

Demographic history shapes North American gray wolf genomic diversity and informs species' conservation

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Research Article

Keywords:

Posted Date: September 6th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3280309/v1>

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Abstract

Effective population size estimates are critical information needed for evolutionary predictions and conservation decisions. This is particularly true for species with social factors that restrict access to breeding or experience repeated fluctuations in population size across generations. Further, if isolated, the only natural process that introduces new variation into the gene pool is *de novo* mutation rate. We investigated the genomic estimates of effective population size along with diversity, subdivision, and inbreeding from 81,595 RADseq SNPs genotyped in 437 gray wolf samples from North America collected between 1986 and 2021. We found genetic structure across North America, represented by three distinct demographic histories of western, central, and eastern regions of the continent. Further, gray wolves in the northern Rocky Mountains have lower genomic diversity than wolves of the western Great Lakes and has declined over time. Effective population size estimates revealed the historical signatures of continental efforts of predator extermination, despite a quarter century of recovery efforts. We are the first to provide molecular estimates of effective population size across distinct gray wolf populations in North America, which ranged between $N_e \sim 141-226$ since 1990. We provide data that informs managers regarding the status and importance of effective population size estimates for gray wolf conservation, which are on average 5.2–9.3% of census estimates for this species. We show that while gray wolves fall above minimum effective population sizes needed to avoid extinction due to inbreeding depression in the short term, they are below sizes predicted to avoid long-term risk of extinction.

Introduction

The theory of the effective population size (N_e) was originally developed by Sewall Wright (1943, 1965) to provide a means for comparing structure across seemingly disparate populations to result in an estimate that represents an idealized population of randomly mating individuals (Crow & Kimura 1970). Thus, social organization and non-random breeding will impact the distribution of genotypes over geographic space and concomitantly N_e estimates. Any factor that results in deviations from random breeding (e.g., social factors, breeding strategies, site availability) or changes population size across generations will result in an effective population size estimate that is a fraction of the census size (N) (Keller & Reeve 1994; Hedrick and Kalinowski 2000; Clutton-Brock 2016; Charlesworth and Willis 2009). For species with high reproductive skew and social structures that repress reproduction in subdominant ranks, the effective population size estimate inferred from sex ratios, dispersal or migration rates, number of reproductive individuals, or genetic assessments is critical information needed for evolutionary predictions (Lanfear et al. 2014; Wang et al. 2016).

Population sizes fluctuate over time, either through natural process or due to anthropogenic activity such as wildlife management (Rowe & Beebee 2004). Any reduction in size, compounded with isolation, will erode genetic variation via random genetic drift to a degree that depends on the severity and duration of these bottlenecks (Fisher 1958). Without inter-population connectivity, the only process that naturally introduces new variation into the gene pool is *de novo* mutations. New mutations are more likely to quickly drift to fixation in isolated small populations, resulting in continuing low levels of genetic diversity

(Fisher 1930; Wright 1931; Coyne et al. 1997; Wade & Goodnight 1998). The potential for a population to respond to evolutionary challenges deteriorates as genomic variation dwindles, thereby limiting adaptive outcomes (Frankham 2005; Allendorf 2016; Hoffmann et al. 2017; Lopez-Cortegano et al. 2019). Anthropogenic effects that reduce population size and impact life history events central to individual-level fitness (e.g., reproduction, dispersal) are well known to degrade genomic variation and adaptive potential (Frankel & Soulé 1981; Reed & Frankham 2003; Frankham 2005; Allendorf et al. 2008; Coltman 2008).

In their recent evolutionary history, gray wolves (*Canis lupus*) in North America have been eradicated from much of their southern continental range through federal and state programs first implemented during the mid-19th century. These programs were highly effective and by the late 1950s had exterminated the wolf from the conterminous United States except for a few individuals on Isle Royale National Park in Lake Superior (Minnesota) and a few hundred individuals in northeastern mainland Minnesota (Young & Goldman 1944; Peterson 1955; Kolenosky & Standfield 1975; Parker 1995; Franzmann & Schwartz 1997; Boitani 2003), and throughout the majority of their historic range by the mid-20th century. In the face of a near total elimination, coupled with social structure of the species and removal of dispersers, there was a growing concern regarding the future survival of the gray wolf species which led to management actions to increase their numbers and distribution, including reintroduction in Yellowstone National Park (YNP) and central Idaho (Adams et al. 2008; Brainerd et al. 2008; Treves et al. 2016; Rick et al. 2017). A targeted study of wolves living within YNP reported a significantly smaller effective population size than the censused population (vonHoldt et al. 2008), emphasizing the critical role of population connectivity to combat genetic drift, inbreeding, and erosion of heterozygosity (Gese & Mech 1991; Mech & Boitani 2003; Jedrzejewski et al. 2005; Allendorf et al. 2008; vonHoldt et al. 2008).

In the United States, gray wolves are managed as three populations with distinct demographic histories: Northern Rocky Mountains, the western Great Lakes, and Southwestern (explicitly for the Mexican wolf *C. l. baileyi* subspecies) regions. Gray wolves in the northern Rocky Mountains were extirpated by the 1920s and were listed under the Endangered Species Act (ESA) in 1973. As such, all gray wolves in the lower 48 United States range were listed as endangered, with the exception of gray wolves living in Minnesota that were listed as threatened. There was no national gray wolf recovery plan; rather, each population received their own unique plan. The northern Rocky Mountain Wolf Recovery Plan (NRMWRP) outlined gray wolf recovery by supporting natural colonization and wolf translocation to central Idaho and Wyoming's YNP, which occurred during the winters of 1995 and 1996 when 66 wolves from Alberta and British Columbia founded two reintroduction areas (59 FR 60266; USFWS 1987). Dispersers from YNP expanded into adjacent Montana, Idaho, and Wyoming counties (collectively referred to as the Greater Yellowstone Ecosystem), and dispersers from central Idaho expanded into adjacent Montana, Wyoming, and Oregon. Beginning in the late 1990s, periodic dispersing wolves from southern British Columbia and the northern Rocky Mountains were documented in the Pacific Northwest states of Washington, Oregon, and northern California. By 2011, the first wolf entered Oregon with confirmed reproduction in 2015. The western Great Lakes population is composed of the eastern portion of the Dakotas, Minnesota, Iowa, Wisconsin, a northern portion of Illinois, and Michigan (lower and upper peninsula). Gray wolves in Minnesota were first protected under the ESA in 1974, with subsequent expansion into Wisconsin and Michigan by the

early 1990s (Refsnider et al. 2009). The Timber Wolf Recovery Plan further considered the historic range to Minnesota eastward to Maine and south to the northern portion of Florida (Refsnider et al. 2009). The southwestern population that encompasses the endangered Mexican gray wolf subspecies was not included in this study.

Effective in January 2021, the U.S. Fish and Wildlife Service (FWS) delisted gray wolves everywhere in the lower 48 United States, except for the Mexican wolf subspecies (final rule 85 FR 69778). On February 10, 2022, a US District Judge vacated the rule and restored ESA protections for all gray wolves in the lower 48 United States except for the Northern Rocky Mountain region. The northern Rocky Mountains wolves were delisted per Congressional action that included a shielding of the action from judicial review. The species remains under their respective states' jurisdictions. The delisting decision relied in part on the lack of information from FWS that the western Great Lakes population could indeed be self-sustaining without federal protection. By January 2023, the Circuit Mediator issued an order temporarily staying the appeals during which a status review for the gray wolf will be conducted.

Our goal was to assess the temporal and spatial variation in genetic signatures over the recent decades of gray wolf protections and recovery across portions of North America and provide information to consider for long-term viability of gray wolves as it pertains to their ESA listing status in the United States. We conducted this genomic surveillance across the North American continent to showcase how demography and genomic signatures are intertwined. This assessment provides a contemporary assessment of genetic parameters important to genomic viability across geographic and regulatory scales for integration into conservation goals for a social carnivore species.

Materials and Methods

Sample collection and genomic library construction

We obtained archived blood or tissue samples collected from 482 gray wolves across their continental range in North America (Canada = 91, USA = 391) from state and federal partners, local trappers, and private genetic collections (Fig. 1A, Table S1). Locations of sample origins varied, from regional identification to counties, parks, or states and provinces. We partitioned samples into two levels of geographic resolution, regional and U.S. managed populations. For the U.S. managed populations, we define the "northern Rocky Mountains" (abbreviated as RM) composed of samples that originated from California, Idaho, Montana, Washington, and Wyoming. We define Michigan, Minnesota, Wisconsin to compose the "western Great Lakes" (abbreviated as GL).

We extracted genomic DNA following manufacturer's protocol (Qiagen DNeasy Blood and Tissue kit). We used the Qubit fluorometer system for DNA quantification to standardize the input amount for use in the modified restriction-site associated DNA sequencing (RADseq) capture protocol (Ali et al. 2015). Briefly, we digested genomic DNA with *SbfI* with a subsequent ligation of unique 8-bp barcoded biotinylated adapters to permit the pooling of 48 DNA samples into a single library. We randomly sheared each library

to 400bp in a Covaris LE220 followed by an enrichment for the adapter ligated fragments using a Dynabeads M-280 streptavidin binding assay. We then prepared the enriched libraries for paired-end (2x150nt) Illumina NovaSeq 6000 sequencing at Princeton University's Lewis-Sigler Genomics Institute core facility using the NEBnext Ultra II DNA Library Prep Kit (New England Biolabs). For any step of purifying or size selection of DNA, we used Agencourt AMPure XP magnetic beads (Beckman Coulter).

Bioinformatic processing

We retained sequence read pairs that contained both our known unique barcodes and remnant *Sbfi* recognition site, which were processed in *STACKS*v2.6 (Catchen et al. 2013; Rochette et al. 2019). We used the *process_radtags* module to rescue our barcoded reads with a 2bp mismatch and excluded reads with a quality score < 10. We next removed PCR duplicates in the *clone_filter* module followed by mapping to the reference dog genome CanFam3.1 assembly (Lindblad-Toh et al. 2005) using *bwa-mem* (Li 2013a). We also included the Y chromosome (KP081776.1; Li et al. 2013b) with the CanFam3.1 reference assembly. After alignment, we excluded mapped reads with MAPQ < 20 and then converted the SAM files to BAM format in *Samtools* v0.1.18 (Li et al. 2009). We implemented the *gstacks* and *populations* modules in *STACKS*v2 with an increase in the minimum significance threshold in *gstacks* and used the maximum-likelihood marukilow model that incorporates uncertainties for low-coverage data (*-vt-alpha* and *-gt-alpha* with $p = 0.01$). We additionally used the flag *-r 60* to retain only newly annotated sites found in at least 60% of the samples in the catalog. In *VCFtools* v0.1.17 (Danecek et al. 2011), we estimated the pre-filtered sequence coverage and then subsequently filtered loci to exclude singleton and private doubleton alleles, removed loci with more than 90% missing data across all samples, and excluded individuals with more than 30% missing data. We removed loci with a minor allele frequency (MAF < 0.03) and required at least an 80% genotyping rate per locus (*-geno 0.2*) in *PLINK* v1.90b3i (Chang et al. 2015).

We used *VCFtools* for individual-level metrics of heterozygosity (observed, H_0 ; expected, H_E) and the two-sample Kolmogorov-Smirnov to test for statistical differences in data distributions and correlations in R (R 2022). We then utilized the *populations* module in *STACKS*v2 to identify alleles private to each canid lineage. We further conducted a rarefaction method for private allele richness per locus while controlling for sample size variation in the number of genomes sampled in the program *ADZE* (Szpiech et al. 2008) with the parameter *G* of sample size set to 100.

Sex inference from sequence coverage of the Y chromosome

As we included the Y chromosome (KP081776.1; Li et al. 2013b) with the CanFam3.1 reference assembly for read alignment, we used t-tests and the two-sample Kolmogorov-Smirnov to determine the sequence coverage differences between the sexes. This provided us an opportunity to establish a threshold of Y-specific sequence coverage to infer sex, with females inferred from falling below the threshold and males above. We then repeated analyses independently for each sex to explore the impact of sex-biased demography.

Population structure and differentiation

For demographic analyses, we constructed a statistically neutral and unlinked dataset of SNPs by excluding sites within 50-SNP windows that exceeded genotype correlations of $r = 0.2$ (*-indep-pairwise* 50 5 0.2; a proxy for linkage disequilibrium or LD) and SNPs that significantly deviated from Hardy-Weinberg Equilibrium (HWE) with the argument *-hwe* 0.001. We conducted both non-model and model-based clustering analysis. We completed the former as a principal component analysis (PCA) in *FlashPCA v2.1* (Abraham et al. 2017) and the latter with an unsupervised maximum likelihood framework with *Admixture* (Alexander et al. 2009). We analyzed the fit of two to 10 partitions (K) with the cross-validation error (*cv*) flag. We also estimated inter-group pairwise genetic differentiation as Weir and Cockerham's F_{ST} in *VCFtools* v0.1.17. We reported average F_{ST} across the genome (autosomes and X chromosome combined).

Inbreeding estimates from autozygosity

We analyzed the minimally filtered SNP set separately for loci on the autosomes and X chromosome. These loci represented a total length (L_{genome}) of 2,202,059,258 and 123,842,264 nucleotides for autosomes and the X chromosome, respectively. The geographic region was used as an identifier for the function *homozyg* in *PLINK* v1.9 (Table S1). To detect autozygosity from runs of homozygosity (ROH), we used the following parameters for low coverage data: homozyg-density 50, homozyg-gap 1000, homozyg-kb 300, homozyg-snp 50, homozyg-window-het 4, homozyg-window-missing 5, homozyg-window-snp 50, and homozyg-window-threshold 0.05 (Ceballos et al. 2018). We converted the ROH segments to an individual-level inbreeding coefficient (F_{ROH}) following Taboada et al. (2014):

$$F_{ROH} = \frac{\sum L_{ROH}}{L_{genome}}$$

where L_{ROH} is the length of an ROH segment in an individual.

Effective population size estimates

We estimated effective population (N_e) sizes and focused on recent (past 200 generations) estimations as presumed to be more accurate. We assumed that the populations analyzed were historically qualitatively similar up to 200 generations ago. Effective population size estimates extrapolate population parameters from genetic diversity metrics. Although dispersal and translocation events are known, the collection of genetic variation is the core of such inference and is bounded by how a population is defined in time and space. Here, we implemented the algorithm in *GONE* (Santiago et al. 2020), which is an LD-based method that accounts for drift (i.e., finite sample size) and makes use of recombination rates but is influenced by both population structure and admixture. *GONE* leverages a genetic algorithm from Mitchell (1998) to search across sequences of possible historical effective

population sizes that best explain the spectrum of observed LD values to minimize the sum of squares of the differences between observed and expected allelic covariances. We estimated N_e sizes at two levels: each major geographic region and population designations for management implications in the United States. However, resulting estimates for the wolf populations in Canada should be interpreted with caution given our limited genotype surveillance across the region. We estimated N_e from autosomal SNP data and translated generations into years using 4 years per generation as the unit of time (vonHoldt et al. 2008; Mech et al. 2016). We believed that only the minimally filtered RADseq data (i.e., missingness and MAF) was appropriate for these estimates used by SFS-based methods (Beichman et al. 2017).

Admixture is part of the history of the western Great Lakes gray wolf population

We rediscovered SNPs with the addition of BAM files from previously published canids: 106 reference western coyotes (*C. latrans*) from vonHoldt et al. (2022) and 30 reference eastern wolves (*C. lycaon*) from Heppenheimer et al. (2018) (Table S1B). The gray wolves in the Great Lakes region are known to have a history of admixture with both coyotes and eastern wolves, with (vonHoldt et al. 2010a; Heppenheimer et al. 2018). The predominant signal described to date is that Great Lakes region gray wolves have partial coyote ancestry with gray wolves of southeastern Ontario carrying more partial ancestries of eastern wolves. These were merged with the BAM files from the population of northern Rocky Mountains and western Great Lakes samples to explore the impact of coyote and eastern wolf admixture on gray wolf genetic estimates. We followed the same analysis and filtering methods as described above to obtain a statistically unlinked and neutral set of SNP loci. We conducted an unsupervised assignment analysis for $K = 2-10$ in *ADMIXTURE*, complemented with genetic differentiation (F_{ST}) estimates using *VCFtools* v0.1.17.

Reliable inferences from reduced representation low-coverage population-level genotype data

Population genomic studies can leverage the affordable technologies of reduced representation data collection methods, such as RADseq, to collect genotype data from hundreds or thousands of individuals. The drawbacks are obvious in terms of missing rare alleles or allele dropout rates due to the nature of the library preparation. Thus, studies have assessed the biases and challenges of low-coverage data (3-6x) compared to whole genome sequence (WGS) and found that the former can be equally informative with careful adjustments to methods and inferences (Ceballos et al. 2018; Duntsch et al. 2021). It is known that some population metrics like ROH are expected to be biased. For example, low-coverage data likely underestimates the frequency of small and overestimates larger ROH fragments (Lavanchy & Goudet 2023).

Results

We sequenced 482 gray wolf samples from North America, collected between 1986 and 2021 when known, with an average fold sequence coverage of $7.3(\pm 3.4)$ to discover 1,099,764 raw, RAD loci that passed our *STACKS* filtering parameters but prior to population level filtering (Table S1). We excluded 45 wolves due to high (> 20%) missingness and repeated the filtering. The result is a dataset of 162,109 SNPs genotyped in 437 wolves gray wolves from Canada (n = 92) and the United States (n = 345), with a subset of 81,595 statistically neutral and unlinked SNPs.

We inferred sex for individuals bioinformatically based on the depth of reads mapped to the Y chromosome. Of the 437 wolves, field-based observations identified 104 females and 118 males. When we presumed these samples having correct sex inference, the average sequence depth on the Y chromosome was significantly enriched in males (females = 3406.9, males = 25587.3, 1-tailed t-test of unequal variance $t = -17.99$, $df = 219.7$, $p < 10^{-16}$) and these two distributions are significantly different (Two-Sample Kolmogorov-Smirnov $D = 0.802$, $p < 10^{-16}$) (Fig. S1A). We inferred 205 females that had a sequence coverage < 10,000x (average Y-chromosome sequence coverage = 594.2) and 232 males with > 18,000x (average coverage = 28454.1), where these two inferred sequence coverage distributions were again statistically divergent (Two-Sample Kolmogorov-Smirnov $D = 1.0$, $p < 10^{-16}$) (Fig. S1B).

Gray wolves are genetically and geographically structured across North America

We presented two levels of genetic structure across the North American continent that reflect the geographic assignment probabilities for two cluster analyses: the PCA (K = 3) and the best supported partition from maximum-likelihood inference (K = 9) (Fig. 1B-C, Fig. S2). Three genetic clusters broadly represent three distinct demographic histories of western, central, and eastern regions of the continent. We divided the western cluster into two subclusters, one to reflect the shared demography of southwestern Canada and western USA through the translocation and colonization of wolves in the northern Rocky Mountains population, and the other representing northern Canada (Table S1). The other two clusters represent northern Quebec and the shared demography of Ontario and the western Great Lakes population (Table S1). Out of these four geographic groupings, we found that only two groups carried private alleles (western USA and southwestern Canada, n = 332; Great Lakes and Ontario, n = 6,801) out of 162,109 SNPs. A rarefaction analysis mirrors the demographic history of each, with the Great Lakes and Ontario regional group showing the highest level of allele richness and mean number of private alleles per locus controlled for sample size differences (Fig. 1D), likely due to their known history of coyote and eastern wolf admixture (Koblmüller et al. 2009; vonHoldt et al. 2016). Finer-scale clustering revealed a stronger role of geographic isolation, with more resolution of substructure within USA's northern Rocky Mountains and the Pacific Northwest regions (Fig. 1C). The shared assignments across three genetic partitions reflect the shared genetic ancestry across large geographic distances due to the translocation of gray wolves in 1995 and 1996 (British Columbia, Alberta, and Montana) to central Idaho and the Greater Yellowstone Ecosystem (mean Q : partition 1 BC = 0.43, ID = 0.14, GYE = 0.22; partition 2 BC = 0.25, ID = 0.40, GYE = 0.07; partition 3 BC = 0.09, ID = 0.13, GYE = 0.65). Populations with shared

demographic histories (northern Canada versus western USA and southwestern Canada, $F_{ST}=0.034$) had the lowest levels of genetic differentiation while the highest was found between opposite coasts of the continent (western USA and southwestern Canada versus northern Quebec, $F_{ST}=0.084$) (Table 1, Fig. S3). We find that all genetic differentiation distributions are significantly distinct (Table S2). We assessed this metric for females and males separately for two geographic regions (western USA and southwestern Canada; Great Lakes and Ontario). While northern Rocky Mountain gray wolves showed variable levels of differentiation within the region (F_{ST} genome = 0.0-0.13, $X = 0.0-0.09$), females were significantly higher levels of genome-wide differentiation to other females (female-female $F_{ST}=0.052$) than males (male-male $F_{ST}=0.032$, 1-tailed t-test of unequal variance $p = 0.01207$) (Fig. S4A). In contrast, western Great Lakes gray wolves had much lower intra-region genetic differentiation (F_{ST} genome = 0.0-0.03, $X = 0.0-0.04$), with no significant differences between males and females (F_{ST} female-female = 0.017, male-male = 0.019, $p = 0.3242$) (Fig. S4B).

Table 1

Average and weighted Weir and Cockerham estimates (above and below diagonal, respectively) of genetic differentiation (F_{ST}) across 81,595 SNPs between geographic regions of gray wolves (see Fig. 1A for population abbreviations).

Geographic group (n)	Population(s)	Northern Canada	Western USA and southwestern Canada	Northern Quebec	Great Lakes and Ontario
Northern Canada (42)	NT, NU, YT	–	0.034	0.052	0.052
Western USA and southwestern Canada (182)	AB, BC, CA, ID, MT, OR, WA, WY	0.033	–	0.084	0.056
Northern Quebec (24)	QC	0.071	0.094	–	0.073
Great Lakes and Ontario (189)	MI, MN, ON, WI	0.054	0.065	0.090	–

Genomic diversity and inbreeding coefficients are variable across continental North America

Northern Canada gray wolves had the highest levels of expected heterozygosity estimates, significantly higher than the western USA and southwestern Canada (Two-sample KS test $D = 0.289$, $p = 0.0066$) as well as the Great Lakes and Ontario ($D = 0.365$, $p = 0.0002$) (Table 2A). Observed heterozygosity estimates, however, were significantly lower than expected for each comparison (Table 2A). Northern Canada had the highest observed heterozygosity across all four geographic regions with statistical significance to all except the Great Lakes and Ontario region ($D = 0.228$, $p = 0.0570$) (Table 2A). Finally, western USA and southwestern Canada carried significant higher observed heterozygosity than northern Quebec ($D = 0.392$, $p = 0.0030$) (Table 2A).

Table 2. Average expected and observed heterozygosity (H_E and H_O , respectively) and effective population size (N_e) estimates (averaged estimates from 1986-2021) from 81,595 statistically neutral and unlinked SNPs for each **A)** major geographic location (p -values are from a two sample KS test between H_E and H_O) and **B)** regional population within the United States. (Abbreviations: n, sample size)

A)

Geographic group (n)	H_E	H_O	KS D, p	N_e
Northern Canada (42)	0.217	0.209	D=0.429, $p=0.000893$	229.0
Western USA and southwestern Canada (182)	0.215	0.203	D=0.560, $p=2.2 \times 10^{-16}$	141.4
Northern Quebec (24)	0.217	0.201	D=0.958, $p=5.4 \times 10^{-10}$	221.4
Great Lakes and Ontario (189)	0.214	0.206	D=0.381, $p=2.5 \times 10^{-12}$	314.8

B)

Population (n)	H_E	H_O	KS D, p	N_e
Northern Rocky Mountains (176)	0.215	0.202	D=0.568, $p=2.2 \times 10^{-16}$	3230.1
California (4)	0.216	0.198		
Idaho (43)	0.216	0.211		
Montana (12)	0.217	0.193		
Oregon (21)	0.214	0.208		
Washington (15)	0.214	0.198		
Wyoming (81)	0.215	0.198		
Western Great Lakes (169)	0.214	0.205	D=0.438, $p=1.7 \times 10^{-14}$	4080.0
Michigan (49)*	0.213	0.193		
Minnesota (64)	0.215	0.212		
Wisconsin (56)	0.213	0.207		

* Includes gray wolves from Isle Royale National Park in Lake Superior

The X chromosome evolves at a different rate than autosomes, with full exposure to natural selection in the haploid state when carried by males. We found that inbreeding levels for autosomes across the 437 gray wolves carried significantly higher inbreeding estimates than the X chromosome (F_{ROH} , autosome = 0.25, X = 0.07, Welch Two Sample $t = 27.9$, $df = 782.1$, $p = 2.2 \times 10^{-16}$) (Fig. 1E). There was an overall

negative linear relationship between effective population size and autosomal inbreeding estimates ($R=-0.9$, $p = 0.1$). We further report the expected positive correlation between the number of autosomal ROH segments and inbreeding estimates ($R = 0.77$), with a weaker yet similar trend for the X chromosome ($R = 0.44$). Autosomal inbreeding levels were highest in the wolves of western USA and southwestern Canada ($F_{ROH}=0.296$), which were not significantly different from northern Canada ($F_{ROH}=0.278$) or northern Quebec ($F_{ROH}=0.267$). Wolves of the Great Lakes/Ontario ($F_{ROH}=0.199$) had significantly lower inbreeding levels ($F_{ROH}=0.278$) than the other geographic regions.

The Northern Rocky Mountain population is genetically distinct

To provide information relevant to ongoing management considerations and decisions, we partitioned the samples to analyze only those belonging to the populations identified in the United States, the northern Rocky Mountains ($n = 188$) and the western Great Lakes ($n = 199$). The preceding analysis identified the distinctiveness between the northern Rocky Mountains and western Great Lakes population segments as per their divergent assignment probabilities ($K = 3$ and $K = 9$) (Figs. 1B-C, Fig. S2). We found that six (4.5%) of the northern Rocky Mountains wolves had assignments to a cluster divergent from their geographic origins at $K = 3$ (when $Q > 0.00001$, $Q = 0.01-0.25$), all of which were individuals sampled in the Pacific Northwest. The misclassification of western Great Lakes wolves is more varied due to assignments to the proximate Canada wolf populations at $K = 3$ ($Q = 0.01-0.86$). This pattern continued at $K = 9$, where the highest non-Rocky Mountains assignments were wolves assigned to Canada's Northwest Territories Province, ($Q = 0.01-0.37$) concordant with a shared demographic history. We identified seven western Great Lakes individuals with assignments (several samples in Isle Royale NP, $Q = 0.01-0.56$) to Canada's Northwest Territories Province, two assigned to Idaho (sampled in MN and WI, $Q = 0.99$), and one to Oregon/California (sampled in MN, $Q = 0.99$), with several demographic processes to explain such signatures (e.g., admixture, shared ancestry, recent dispersal, statistical noise).

As with the geographic regions, expected heterozygosity was significantly higher than observed in each population (Table 2B). We found that the northern Rocky Mountains wolves had significantly lower observed heterozygosity than wolves in the western Great Lakes population ($D = 0.158$, $p = 0.02744$) (Table 2B). We restricted the analysis to samples only with known years of sample collection between 1990 and 2020 within the population of the northern Rocky Mountains ($n = 137$) and western Great Lakes ($n = 86$) to survey changes of diversity over time. Using Pearson's product-moment correlation, we found that all heterozygosity estimates for the northern Rocky Mountains population significantly declined over the 30 years surveyed ($H_0: R=-0.41$, $t=-5.2$, $df = 135$, $p = 5.9 \times 10^{-7}$; $H_E: R=-0.42$, $t=-5.4$, $df = 135$, $p = 3.6 \times 10^{-7}$) (Fig. 2A). Although the WGL population shows a similar albeit weaker pattern of decline, there was no statistical significance ($H_0: R=-0.12$, $t=-1.1$, $df = 84$, $p = 0.2925$; $H_E: R=-0.12$, $t=-1.1$, $df = 84$, $p = 0.2933$) (Fig. 2B). Females in the northern Rocky Mountains population were significantly more differentiated from each other than males across the genome (mean $F_{ST}=0.052$ and 0.032 , respectively; 1-tailed t-test of unequal variance $p = 0.01207$) and the X chromosome ($F_{ST}=0.051$ and 0.029 ; $p = 0.0051$) (Fig. S4). This

pattern was not found in the females of the western Great Lakes population (genome: $F_{ST}=0.017$ and 0.019 ; $p = 0.3242$; X chromosome: $F_{ST}=0.016$ and 0.012 ; $p = 0.1876$).

The northern Rocky Mountain gray wolves had significantly higher autosomal inbreeding coefficients compared to the western Great Lakes, which differences across the X chromosome were not significant (F_{ROH} , autosomes: RM = 0.299, GL = 0.211, $t = 8.5$, $df = 309.6$, $p = 8.67 \times 10^{-16}$; X chromosome: RM = 0.076, GL = 0.070, $t = 0.8$, $df = 260.3$, $p = 0.4473$) (Fig. S5). The outlier inbreeding coefficients for western Great Lakes can be attributed to the small and isolated gray wolf population living on Isle Royale National Park.

Population effective size estimates show the continental history of extermination and recovery

We inferred population effective sizes for the past 200 generations from autosomal SNPs for each of the four regional genetic clusters that carried genetic distinction. We estimated N_e ranged between 23.6 and 25,864.5 over the past 200 generations at a regional scale (Fig. 3A; Table S3). Northern Canada had the highest historical size estimated at 30,000 wolves 200 generations ago, while northern Quebec was stable over all 200 generations at very low estimates (range = 23.6-510.3, average = 236.9, mode = 63.0). The regional populations of western USA/southwestern Canada and Great Lakes/Ontario had similar patterns of precipitous decline from $N_e \sim 9,000$ wolves 200 generations ago to 152.4 and 266.3 wolves, respectively, in the present day. When we restricted our analyses to the two populations, we found both the northern Rocky Mountains and western Great Lakes displayed a steep and rapid decline (Fig. 3B). Both histories were collectively qualitatively similar with the earliest estimate of size at $N_e \sim 9,400$ and 9,100, respectively and both declined precipitously with the effective rate of loss of $m_{RM} = -34.8$ and $m_{GL} = -57.2$ wolves per generation. The western Great Lakes population showed a short-term shallower slope between 100 and 50 generations ago ($m = -38.1$). Considering each population, both were estimated at a present day (0–2 generations ago) effective population size of 141.1 and 228.3, northern Rocky Mountains and western Great Lakes, respectively (Fig. 3B; Table S3).

We further compared population estimates for the northern Rocky Mountains and western Great Lakes populations obtained from management, agency, and public reports between 1982–2015 (Table S4). Both regional populations have a history of substantial expansion in census population sizes between 1982 and 2010 when the northern Rocky Mountains were estimated to have $N \sim 1,723$ and western Great Lakes at $N \sim 4,321$ wolves, remaining mostly stable to the present-day estimates of $N \sim 1,881$ and 3,025, respectively (Fig. 3C). We estimated that the western Great Lakes effective population size has remained stable since 1990 with an average rate of growth larger than that of the northern Rocky Mountains (GL $m = 0.23$; RM $m = 0.07$), with significantly higher effective population estimates for western Great Lakes ($N_e = 226.3$) than the northern Rocky Mountains ($N_e = 141.7$) (t-test unequal variance $p = 1.709 \times 10^{-14}$). Lastly, we estimated the temporal trend of N_e/N collectively for the northern Rocky Mountains and the western Great Lakes and found the effective population size remained at 5.2–9.3% of the census size since early 2000s (Fig. 3C).

Admixture with coyotes and eastern wolves is unique to the Great Lakes gray wolves

We created a second dataset that included western coyotes and eastern wolves to explore signatures of admixture in the gray wolves of the Great Lakes region. We discovered 163314 genomic loci genotyped in 465 canids (179 gray wolves from the northern Rocky Mountains population, 184 from the western Great Lakes population, 74 western coyotes, and 28 eastern wolves). We also constructed a statistically neutral and unlinked dataset of 80,655 SNPs. At the highest level of partition ($K = 10$), we found that gray wolves of the western Great Lakes population had the highest average (\pm sd) probability assignment to clusters of other Great Lakes gray wolves ($Q = 0.64 \pm 0.4$) and $< 10\%$ to any other wolf group ($3.4 \pm 0.1\%$ assignments to eastern wolves; $< 2\%$ to Rocky Mountain gray wolves), with minimal assignments to western coyotes ($Q = 0.01 \pm 0.1$) (Fig. S6; Table S5). Rocky Mountain gray wolves similarly formed their own cluster ($Q > 0.97$) with low, albeit detectable, partial assignments of Wyoming gray wolves with coyotes ($< 2\%$) and $< 1\%$ to all other canid groups. The unsupervised cluster analysis was further supported by western Great Lakes population gray wolves having the lowest genetic differentiation estimates with eastern wolves ($F_{ST} = 0.06$ and weighted $F_{ST} = 0.08$) and western coyotes ($F_{ST} = 0.09$ and 0.12), in contrast to the estimates between northern Rocky Mountains population gray wolves and eastern wolves ($F_{ST} = 0.10$ and 0.10) or western coyotes ($F_{ST} = 0.12$ and 0.15).

Discussion

An estimate of the effective population size provides a means by which conservation practitioners can accurately use theory to predict forward-in-time outcomes for various viability scenarios for an endangered species (Lacy 1995). These estimates permit one to estimate the number of generations until gene flow is required to boost the genetic diversity and concomitantly reduce inbreeding coefficients. The application of this theory to wild endangered or threatened populations has remained challenging but is centrally needed for conservation planning and simulating evolutionary outcomes (Frankham et al. 2019). One complication in the interpretation of effective population sizes is the sensitivity of these estimates to population structure (Ellegren & Galtier 2016). Gray wolves inhabiting North America represent a diversity of demographic histories and contemporary dynamics that manifest as distinct genomic signatures. Local adaptation, compounded with social structure of gray wolves, generates population structure, and increases the rate at which random genetic drift depletes their genomic variation and evolutionary potential. When geographic regions experience local extinctions from over-exploitation, dispersals will re-populate the new vacancy and genetically homogenize across proximal subpopulations over time (Ausband & Waits 2020). Despite these recent demographic events of reintroduction or re-population, observed heterozygosity is lower than expected with significant genetic structure across the continent. As per theory, this suggests that the effective population sizes calculated here for each gray wolf population are impacted (Ellegren & Galtier 2016).

The comparison of the census and effective population sizes provides a more valuable metric beyond census size alone. For species with social organization, substructure, and non-random breeding, theory expects that effective population size will be a fraction of the census size (Frankham 1995; Ellegren & Galtier 2016). Although there are many field methods for estimating the ratio of census size to N_e , these are often challenging and require an immense effort in the field. For example, using wolf dispersal and density data on the Perch Lake pack ($N_m=5$, $N_f=5$) in Minnesota, Chepko-Sade and colleagues (1987) estimated effective population size with two methods: the root mean square (variance) method ($N_e=804$) and the 85th percentile distance of the original dispersal distribution method ($N_e=1660.7$). Further, earlier population estimates from 26 microsatellite data of Yellowstone National Park wolves reported N_e ranging between 6 and 22.6 for 1995–2004 and the respective census sizes of 21 and 80 (range $N_e/N = 0.10–0.37$) (vonHoldt et al. 2008). Genomic-based inferences still face challenges albeit different from field-base inferences; regardless, estimates are critical for shaping appropriate conservation management plans. Understanding this relationship is important because management applies to actual populations which are observed and managed based on census size, not effective population size. Using genomic data from these populations, we show that this ratio is different in different parts of the distribution. Overall, the census and effective population sizes differ by approximately an order of magnitude.

We conducted a population genome-level survey of three genetic groups of gray wolves across North America and resolved deeper fine-scale resolution that was reflective of geography and demographic history. These groups correspond to the Great Lakes region, northern Quebec, and the western region of Canada and the United States. While all the populations we studied have a history of overexploitation, each group has unique aspects to their population histories. The gray wolves of the Great Lakes carry a genetic signature of historic admixture (Leonard & Wayne 2008; Koblmüller et al. 2009; Rutledge et al. 2010; vonHoldt et al. 2011, 2016; Heppenheimer et al. 2018), and habitat loss has been of consequence to wolves in northern Quebec (Larivière et al. 2000). The genetic cluster composed of the continent's western region is likely due to the shared ancestry when wolves were translocated from west central Canada as founders for the populations in the northern Rocky Mountains with recent dispersal across the region (vonHoldt et al. 2010b; Hendricks et al. 2019).

Northern Rocky Mountain gray wolves have declining genetic diversity

Gray wolves were restored in the northern Rocky Mountains through a reintroduction program in the mid-1990s and a handful of dispersing wolves southward from Canada into northwestern Montana, which successfully established several populations that contributed towards the first of many delisting proposals for this population in 2003. A study by vonHoldt et al. (2010b) provided the first evaluation of genetic structure, diversity, and connectivity over the initial 10-year recovery period (1995–2004) inferred from microsatellite markers and reported no immediate concerns for genetic variability. However, genome sequencing advances have enabled provided the gray wolf with a plethora of new genetic methods that avoid some central and limiting concerns when using microsatellite markers (Väli et al. 2008). As such,

we encourage genetic surveys of gray wolves consider a genome-wide reduced representation sequence-based method for large-scale population studies, which are feasible for any sample type and are less prone to calibration and ascertainment concerns of microsatellites collected across facilities, platforms, and research groups (Bonin et al. 2004; Pompanon et al. 2005).

We found genetic evidence of dispersal patterns in the Pacific Northwest, where genetic signatures clearly identified that these western continental wolf populations relied upon male-mediated dispersal for gene flow. We also detected signatures that female wolves across the western USA and southwestern Canada were significantly more differentiated from each other than males. In contrast, this pattern was not found in the females of the Great Lakes and Ontario region, likely an interaction between the population never being fully eradicated and an evolutionary history of genetic admixture with coyotes. Further, we report evidence of both significantly lower levels of genomic diversity in the northern Rocky Mountains paired with eroding diversity and higher inbreeding coefficients since 1990. This temporal decline in genetic diversity was not found in the western Great Lakes wolves. One limitation is that our genetic focus does not explore the fitness effects of such trends; however, such metrics are often central in conservation strategies. Although we currently do not report on fitness-related consequences, evaluations of such have been conducted on highly bottlenecked and inbred populations like Isle Royale and Scandinavia (Hagenblad et al. 2009; Robinson et al. 2019; Åkesson et al. 2022). The wolves of the northern Rocky Mountains currently have an increased mortality rate due to relaxed regulation. Notwithstanding, gray wolf life history of short time to sexual maturity, large litters, and dispersal can mitigate population-level risks from human-related mortality (Fuller et al. 2003; Adams et al. 2008). However, Cassidy and colleagues (2022) recently found significant effects of human-caused mortality on other important biological processes in wolves (e.g., pack persistence and pup production) that have implications for breeding and gene flow. Given the difficulty states have faced in meeting their goals of significant population reduction (e.g., Idaho's goal of 500 wolves with an estimated 1270 census size, Idaho Fish and Game Gray wolf management plan draft January 2023), the effective population size estimates are then interpreted to be strongly influenced by the number of breeding wolves and gene flow, less from census size. Current management actions that seek to reduce overall populations and permit hunting during the breeding season have the greatest potential to have consequences on effective population sizes.

Great Lakes gray wolves have a unique demographic history

Following theoretical expectations, the level of genetic richness and uniqueness is correlated with the western Great Lakes wolf demographic history of colonization and admixture (Allendorf et al. 2001). In agreement with previous findings, western Great Lakes wolves carry the lowest levels of inbreeding and the highest levels of allelic richness and private alleles. This is explained by their historic genetic exchange with other sympatric canid lineages, supported by both genetic cluster analysis and the lowest genetic differentiation with eastern wolves ($F_{ST}=0.06$ and weighted $F_{ST}=0.08$) and western coyotes ($F_{ST}=0.09$ and 0.12), in contrast to the estimates between northern Rocky Mountains population gray wolves and eastern wolves ($F_{ST}=0.10$ and 0.10) or western coyotes ($F_{ST}=0.12$ and 0.15). This demography is unique and provides an immediate mechanism by which these populations can respond

to a rapidly changing world both in terms of climate and of anthropogenic activity (Rius & Darling 2014; vonHoldt et al. 2017; Kagawa & Seehausen 2020; Ottenburghs 2021).

Conservation decisions in light of effective size estimates

We compiled reported population sizes across the states that compose the northern Rocky Mountains and western Great Lakes population between 1982–2015 from public data and found that gray wolf effective population sizes were 5.2–9.3% of the census size. Peterson et al. (1998) used demographic models of N_e (by Nunney) and demographic data from Isle Royale (which is typical of many wolf populations) and estimated an N_e/N ratio of 16%. Further, many wild canid species will avoid mating with relatives (vonHoldt et al. 2008; Geffen et al. 2011; Sparkman et al. 2012; Ausband 2022), and this inbreeding avoidance mechanism will increase N_e . Our estimates are comparable to those for the cooperative breeding African wild dog (*Lycaon pictus*) where effective population sizes are 8.7–11.3% of the census size (Marsden et al. 2012). According to international conservation goals of the ‘50/500 rule’, the genetic consequences of population subdivision are strongest in small ($N_e < 500$) isolated populations where inbreeding depression occurs, and genomic diversity erodes due to drift. Thus, successful short-term conservation efforts can target $N_e \sim 50$ but should target $N_e > 500$ for the long-term survival of a species (Jamieson & Allendorf 2012). As per this rule, we show that gray wolves fall above minimum effective population sizes needed to avoid extinction due to inbreeding depression in the short term but face long-term risk of extinction on their own given their present day (0–2 generations ago) effective population sizes ($N_e \sim 141–228$). A similar situation was also found for Scandinavian wolves, with realized N_e below advised conservation goals (Laikre et al. 2016). Their ultimate suggestion was to increase N_e and promote methods that would increase genetic exchange 3–5 effective migrants per generation with neighboring populations. Notably, such goals are clearly possible within the ESA framework which defines “conservation” in section 3 to include “the use of all methods and procedures which are necessary to bring any endangered species or threatened species to the point at which the measures provided pursuant to this Act are no longer necessary.”. There are known dispersers, albeit unknown if they are effective dispersers, between southwestern Canada and the U.S. Rocky Mountains. Combined with the shared ancestry due to translocation from the western Canada and northern Rocky Mountain gray wolf populations, demography is a core feature that shapes conservation-relevant metrics. Further, wolves in North America can originate from dramatically different regions with distinct collections of local adaptations and ecotypes (Schweizer et al. 2016; Hendricks et al. 2018; Carroll et al. 2021). The suggested effective migrant strategy would require more consideration of regional signatures of adaptive variation (Carroll et al. 2020). We envision this study as a baseline for future assessments.

Genetic conservation of gray wolves

Species recovery plans are constructed around a core conservation biology framework referred to as “The Three R’s” (representation, resiliency, and redundancy) for reducing the risk of extinction (Shaffer & Stein 2000). Under the ESA, this can be satisfied by maintaining multiple large, genetically robust populations across the historic range that are self-sustaining. Gray wolves have already met many of these aspects,

with several populations found across the United States, and natural dispersal occurring to help occupy portions of their historic range, although the species still only occupies approximately 10–15% of its historical range (Carroll et al. 2006). With fluctuating federal protection, populations can recover, be delisted, experience reductions through human-caused mortality, and then return to federal protection, thus restarting the cycle. In addition to jurisdictional issues within the United States (Smith et al. 2016), there are also international challenges. Both populations considered here are part of a larger gray wolf population that is distributed across the United States and Canada border, making their conservation status dependent upon biological and social conditions in both countries. Joint US-Canada conservation plans and action have been successfully executed in the past (Bangs & Fritts 1996), but international coordination can be complicated to maintain (Quevedo et al. 2019). Any disruption of dispersal across this international line, or decline in one country, would impact the population viability of the wolves. The Assistant Secretary of the Interior is quoted, regarding the ESA that “...it is in the best interests of mankind to minimize the losses of genetic variations. The reason is simple: they are potential resources. They are keys to puzzles which we cannot solve, and may provide answers to questions we have not yet learned to ask” (H.R. Rep. No. 93–412, pp. 4–5, 1973). Such Congressional intent clearly displays the intent of including all means for the conservation genetic variation. Further, human activity homogenizes the landscape in which endangered species rely, and such activities “...threaten their – and our own – genetic heritage. The value of this genetic heritage is, quite literally, incalculable.” (93D Congress Report, 1st Session, No 93–412, page 143).

The minimum effective population size of 500 necessary to ensure long-term population viability has been difficult to apply in practice. There are many reasons for this. One reason is the abstractness- it can be hard for a manager to know what the effective population size of the population they are managing is when what they can count is the census size. In 2021, the northern Rocky Mountains had a census size estimated at 3,354 and western Great Lakes at 4,526. However, we can then translate these values to an effective population size ranging between 201 and 335 wolves for the northern Rocky Mountains and 272 and 453 for the western Great Lakes. Given the strong skew in the effective-to-census size ratio in gray wolves, larger wolf populations are necessary to ensure long term adaptation and survival. Disperser success is an additional critical factor for long-term survival of the species, promoting gene flow that will reduce inbreeding and elevate effective population sizes through increased allelic variation and demographic rescue (Newmark et al. 2023). Dispersers are often challenged by utilizing lower quality corridors with high mortality risk to find suitable areas for establishing new territories (Oakleaf et al. 2010). The protection of gray wolf dispersers between wolf populations is thus important to improve their effective population sizes for long-term persistence.

Declarations

Acknowledgements

We acknowledge the following for samples used in this study: L. David Mech, Michel Crête, and the Michigan Department of Natural Resources. We are appreciative of manuscript comments also provided

by Stephen Gaughran. We are grateful to the Turner Endangered Species Fund for support.

Data Accessibility Statement

We have deposited all mapped and sorted BAM files on NCBI SRA under the accession PRJNA961038 and all accession numbers are listed per sample in Table S1.

Conflict of Interest

DRS is the Leader for the Yellowstone Wolf Project. MP is the Director of the Turner Endangered Species Fund and advisor to Turner Biodiversity Divisions. JS is a wildlife biologist for the Grand Teton National Park. KL is a senior environmental scientist specialist for the Northern Region of the California Department of Fish and Wildlife. ME is. A senior wildlife forensic specialist at the California Department of Fish and Wildlife.

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Figures

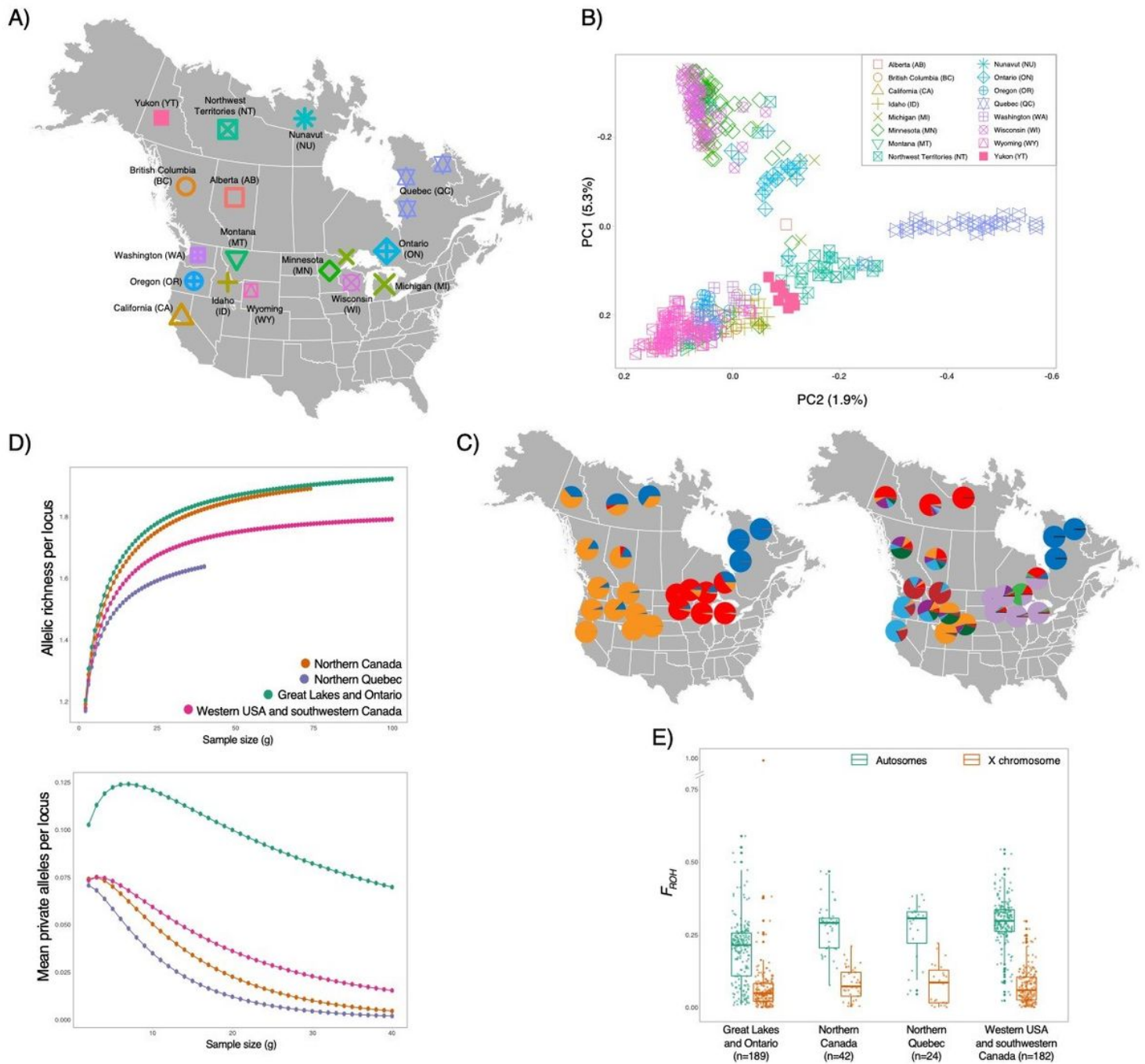


Figure 1

Population genetic structure of 437 gray wolves from **A)** North American populations genotyped at 81,595 statistically neutral and unlinked SNPs inferred from **B)** principal component analysis and a maximum-likelihood approach for **C)** three and nine partitions (map credit: Free Vector Maps WRLD-NA-01-0007). **D)** Rarefaction of allelic richness and private alleles for each major geographic region of gray wolves (see Table S1). **E)** Box-and-whisker plots of inbreeding median estimates derived from autozygosity (F_{ROH}) across major geographic regions for each chromosome type.

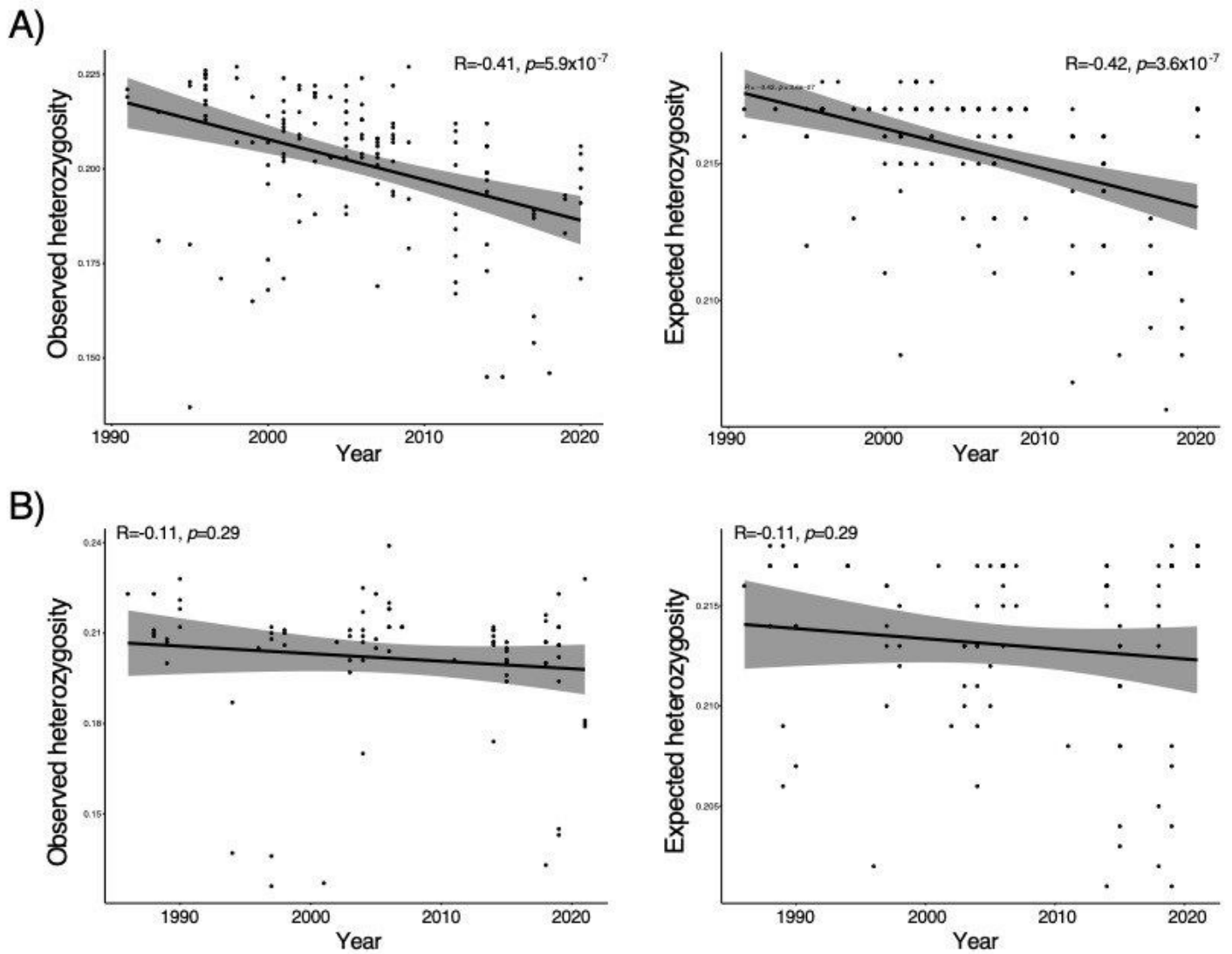


Figure 2

Heterozygosity (observed and expected) trends for the **A)** northern Rocky Mountain (n=137) and **B)** western Great Lakes (n=86) distinct population segments in the United States for a 30 year period between 1990 and 2020 (Y-axis). Pearson correlation coefficients and significance values are provided.

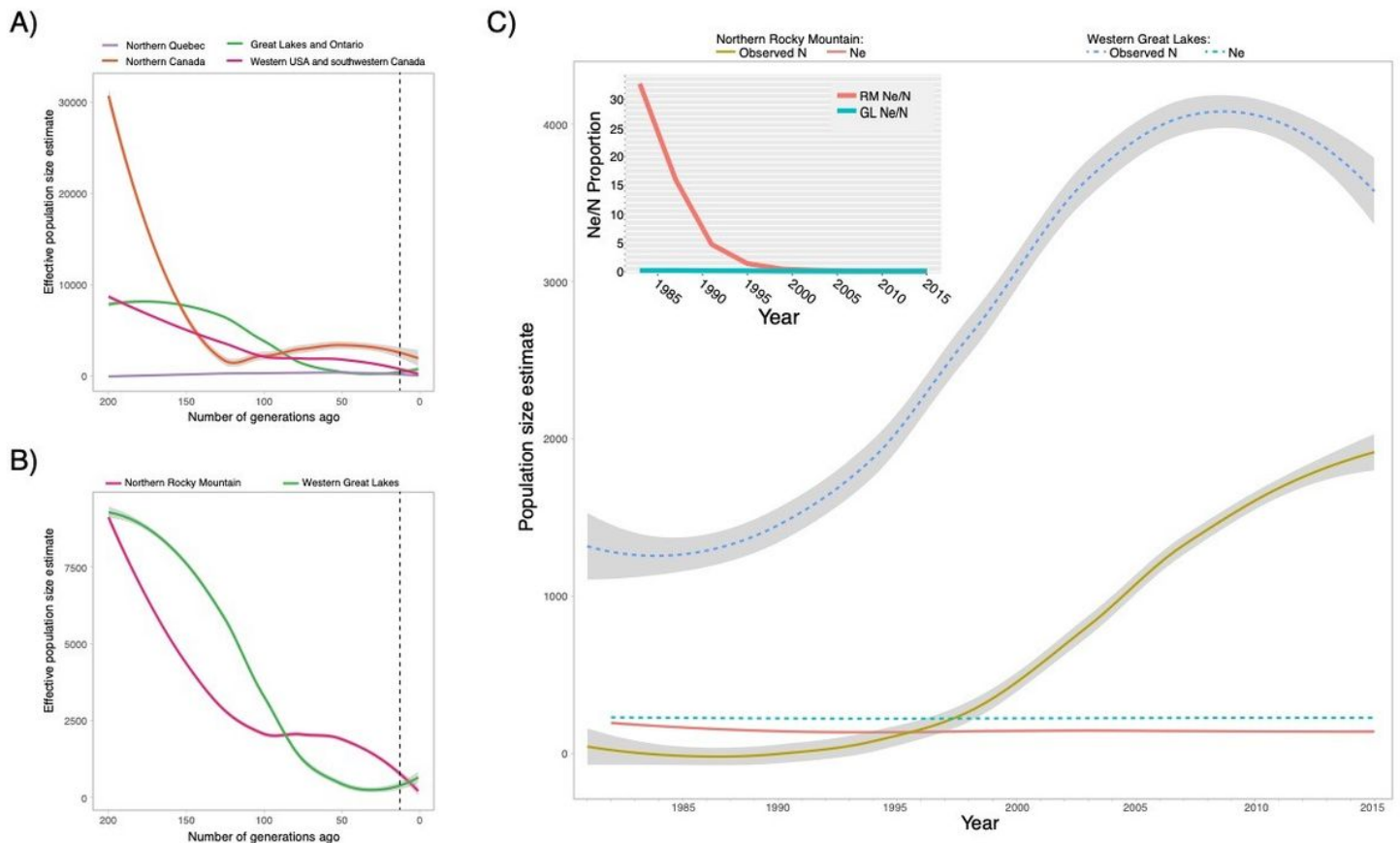


Figure 3

Locally estimated scatterplot smoothed (loess) trend lines of population effective size (N_e) histories for **A)** each of the four identified regional genetic clusters and **B)** each of the regional populations in the United States. The vertical dashed line in each panel indicates the acceptance of the U.S. Endangered Species Act into law in 1973. **C)** Observed (N) and inferred population effective size (N_e) histories for the northern Rocky Mountain (RM) and the western Great Lakes (GL) populations in the United States. We assumed 4 years per generation. The inset displays the ratio of N_e to N since 1982-2015 for each of the two populations.

Supplementary Files

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