within a century (Koven 2013). Finally, we discuss similarities in findings between snowshoe hares and other North American hare species.

Materials and methods

We analysed 975 snowshoe hare samples from 16 U.S. states and 12 Canadian provinces and territories (Appendix S1, Supporting information). Nearly all samples were ear tissue collected from road kill, game harvests, and live-trapping during 1989–2010. Ten samples were faecal pellets collected in Isle Royale, Michigan, in 2009. Eleven samples were tissue from specimens collected near Vancouver, British Columbia, from 1929 to 1970, held at the University of British Columbia Cowan

Museum. For phylogenetic analyses, we additionally analysed one white-tailed jackrabbit (*Lepus townsendii*) tissue sample obtained from GenBank (Accession no. AY292729; Matthee *et al.* 2004) and seven black-tailed jackrabbit (*L. californicus*) tissue samples collected from three U.S. states (California, New Mexico, Nevada).

Microsatellite analysis

Samples were grouped into populations on the basis of two geographic criteria: (i) no potential genetic barriers such as large lakes or rivers, mountain ranges, or non-forested regions bisecting populations (Burton *et al.* 2002; Shafer *et al.* 2010b); and (ii) a maximum of 260 km between any two samples in a population. The second criterion represents a coarse spatial scale much greater than the distance hares disperse (up to ~20 km, Gillis & Krebs 1999) but within the scale of reported gene flow in northern snowshoe hare populations (~600 km, Burton *et al.* 2002). After grouping samples, we limited genetic analyses to groups with at least seven samples. This minimum threshold was arbitrary, but has precedence in other population genetic studies (Schwartz *et al.* 2003; Tracy & Jamieson 2011).

We selected eight polymorphic microsatellite markers developed in the European rabbit, *Oryctolagus cuniculus*, and successfully used with snowshoe hares (Burton *et al.* 2002; Schwartz *et al.* 2007): 7L1D3 (Korstanje *et al.* 2003); SAT02, SAT12, SAT13, SAT16 (Mougel *et al.* 1997); SOL08, SOL30 (with 'GTGTCTT' tail added) (Rico *et al.* 1994); and SOL33 (Surridge *et al.* 1997) (Appendix S2, Supporting information). DNA extraction and genotyping methods are detailed in Appendix S3 (Supporting information).

Allelic dropout and false allele rates were calculated with 10 000 search iterations in Pedant version 1.0. (Johnson & Haydon 2007). For each population, we used Genepop version 4.0.11 (Rousset 2008) to test for Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium. Markov chain parameters for exact tests were set at 10 000 dememorizations, 100 batches, and 5000 iterations per batch (Raymond & Rousset 1995). We used the false discovery rate approach (FDR; Benjamini & Hochberg 1995) in the R software package 'fdrtool' (Strimmer 2008; http://cran.r-project.org/) to correct for multiple significance testing type I error. Potential null alleles and scoring errors due to stuttering and allelic drop-out were identified by Monte Carlo simulation in Micro-Checker version .2.2.3 (van Oosterhout *et al.* 2004).

We performed Bayesian analyses in STRUCTURE version 2.3.3 (Pritchard *et al.* 2000) and Geneland version 4.0.3 (Guillot *et al.* 2005) to partition microsatellite data into genetic clusters, and to assign individuals to their likely cluster of origin. In STRUCTURE, we applied an admixture model with the 'locprior' option, using a burn-in period of 20 000 generations and 100 000 MCMC iterations after burn-in. We compared results from correlated vs. uncorrelated allele frequency models. To check for MCMC consistency, we performed 20 replicates for each K (number of clusters) from 1 to 40. The most likely K value was determined in Structure Harvester version 0.6.93 (Earl & vonHoldt 2011) as the likelihood model with the highest ΔK (Evanno *et al.* 2005), unless the maximum lnP (D) was for $K = 1$ (which would indicate no substructure). In the presence of substructure, the ΔK method detects the highest hierarchical structure (Evanno *et al.* 2005). We assigned each individual to its most probable cluster and repeated the analysis for each cluster separately, until further substructure could not be detected. Cluster assignment was based on outcomes from the run with highest lnP(D) among 20 replicates. Following Coulon *et al.* (2008), only individuals with at least 60% membership in a cluster were included in subsequent analyses. For each subsequent analysis, model parameters remained the same, but maximum K was set at one greater than the number of sampled populations in that cluster.

In Geneland, we evaluated results from three spatially explicit model combinations. We examined both correlated and uncorrelated allele frequency models without filtering null alleles. We additionally examined an uncorrelated frequency model while filtering null alleles. For each of the three model combinations we ran 20 independent replicates of 1 000 000 MCMC iterations with a thinning of 1000 and burn-in of 200 000 iterations. K was allowed to vary from 1 to 40. Following program recommendations for our sample size, we set maximum rate of Poisson process = 853 and maximum number of nuclei in the Poisson-Voronoi tessellation = 2559. We allowed a 15-km uncertainty in spatial coordinates. MCMC convergence was assessed for each model combination by comparing estimated K and cluster assignments across replicate runs.

GENALEX version 6.3 (Peakall & Smouse 2006) was used to calculate number of alleles and expected heterozygosity (Nei 1978) for each population. For all pairs of populations, we estimated Nei's D (Nei 1972) and Weir & Cockerham's (1984) F_{ST}, with the latter calculated in ARLEQUIN version 3.5.1.2 (Excoffier *et al.* 2005). Significance was determined with 1000 permutations of samples among populations and FDR correction for multiple comparisons.

We used rarefaction, implemented in HP-RARE version 1.0 (Kalinowski 2005), to calculate private allelic richness (PAR) for each population, standardized to the smallest sample size (seven individuals) in this study. To minimize biases due to uneven sampling

density, all populations within a 350 km radius were excluded from the calculation of PAR for each sampled population (we also tested 500 km and results were similar; data not shown). We examined scatterplots of genetic metrics against latitude and longitude to identify signatures of genetic drift at the current range periphery and to understand geographic patterns of diversity.

Mitochondrial DNA analysis

We amplified a 468 bp fragment of the mitochondrial control region (CR) with primers LCRSEQ (Melo-Ferreira *et al.* 2007) and LepD2H (Pierpaoli *et al.* 1999) in all snowshoe hare samples. A fragment with 633 bp of the cytochrome b (Cytb) gene was also sequenced in a subset of 80 snowshoe hare and seven black-tailed jackrabbit samples, using primers LGCYF (Alves *et al.* 2003) and LCYTBR (Melo-Ferreira *et al.* 2005), as detailed in Appendix S3 (Supporting information). The Cytb subset comprised at least one snowshoe hare sample from each population and additional samples from regions of high CR genetic structure. We visually aligned sequences in CodonCode Aligner version 3.5.4 (CodonCode Corporation, Dedham, MA, USA).

Phylogenetic trees were constructed in BEAST version 1.7.4 (Drummond *et al.* 2012) based on the Cytb gene, which has a slower mutation rate and thus lower tendency than CR for homoplasy over long timescales (Baker & Marshall 1997). We used jModelTest version 2.1.3 (Darriba *et al.* 2012) and the Bayesian information criterion to assess the best-fit model of sequence evolution. Posterior probabilities were determined from three independent runs of 250 million generations, using the selected mutation model, the Yule tree prior and a random local clock (Drummond & Suchard 2010), excluding the initial 10% of each run as burn-in. The stability of the runs and convergence of the MCMC were assessed with Tracer version 1.5 (http://beast.bio.ed.ac.uk/Tracer). Results from the three runs were concatenated in LogCombiner version 1.7.4 and trees annotated using TreeAnnotator version 1.7.4. The annotated phylogenetic tree and posterior probability estimates were visualized in FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). To estimate lineage divergence times, we used a mutation rate of 0.02 substitutions per site per million years (Brown *et al.* 1979), which has been used to estimate divergence in other hare studies (Pierpaoli *et al.* 1999; Melo-Ferreira *et al.* 2007).

The demographic history of the major mtDNA lineages was inferred from control region sequences using the Bayesian Skyline Plot (BSP) (Drummond *et al.* 2005) implemented in BEAST. Three replicate runs of 100 million generations were performed using the appropriate mutation models (for Boreal, TrN+I+G; for Greater Pacific Northwest and U.S. Rockies, HKY+G) selected using the procedure described above and a random local clock (Drummond & Suchard 2010). Tracer version 1.5 was used to assess stability of the MCMC and the initial 10% of each run was discarded as burn-in. We used LogCombiner version 1.7.4 to concatenate results of the three replicate runs. A CR mutation rate of 0.156 substitutions per site per million years (derived from Melo-Ferreira *et al.* 2007) was used to calibrate the BSP.

We examined within-lineage structure with the CR gene, because its fast rate of evolution makes it suitable for intraspecific studies (Vigilant *et al.* 1991). An unrooted median-joining network (NETWORK version 4.5.1.6, http://www.fluxus-engineering.com/) was generated from CR haplotypes identified in DnaSP version 5.10 (Librado & Rozas 2009). Transversions were weighted three times as high as transitions, following software recommendations. For K = 1–10, SAMOVA version 1.0 (Dupanloup *et al.* 2002) identified the partitioning of CR haplotype variance due to differences among groups. We ran SAMOVA with 500 initial population partitions and 10 000 iterations for each K. Significance of variance components was evaluated by 1000 permutations of populations among groups.

We used ARLEQUIN v.3.5.1.2 (Excoffier *et al.* 2005) to calculate haplotype and nucleotide diversities. As with microsatellite data, scatterplots were used to assess latitudinal and longitudinal patterns in genetic diversity. To evaluate genetic differentiation, we calculated pairwise control region F_{ST}. ARLEQUIN v.3.5.1.2 was used to determine significance of tests with 10 000 bootstraps and FDR control for multiple comparisons.

Results

Microsatellite analysis

With an average of 2.2 PCR replicates per sample, we successfully genotyped eight microsatellite loci for 922 snowshoe hares. The mean allelic dropout rate per allele was 0.0070 and mean false allele rate was 0.0035, for all loci combined. After excluding populations with <7 individuals, 853 samples in 39 populations remained for analyses (Fig. 1). Only 4% of 312 population-loci combinations significantly deviated from Hardy-Weinberg Equilibrium, generally due to heterozygote deficit. Slightly over 5% of 1026 tests for linkage disequilibrium were significant. Micro-checker identified potential null alleles in 8% of 312 population-loci tests. However, null alleles and deviations from HWE were not associated more frequently with any particular locus, and genotypic disequilibrium was not consistently attributed

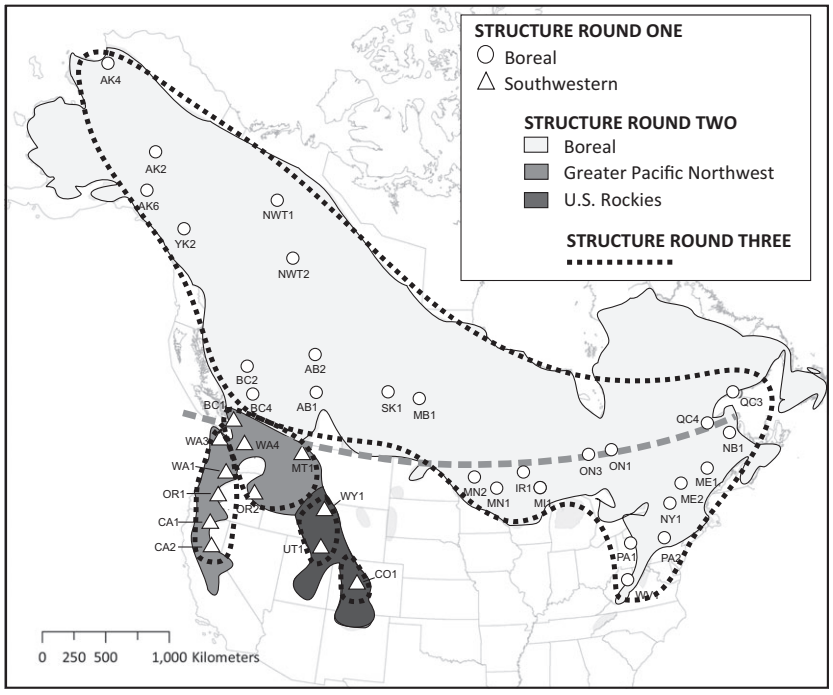

Fig. 1 Sampling locations and geographic distribution of major snowshoe hare microsatellite clusters, as defined by STRUCTURE version 2.3.3 hierarchical analysis (Pritchard *et al*. 2000). The first run of STRUCTURE distinguished Boreal (circles) from southwestern (triangles) populations. The second run further split the southwestern cluster (grey shades). By the third run, five distinct genetic clusters were identified (dotted ovals). The approximate southernmost latitude of the LGM is marked with a grey dashed line. Study results suggest extant snowshoe hare populations likely expanded from refugia south of this latitude and from the current eastern hare range.

to a particular locus pair. Therefore we retained all loci for subsequent analyses.

STRUCTURE analyses identified hierarchical population division (Fig. 1 and Appendix S4, Supporting information). In the first round of STRUCTURE runs, the highest likelihood model ($K = 2$) identified a Boreal cluster comprising the entire northern and eastern range of the species, and a southwestern cluster comprising remaining populations. The second round of STRUCTURE further splits the Boreal cluster into two subclusters. However, proportion membership in the subclusters transitioned from west to east (Appendix S5, Supporting information), suggesting an effect of isolation by distance rather than historical isolation (Meirmans 2012). A Mantel test (Mantel 1967), following Rousset's (1997) method, confirmed a significant correlation between geographic and genetic distance in the Boreal cluster ($P < 0.001$). Because other analyses and markers also supported a single Boreal cluster, we did not continue STRUCTURE analyses to further subset the Boreal cluster. In contrast to the Boreal cluster, the second round of STRUCTURE clearly divided the southwestern group into two genetic clusters, corresponding to the Greater Pacific Northwest region and to the U.S. Rockies. Further rounds of the hierarchical STRUCTURE analysis subdivided the Greater Pacific Northwest and U.S. Rockies groups into many subclusters. Ultimately, by the fifth round of analysis, all southwestern populations were identified as distinct subclusters except for WA1 and WA4 in Washington. For these two populations, some individuals could not be assigned to a cluster with at least 60% probability, and other individuals grouped with other populations. Hierarchical cluster patterns were identical for the correlated and uncorrelated allele frequency models.

Using Geneland, all replicates of the uncorrelated frequency models (with and without filtering null alleles) consistently identified a single Boreal cluster and 4–7 distinct clusters in the species' southwestern range. With the uncorrelated model and null alleles filtered, the highest mean posterior density across 20 replicates was obtained for $K = 5$, with clusters almost identical to those identified from the first three rounds of STRUCTURE hierarchical analysis (Appendix S6, Supporting information). All replicates of the correlated frequency model in Geneland inferred 39–40 genetic clusters, likely due to known instabilities of this model in the presence of isolation by distance (Guillot 2008).

Measures of F_{ST} and Nei's D were highly correlated across populations ($r = 0.93$, $P < 0.001$). We found high F_{ST} pairwise estimates (>0.20) between the three genetic clusters identified in the first two rounds of STRUCTURE. These clusters were the most congruent across markers (microsatellite and mtDNA) and analyses. Pairwise F_{ST} was high within the Greater Pacific Northwest and U.S. Rockies clusters, but was usually below 0.20 within the Boreal cluster (Appendix S7, Supporting information).

Most snowshoe hare populations were characterized by high genetic diversity (Table 1). On average, populations in the Boreal cluster exhibited the highest allelic richness and heterozygosity, but the lowest uniqueness

Table 1 Microsatellite diversities averaged across 8 loci for each of 39 sampled populations. Populations are grouped into three genetic clusters identified by the first two rounds of STRUCTURE hierarchical analysis

Population	*N*	A	AR	H_0	H_E	PAR
AB1	9	7.00	6.20	0.69	0.76	0.13
AB2	18	8.63	5.95	0.69	0.77	0.14
AK2	28	7.50	4.79	0.56	0.62	0.02
AK4	15	5.75	4.63	0.56	0.64	0.07
AK6	9	4.75	4.38	0.61	0.64	0.19
BC2	25	7.88	5.09	0.57	0.67	0.07
BC4	9	4.75	4.33	0.56	0.65	0.08
IR1	10	5.88	5.13	0.66	0.71	0.05
MB1	13	6.50	5.12	0.59	0.71	0.10
ME1	40	9.75	5.89	0.72	0.77	0.06
MI1	8	6.50	6.10	0.72	0.80	0.33
MN1	34	9.63	5.62	0.58	0.73	0.07
MN2	12	7.50	5.97	0.70	0.79	0.04
NB1	20	9.00	5.86	0.68	0.74	0.17
NWT1	9	6.50	5.83	0.69	0.74	0.11
NWT2	18	7.63	5.59	0.67	0.72	0.01
NY1	13	6.75	5.58	0.70	0.79	0.10
ON1	11	7.00	5.86	0.67	0.75	0.02
ON3	19	9.25	5.85	0.66	0.75	0.15
PA1	10	5.25	4.63	0.63	0.65	0.15
PA2	13	6.63	5.23	0.70	0.68	0.07
QC3	20	8.50	5.80	0.70	0.77	0.09
QC4	17	7.00	5.38	0.71	0.76	0.05
SK1	8	6.13	5.78	0.56	0.72	0.20
VT1	10	5.63	4.97	0.70	0.73	0.03
WV1	14	4.50	3.90	0.50	0.63	0.10
YK2	30	8.50	5.10	0.62	0.65	0.11
Boreal	*Total N = 442*	*7.05 (1.50)*	*5.35 (0.61)*	*0.64 (0.06)*	*0.72 (0.06)*	*0.10 (0.07)*
BC1	15	4.25	3.30	0.46	0.51	0.02
CA1	12	4.88	4.16	0.59	0.61	0.13
CA2	7	3.63	3.63	0.48	0.52	0.01
MT1	100	12.13	5.58	0.70	0.73	0.17
OR1	32	9.00	5.29	0.64	0.68	0.23
OR2	17	5.88	4.48	0.62	0.61	0.23
WA1	30	9.25	5.91	0.71	0.77	0.38
WA3	9	5.13	4.64	0.71	0.65	0.29
WA4	29	9.38	5.73	0.67	0.76	0.35
Greater Pacific Northwest	*Total N = 251*	*7.06 (2.94)*	*4.75 (0.94)*	*0.62 (0.09)*	*0.65 (0.10)*	*0.20 (0.13)*
CO1	58	7.75	4.25	0.55	0.57	0.14
UT1	25	4.88	3.66	0.50	0.54	0.27
WY1	77	7.25	4.06	0.53	0.55	0.10
U.S. Rockies	*Total N = 160*	*6.63 (1.53)*	*3.99 (0.30)*	*0.53 (0.03)*	*0.55 (0.02)*	*0.17 (0.09)*

N, number of individuals; A, number of different alleles; AR, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; PAR, population private allelic richness.
Cluster averages and standard deviations (in parentheses) are italicized.

(PAR). Greater Pacific Northwest populations exhibited high diversity and the highest uniqueness of the three major clusters.

Genetic diversity was highest at mid-latitudes (i.e. near 49°N latitude; Fig. 2 and Appendix S8, Supporting information) and increased from west to east across the species' range. For the Greater Pacific Northwest cluster, PAR increased with latitude up to 49°N latitude (Fig. 3). There were no apparent longitudinal trends in PAR.

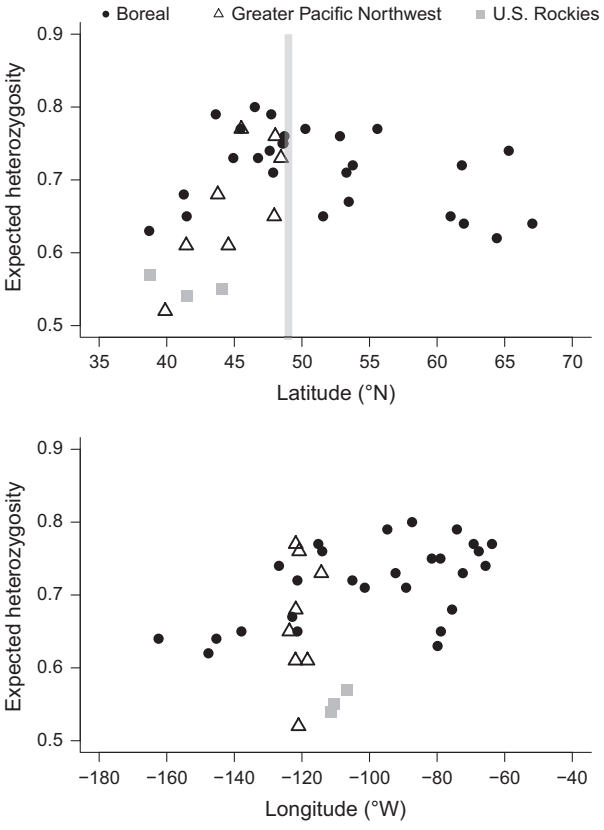

Fig. 2 For each sampled population, expected heterozygosity plotted against latitude (top) and longitude (bottom). The grey vertical bar marks the approximate southernmost latitude of the LGM.

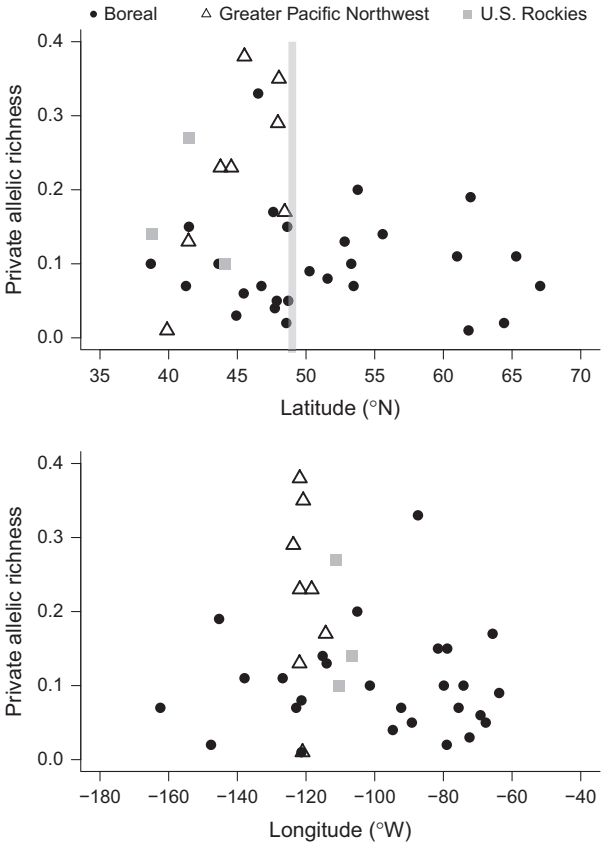

Fig. 3 For each sampled population, private allelic richness (PAR) plotted against latitude (top) and longitude (bottom). The grey vertical bar marks the approximate southernmost latitude of the LGM.

Mitochondrial DNA analysis

The final data set for CR analyses comprised 893 snowshoe hare samples represented by 365 haplotypes. For phylogenetic tree construction, the subset of 80 snowshoe hare Cytb sequences comprised 43 haplotypes.

The best-fit model of nucleotide substitution for Cytb phylogeny was HKY+G. Three highly divergent lineages were identified (Fig. 4), broadly corresponding with the major genetic clusters identified in the first two rounds of STRUCTURE analysis of microsatellites (Fig. 1 and Appendix S4, Supporting information). MtDNA analysis also identified two sublineages (with >95% posterior probability) that corresponded with the finer scale splitting of southwestern populations from hierarchical STRUCTURE analysis: (i) WA3 in Olympic National Park, Washington, was a sublineage of the Greater Pacific Northwest lineage; and (ii) CO1 in Gunnison, Colorado, was a sublineage of the U.S. Rockies lineage. Within the Boreal lineage, a basal group comprised samples from populations near the lineage's southern range (MT1, Montana; MB1, Manitoba; ON1, Ontario; Fig. 4). The Cytb topology indicated that snowshoe hares in the Greater Pacific Northwest lineage are more closely related to black-tailed jackrabbits than to other snowshoe hare populations.

Divergence estimation between the Boreal and U.S. Rockies snowshoe hare lineages is 1.30 mya (95% CI 0.88–1.74 mya). The CO1 sublineage split off from the U.S. Rockies major lineage more recently (0.78 mya; 95% CI 0.42–1.18 mya). The clade comprising most of the snowshoe hares in the Greater Pacific Northwest lineage diverged from BTJR about 0.59 mya (95% CI 0.34–0.90 mya; Fig. 4). The Bayesian skyline plot provided strong evidence of a recent demographic expansion of the Boreal lineage (Fig. 5), whereas expansion of the Greater Pacific Northwest and U.S. Rockies groups was not supported.

NETWORK and SAMOVA analyses, based on the CR gene, accorded with the Cytb phylogeny. In an unrooted median-joining network, the Cytb lineages and sublineages were reciprocally monophyletic and separated from each other by ≥10 CR base pair substitutions (Appendix S9, Supporting information). By these criteria, the OR2 population of Malheur National Forest,

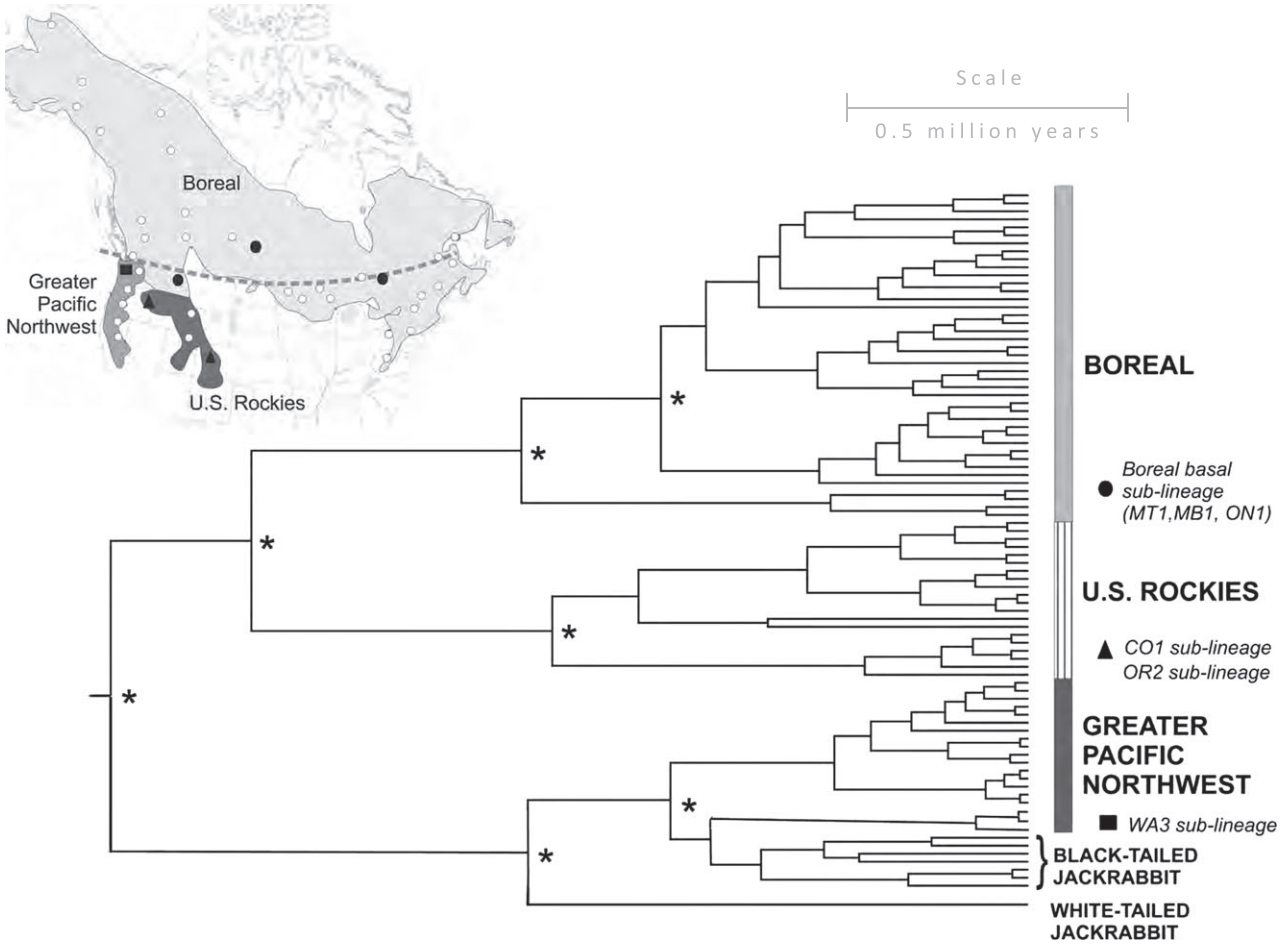

Fig. 4 Snowshoe hare phylogenetic relationships, as constructed in BEAST version 1.7.4 (Drummond *et al.* 2012), based on the Cytb gene. Asterisks (*) indicate lineages and sublineage divisions with ≥95% posterior probability support. Map shows the geographic distribution of the three major Cytb lineages. Filled symbols identify sublineages.

Oregon, was also identified as a distinct sublineage of the U.S. Rockies lineage.

Differences between the Greater Pacific Northwest lineage and all other snowshoe hare lineages explained 77% of total genetic variation in the CR gene (SAMOVA; Dupanloup *et al.* 2002). If the Greater Pacific Northwest snowshoe hare lineage is introgressed from black-tailed jackrabbits, this deep genetic division when $K = 2$ is an artefact of interspecific hybridization rather than snowshoe hare demographic history. Therefore, we also analysed the data with the Greater Pacific Northwest lineage excluded. In this analysis, 66% of variation was explained by differences between four groups ($K = 4$, $P < 0.001$): the Boreal lineage, U.S. Rockies lineage, and the sublineages CO1 and OR2. Control region pairwise F_{ST} was high between and within all lineages except within the Boreal lineage (Appendix S10, Supporting information).

Only two populations (MT1 and WA4; Fig. 1) contained haplotypes that could be ascribed to more than one lineage, with both showing ties to Boreal and Greater Pacific Northwest lineages. Most populations were characterized by high haplotype and nucleotide diversities, with exceptions in British Columbia (BC1, near Vancouver) and in southern populations (CA1 and CA2 in California; UT1 in Utah; WV1 in West Virginia; Appendix S11, Supporting information). Haplotype and nucleotide diversities increased with latitude up to ~49°N latitude (Appendices S12 and S13, Supporting information). MtDNA diversity did not exhibit any clear longitudinal pattern.

Discussion

In this range-wide study of a species distributed across boreal North America, we found that snowshoe hares formed three major genetic groups with well-defined distributions, coincident with patterns observed in other North American boreal species (Arbogast 1999; van Els *et al.* 2012). The entire northern and eastern range of the

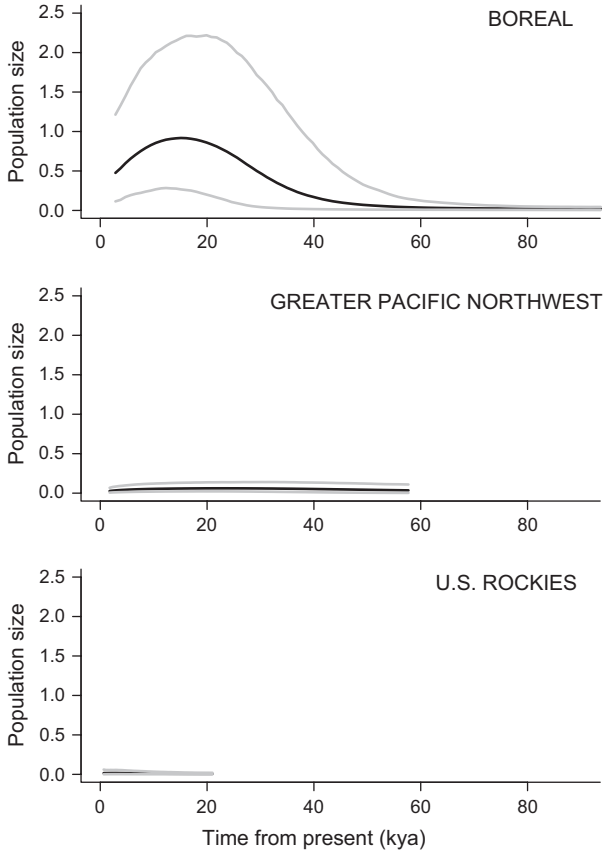

Fig. 5 Bayesian skyline plots (BEAST version 1.7.4; Drummond *et al.* 2012) for three snowshoe hare genetic lineages. Relative population sizes are in units of Ne x mutation rate. Grey lines represent the 95% CI.

snowshoe hare, spanning 6000 km across Canada and the eastern U.S., constituted a single Boreal group characterized by high genetic diversity and gene flow. Two geographically confined groups—in the Greater Pacific Northwest and U.S. Rockies—exhibited lower gene flow and high genetic uniqueness. The three major groups are coherent from microsatellite and mtDNA analyses. Both markers further identified genetic subdivision within the Greater Pacific Northwest and U.S. Rockies, of which the separation of CO1 (Colorado) from the U.S. Rockies group was congruent across all markers and analyses. Modern populations of snowshoe hares likely derived from refugial populations that persisted through the Quaternary ice ages in eastern and southern refugia.

We found high genetic diversity in most sampled populations, but reduced diversity at current range edges, especially for populations at the species' fragmented southern edge. Southern range populations below 49°N had high genetic uniqueness with minimal gene flow with their northern neighbours, suggesting snowshoe hares could lose considerable genetic diversity if southern boreal habitats are lost.

Evolutionary history and refugial origin

This work revealed strong genetic structure at different hierarchical levels and a remarkable coincidence of the inference of three major geographically explicit groups of snowshoe hares based on mtDNA sequences and microsatellite data. Further, these markers also coincide in the suggestion of additional genetic fragmentation in the species' southwestern range. Based on the mtDNA phylogeny, we estimated divergence of the three major groups to be 1.30–0.78 mya, long before the height of the LGM ~18 kya. Regional mixing among groups was sufficiently low during subsequent interglacial warm periods, including the current one, that deep genetic divisions are still maintained in the mtDNA and microsatellite data.

Many co-occurring forest species for which continent-wide genetic data are available share this phylogeographic pattern—a large genetic cluster across Canada and the eastern U.S. and one or more smaller genetic clusters in the western USA. Examples include the gray jay (*Perisoreus canadensis*, van Els *et al.* 2012), northern flying squirrel (*Glaucomys spp.*, Arbogast 1999), black bear (*Ursus americanus*, Wooding & Ward 1997) and hairy woodpecker (*Picoides villosus*, Klicka *et al.* 2011). Genetic groups in these species diverged an estimated 2.97–0.69 mya, a range that encompasses our divergence estimates for the snowshoe hare.

Boreal snowshoe hare lineage. The height of the LGM in North America occurred ~18 kya, and by 6 kya, the glaciers had largely disappeared (Pielou 1991). Given the Boreal lineage diverged from other snowshoe hare lineages an estimated 1.30 mya, colonization of newly available boreal habitats after the LGM must have occurred primarily from refugial populations within the Boreal lineage. We had hypothesized a Beringian refugium for snowshoe hares, as reported for several other North American boreal species (Shafer *et al.* 2010b), but we did not find genetic diversity or uniqueness patterns indicative of a major Beringian refugium for snowshoe hares. A few snowshoe hare fossils are documented from Alaska and Yukon from 20 to 10 kya, but the majority of hare fossils from this period are from the lower 48 U.S. states (FAUNMAP Working Group 1994). Thus, any relict snowshoe hare populations that survived the LGM in Beringia may have been too small or isolated to be heavily represented in contemporary snowshoe hare genetic structure.

Instead, genetic patterns suggest that the Boreal lineage primarily expanded from refugia near the southern

edge of the ice sheets and from eastern refugia. This idea is supported by the basal position of snowshoe hare Cytb haplotypes sampled from locations close to current southern limits of the Boreal lineage. In addition, microsatellite diversity was highest in eastern populations of the Boreal lineage. Results are consistent with fossil pollen data, which indicate that at the LGM, boreal forests in North America persisted in at least two major pockets—the Pacific Northwest and the southeastern USA (Williams *et al.* 1993).

The overall high genetic diversity through much of the Boreal lineage, and the significant pattern of IBD across the Boreal lineage range, suggests cross-continental expansion may have proceeded slowly or from a broad refugial front (Hewitt 1996). Signals of demographic expansion revealed in the Bayesian skyline plot indicate Boreal lineage expansion may have begun ~48 000 years ago.

Greater Pacific Northwest and U.S. Rockies snowshoe hare lineages. The Greater Pacific Northwest and U.S. Rockies lineages occur in the species' southwest range, which was largely ice free during the Pleistocene. The high genetic uniqueness and strong genetic subdivisions in mtDNA of these lineages indicate that they arose from at least two discrete refugia. Comparative phylogeographic studies have identified the northwestern USA as an area of exceptionally high genetic differentiation for boreal and temperate species, due to the complex physiography of the region and its relative stability as a glacial refugium (Soltis *et al.* 1997; Swenson & Howard 2005; Shafer *et al.* 2010b). Our Bayesian estimates of temporal fluctuations of effective population size suggested these evolutionary groups remained relatively stable through evolutionary time (Fig. 5).

The mtDNA analyses indicate the Greater Pacific Northwest snowshoe hares are more closely related to black-tailed jackrabbits than to other snowshoe hares. Two competing hypotheses may explain this result: (i) mitochondrial DNA introgression (through hybridization) from *Lepus californicus* into *Lepus americanus* in the southwestern range of the latter or (ii) retention of an ancestral polymorphism shared between the two species (Moore 1995). Extensive mtDNA introgression occurs among other species of hares, resulting from ancient or current contacts among species and sometimes causing extensive replacements of lineages (Alves *et al.* 2008). Even though the geographic restriction of the shared variants and the remarkably close phylogenetic relationship with current *L. californicus* variants support the introgression hypothesis, the inference of such phenomena would require reconstruction of the speciation history of the taxa, using genealogical information from nuclear loci (Melo-Ferreira *et al.* 2012). It is nevertheless important to note that if introgression caused this interspecific sharing of lineages, it was remarkably pervasive and may have completely replaced the mtDNA variation in the Greater Pacific Northwest evolutionary group identified using microsatellites.

Genetic diversity of core vs. peripheral populations

Genetic diversity of snowshoe hares was highest in mid-latitude populations, near the southernmost edge of the LGM. From here, diversity clearly decreased towards the south and less dramatically towards the north. The southern range edge for snowshoe hares is highly impacted by natural and anthropogenic habitat fragmentation (Hansen *et al.* 2010). The observed genetic pattern is consistent with the core-periphery hypothesis, with populations in the fragmented southern periphery exhibiting the lowest genetic diversity and gene flow. Further, high amplitude population fluctuations may promote gene flow and genetic diversity (Ehrich *et al.* 2009). Snowshoe hare populations across their northern range undergo large population cycles, whereas southern populations may have reduced cyclicity (Hodges 2000).

Anticipated genetic consequences of southern population loss

Our study provides important insights on how potential loss of southern hare populations (below 49°N) may affect genetic diversity. The strong genetic subdivisions and uniqueness of snowshoe hare populations suggest that anticipated fragmentation and loss of these habitats due to climate change and human activities may greatly reduce overall species genetic diversity, with possible negative implications for future adaptive potential. For example, we identified at least three snowshoe hare evolutionarily significant units (ESUs), using Moritz's (1994) criteria of reciprocal monophyly for mtDNA and significant divergence in the frequencies of nuclear alleles. Two ESUs occurred wholly in the species' southern range. Three snowshoe hare sublineages, reciprocally monophyletic and separated from each other by ≥10 CR base pair substitutions, were also found in the southern range. Additional ESUs may occur in parts of the southern range not sampled in this study: for example, we did not sample hares in New Mexico or in northern Idaho, an area with high endemism hypothesized to be the 'Clearwater refugium' (Daubenmire 1975; Soltis *et al.* 1997).

A limitation of this study is its reliance on neutral genetic variation, without complementary information on adaptive potential, for identifying ESUs (Funk *et al.*

2012). We identified ESUs on the basis of Moritz's definition because it can be operationally applied from neutral genetic markers (de Guia & Saitoh 2007). Other definitions of ESU emphasize conserving adaptive variation, by incorporating adaptive genetic variation, life history traits, morphology and species distribution (Ryder 1986; Vogler & DeSalle 1994). An additional question that should be addressed is, 'How much would loss of southern populations impact the species' ability to adapt to global warming?' Such studies would require evaluation of quantitative genetic trait variations directly linked to traits with adaptive value under altered climate regimes. For snowshoe hares, adaptive variation may include phenology of seasonal coat colour moult confronting decreased snow pack, especially in the southern part of the range (Mills *et al.* 2013).

Concomitant with the predominantly southern distribution of ESUs and sublineages, a large proportion of snowshoe hares' neutral private allelic richness (PAR) occurs in the U.S. Rockies and Greater Pacific Northwest, where isolation and relative stability over evolutionary time were likely responsible for their accumulation of mutations and unique genetic structure. On average, populations in the U.S. Rockies and Greater Pacific Northwest lineages had almost twice the PAR of populations in the Boreal lineage. In contrast to the highly connected Boreal populations, loss of a population in the U.S. Rockies and Greater Pacific Northwest lineages could mean complete loss of many unique alleles. At neutral markers, this loss would not be a major conservation concern, but it portends an analogous loss of diversity at evolutionarily significant loci.

The high genetic structure and uniqueness in the southern range of the snowshoe hare reflect a common phylogeographic pattern among North American species. Regional comparative studies emphasize that the Pacific Northwest and U.S. Rockies are hotspots of genetic diversity for many species (Soltis *et al.* 1997; Swenson & Howard 2005; Shafer *et al.* 2010a). Although there are few rangewide studies for boreal species, they typically corroborate the cryptic genetic distinctiveness of these southern populations in the context of the species' entire North American range (Wooding & Ward 1997; Arbogast 1999; Arbogast & Kenagy 2001; Klicka *et al.* 2011; van Els *et al.* 2012). Collectively, these findings support Hampe and Petit's (2005) call for prioritizing conservation of southern edge populations of boreal species.

For snowshoe hares and many other boreal species in North America, southern populations may already be losing genetic diversity due to anthropogenic change such as habitat fragmentation (desert bighorn sheep, *Ovis canadensis nelsoni;* Epps *et al.* 2005) and climate change (alpine chipmunk, *Tamias alpinus;* Rubidge *et al.* 2012). The range of snowshoe hares has contracted northward throughout the previous century, primarily related to habitat loss and conversion, with potential contributions from harvest and climate change (Hodges 2000; NatureServe 2014). Populations in West Virginia, North Carolina, Tennessee and Virginia have declined. Snowshoe hares are extirpated from Ohio, New Jersey and North Carolina and possibly extirpated from Maryland (NatureServe 2014). They are listed as critically imperilled (S1) in Virginia, imperilled (S2) in New Mexico and vulnerable (S3) in Pennsylvania, Utah and Nevada. In California, the subspecies *L. a. tahoensis* is a state-listed Species of Special Concern.

In the face of certain climate change with uncertain impacts, it is difficult to predict how species conservation efforts can best be prioritized to maximize long-term persistence. Although we cannot anticipate the unforeseen, we can use our understanding of the present to heed the advice of geneticist Otto Frankel (1974) that 'at this point of decision-making it may be our evolutionary responsibility to keep evolutionary options open so far as we can'. Using historical processes as a guide, an emphasis on conserving southern edge populations seems prudent for this strongly interacting prey species.

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E.C., L.S.M. and K.E.H. designed the research; E.C. conducted field sampling and laboratory work; L.S.M. and K.E.H. contributed some hare genetic samples; P.C.A. and J.M.F. provided laboratory training and assistance; E.C. and J.M.F. analysed data and wrote this work with direction, assistance and editorial review from L.S.M., K.E.H. and P.C.A.

Data accessibility

All Cytb and CR sequences from this project have been deposited in GenBank (Accession nos KF781351–KF781437; KF804153–KF805042; HM771306–HM771308). Sample details, sequence alignments, microsatellite genotypes, and input files have been deposited in the Dryad Digital Repository (Conservation implications of the evolutionary history and genetic diversity hotspots of the snowshoe hare. http://dx.doi.org/10.5061/dryad.dh63p).

Supporting information

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

Appendix S1 Sources of genetic samples analysed in this study.

Appendix S2 Microsatellite loci diversity.

Appendix S3 DNA extraction and genotyping methods.

Appendix S4 STRUCTURE hierarchical analysis results under a model of admixture and uncorrelated allele frequencies.

Appendix S5 Analysis of the Boreal cluster in the second round of STRUCTURE hierarchical analysis.

Appendix S6 Highest mean posterior density Geneland results for a model with uncorrelated allele frequencies and null alleles filtered.

Appendix S7 Pairwise F_{ST} and Nei's D calculated across eight microsatellite loci.

Appendix S8 Population allelic richness plotted against latitude and longitude.

Appendix S9 Mitochondrial control region median-joining network.

Appendix S10 Mitochondrial control region pairwise $F_{ST.}$

Appendix S11 Mitochondrial control region diversity statistics.

Appendix S12 Haplotype diversity plotted against latitude and longitude.

Appendix S13 Nucleotide diversity plotted against latitude and longitude.