# Genetic structure of a large recolonizing carnivore: the case of the northern cougars (*Puma concolor*)

By

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# ABSTRACT

Anthropogenic activities have pressured and altered landscapes resulting in extinctions and extirpations. However, increased conservation efforts and changing management strategies in some large carnivores have resulted in population and range expansion. Population growth and range expansion are detectable using population genetic techniques by characterizing population structure, and clinal patterns in relatedness (isolation by distance, IBD). Using 902 single nucleotide polymorphisms genotyped via double digestion restriction-site associated digestion from 119 cougars (*Puma concolor*) – both live-captured and harvested cats – in Alberta, Canada we assessed and characterized the genetic structure of this expanding population using spatially explicit and agnostic means. Each predicted and tested subpopulation (based on geographic attributes of sampling location) was characterized and mapped. Predicted subpopulations were inconsistent and not biologically informative; additionally, no significant correlation was detected between genetic and geographic distance (IBD). In all tested and predicted subpopulations, observed heterozygosity was lower than expected. Our findings confirm that the Albertan population of cougars has recently undergone expansion detectable from a genetic perspective (as suggested by the lower-than-expected heterozygosity). The panmictic nature of this population is unique in modern cougars and of importance for managers both of this population and of other predator species.

# PREFACE

This research is an original work by Kimberley G. Barrett, no part of this thesis has been previously published. Samples were provided by the Province of Alberta (Alberta Fish and Wildlife Stewardship, Edmonton, Canada, and the Alberta Fish and Wildlife Enforcement Branch Edmonton Canada). Library preparation and sequencing of DNA was conducted by the Molecular Biology Services Unit at the University of Alberta, Edmonton, Canada.

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# INTRODUCTION

Species' response to anthropogenic activities and effects is a key concern in conservation and management. Changing climatic patterns, urbanization, and land conversion are expected to continue resulting in extinctions and extirpations around the world (Cudmore et al. 2010; Dawe and Boutin, 2016; Schipper et al. 2008). These altered landscapes and attendant reduced competition can allow invasive species to spread, and for previously low-abundance species to increase in population size and range (Hobbs and Huenneke 1992; Fischer and Lindenmayer 2007). After years of persecution, some large carnivore populations are recovering and growing in population size and range (Miller et al. 2013, Wilton et al. 2014). Generally, these changes can alter the population structure and genetic diversity (Allentoft and O'Brien 2010; González et al. 2020; Lino et al. 2019; Wright 1931; Wright 1984).

Historically, predators like bears (*Ursus*), wolves (*Canis lupus*), and cougars (*Puma concolor*), were considered a threat to colonial life and were managed as pests to reduce population size (Miller et al. 2013). In North America, large carnivores decreased in abundance to the point of extirpation in some regions (Laliberte and Ripple 2004). The recent and ongoing recovery of some large carnivores can be partially attributed to the scientific understanding of the role of predators in ecosystems, increased interest in maintaining diverse ecosystems, and growing public intolerance for lethal management strategies (Manfredo et al. 2016; Ripple and Beschta 2012; Slagle et al. 2017). Reduced human-caused mortality has allowed many species to grow in number and begin to re-establish throughout their historic range (Ripple and Beschta 2012; Wilton et al. 2014).

Range expansion can occur after natural disturbances, such as landslides and forest fires, and human activity, such as the introduction of invasive species, land conversion, unsustainable resource consumption, and climate change (Geertsema and Pojar 2007; Hughes et al. 2020; Zeidberg and Robison 2007). Genetically, range expansion can result in loss of genetic diversity arising from genetic drift, reduced gene flow, and successive founder events between the source and the newly occupied areas (Allentoft and O'Brien 2010; González et al. 2020; Heppenheimer et al. 2018; Lino et al. 2019; Wright 1931; Wright 1984). Given enough time and reduced gene flow, allele frequencies will change and result in populations becoming genetically distinct (Wright 1931; Wright 1984). However, if gene flow is maintained and mating is random across

the range (i.e., panmictic), genetic diversity is unlikely to decrease and population structure is unlikely to form (Wright 1931; Wright 1984).

Population structure can be difficult to detect, especially when barriers are new or populations are actively expanding, as allele frequencies take time to change (Wright 1931; Wright 1984). Using many genetic markers spread across the genome is essential to detect subtle changes in allele frequencies (Dufresnes et al. 2023; Zimmerman et al. 2020). Genomes contain many single nucleotide polymorphisms (SNP), and the rise of next-generation sequencing techniques has made SNP more technically and financially feasible for population genetic studies (Andrews et al. 2016). Further, double digest restriction-site associated digestion sequencing (ddRAD-Seq) is becoming increasingly common as it can produce tens of thousands of genome-wide SNPs, suitable for phylogenomic, association mapping, and population genetic studies, without the need for species-specific genomic information or large quantities of high-quality DNA (Andrews et al. 2016; Peterson et al. 2012). The use of two digestion steps with different enzymes allows for the incorporation of a barcode on the primers of both cut sites allowing for upwards of a hundred individuals to be pooled for a single run of DNA sequencing with careful indexing (Andrews et al. 2016; Peterson et al. 2012). Each sample's primer codes are transcribed during library preparation and become a part of the sequenced DNA fragments allowing for bioinformatic sorting after sequencing (Andrews et al. 2016; Peterson et al. 2012). These pooled solutions are then sequenced using next-generation sequencing technologies (Andrews et al. 2016; Peterson et al. 2012). Using ddRAD-Seq can be challenging when studying wildlife, as high-quality DNA is required. Hair and dried tissue samples can fail to produce enough high-quality DNA required. Lower-quality DNA can result in sequence data with low read depths or coverage (O'Leary et al. 2018). In such cases, mapping reads to a complete reference genome reduces depth requirements as compared to *de novo* (without a reference genome) variant calling, because there are fewer sources of error (O'Leary et al. 2018). High-quality reference genomes are more commonly available for model, crop, or domestic species, but when unavailable, the use of a related species reference genome is common across taxa (Gooley et al. 2022; Janjua et al. 2020; Li et al. 2021). For example, the domestic cat (*Felix catus*) genome is useful for studying wild felids as they evolved and radiated recently (Johnson et al. 2006), with many introgression and hybridization events (Figueiró et al. 2017; Li et al. 2016) making them genetically similar.

All extant cats have a common ancestor approximately 10-15 million years ago before radiating into the greater than 30 species alive today (Collier and O'Brien 1985; Sunquist and Sunquist 2017). Cougars diverged from other *Puma* species approximately 390 thousand years ago in South America before spreading northward 300 000–100 000 years ago (Culver et al. 2000). Present day cougars range from southern Chile to northern Canada across a diversity of ecosystems, ranging from lowland swamps and tropical forests to deserts and highland mountains (Chimento and Dondas 2018; Culver et al. 2000; Nielsen et al. 2015; Saremi et al. 2019). Cougars are solitary carnivores that commonly target large mammals as prey, but will also consume fish, birds, small mammals, and domestic species (Karandikar et al. 2022; Prude and Cain 2021). Depredation in conjunction with human safety concerns resulted in the institution of cull programs to reduce their population size throughout the twentieth century (Miller et al. 2013). However, by the 1970s, Canadian provinces managed cougars as a game species allowing for a regulated hunting season (AESRD 2012; FWB 1980). Population estimates at the end of the cull in Alberta, Canada, suggested their abundance at approximately 700 cougars (AESRD 2012). Since then, the number of cougars in the province has increased, with a 2012 estimate of approximately 2000 individuals (AESRD 2012), the last time robust da were gathered.

Cougars have also been expanding northward and eastward, re-establishing their historical range, and beyond (Knopff et al. 2014). While numerous population genetic studies have been conducted on cougars, none has been done in this population, nor in a population of cougars actively expanding in number and range. Previous studies have found diversity and population structure differ greatly by region (Caragiulo et al. 2014; Gustafson et al. 2019), with some degree of population structure (Anderson et al. 2004; Ernest et al. 2014; Gallo et al. 2020, 2021; Gustafson et al. 2022; McRae et al. 2005; Saranholi et al. 2017; Sinclair et al. 2001; Trumbo et al. 2019; Wultsch et al. 2023). The sole exceptions were studies conducted on cougars from southern Brazil where one study of 25 individuals found no structure (Miotto et al. 2011) and another study of 37 found no structure but significant isolation by distance (Castilho et al. 2011), in both cases more individuals may be required to confidently identify population structure. Generally, genetic diversity and the number of distinct subpopulations decrease northward across the Americas, likely as a product of the species' recent evolutionary expansion north (Gallo et al. 2020, 2021; Gustafson et al. 2022; Saremi et al. 2019; Trumbo et al. 2019).

Anthropogenic effects like hunting pressure, lack of corridors, and habitat conversion have been associated with reduced genetic diversity and increased numbers of genetically distinct subpopulations across all studies (Anderson et al. 2004; Ernest et al. 2014; Gallo et al. 2020, 2021; Gustafson et al. 2022; McRae et al. 2005; Saranholi et al. 2017; Sinclair et al. 2001; Trumbo et al. 2019; Wultsch et al. 2023). Recent studies highlight subpopulations or regions that are distinctly lacking in genetic diversity (Gustafson et al. 2022; Wultsch et al. 2023) highlighting the importance of local context.

Elucidating the genetic structure of the population of cougars in Alberta Canada can provide insights into the source-sink dynamics of an expanding predator and provide a baseline for monitoring genetic diversity. The source-sink dynamic framework is the management lens used in our study area. This framework recognizes variability in habitat quality can lead to source regions (a habitat producing more individuals than it can sustain) and sink regions (a habitat producing a deficit of individuals which can receive migrants; AESRD et al. 2012; Stoner et al. 2013). The impact of this framework and ongoing recolonization on the population structure of the cougars in this region can inform management strategies locally in terms of hunting allowances and potentially whether surrounding regions can expect migrant cougars. In this study, I developed and used ddRAD-Seq-derived SNP genotypes isolated from live-captured and harvested cougars' across Alberta to asses and characterize population structure. Specifically, I assessed population structure using both spatially agnostic or traditional means (does not incorporate sample location) and explicit means (incorporates sample location) of predicting genetic subpopulations and characterizing these putative groups. I employed a variety of programs using different statistics, with and without priors, and with different means of processing the data, to accurately characterize this population while accounting for variability between programs. Given recent and ongoing recolonization and population growth I expected to identify a panmictic population with subtle isolation by distance effects.

# MATERIALS AND METHODS

## Study Area and Sample Collection

I examined cougar population structure in three regions of Alberta (boreal, mountain, and foothill) making up approximately 450 000 $km^2$ (Figure 1; Province of Alberta n.d.). The boreal

region is characterized by flat expanses of quaking aspen (*Populus tremuloides*), balsam poplar (*P. balsamifera*), white birch (*Betula papyrifera*), jack pine (*Pinus banksiana*), and lodgepole pine (*P. contorta*) stands as well as mixed forests (Beckingham and Archibald 1996; ESWG 1995). The mean annual temperature is approximately 2°C with cold winters (average -12°C) and mild summers (average 14°C). The mountain region comprises a variety of single and mixed species stands including Douglas-fir (*Pseudotsuga menziesii*), lodgepole pine, white spruce (*Picea glauca*), and quaking aspen in addition to grasslands (Beckingham et al. 1996). Annual mean temperature ranges between 1°C and 3°C with mild winters (ranging from -5°C to -9°C on average) and cold summers (averaging approximately 10°C) along strongly rolling ridges and rugged mountainous areas. The foothill region contains rolling ridges of aspen, white spruce, lodgepole pine, and balsam poplar (Archibald et al. 1996). As above, the foothills are characterized by mild winters (mean of -7°C), and mild summers (mean of 12°C) for an overall annual mean of 3°C. Agricultural and urban activity and consequent land conversion are intense in the foothill region (ESWG 1995; Haarsma and Qiu 2017). Foothill and mountain regions are recreation and tourism hubs (EP 2021). Mineral extraction activity is present in foothill regions but more intense in the boreal region (AE 2019), with oil and gas development in the boreal and foothill regions (AER 2023; CAEC 2023).

Each region is separated into many management units (management units; Figure 1) used to regulate hunting (Province of Alberta n.d.). In the case of cougars, each management unit allows for a maximum number of harvested animals of each sex including licenced harvest and landowner kills (AESRD et al. 2012). The regulated harvest season extends from September 1st through the end of March or until the quota is filled (Wildlife Act 2000). All harvests must be registered with the province including the sex of the cat and harvest location. All hair and tissue samples included in this study were sourced from hunters, collaring activities, roadkill and conflict kills provided by the Province of Alberta (Figure 1). Live capturing activity occurred primarily in the foothill region where mother cats and offspring were disproportionately targeted.

## DNA Extraction and Sequencing

DNA was extracted from hair and tissue using single-column Qiagen DNA Blood and Tissue extraction kits using a modified version of the manufacturer-recommended protocol to increase yield (Qiagen, Valencia, California, USA; Appendix A.1). DNA was eluted and stored

in AE buffer at -20°C while remaining samples were extracted. Each extract was quantified and quality assessed, by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for initial assessment of quality and by Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using dsDNA High Sensitivity assay kits for standardization. Elutions were concentrated via Speedvac Plus (Savant SC110A, Thermo Fisher Scientific, Waltham, MA, USA) or diluted with water until standardized to 10 $\mu$L of 20 ng/ µL by fluorometry described above. ddRAD-Seq library preparation followed MacDonald et al. (2020) using EcoRI and Sbf1 restriction enzymes (Appendix A.2) and was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) to ensure sufficient quality before sequencing. Samples were split into two runs for single-end 75 base pair (bp) sequencing on a NextSeq 500 (Illumina, San Diego, CA, USA). Each sequencing run was conducted with a NextSeq V2.5 high-output 75-cycle kit (Illumina, San Diego, CA, USA). Both the library preparation and sequencing was conducted by the Molecular Biology Services Unit at the University of Alberta, Edmonton, Canada.

## SNP Calling and Filtering

Raw sequence data were downloaded from Illumina BaseSpace and concatenated into FASTQ files on the Digital Research Alliance of Canada's Cedar cluster to prepare for processing and filtering (Figure 2). These were then demultiplexed into files specific to samples using 'process_radtags' in stacks/2.3 (--renz_1 sbfI --inline_null -t 67 -w 0.15 -s 20 -c -r -D –filter_illumina -E phred33; Catchen et al. 2011, 2013). Cutadapt was then used to ensure all primers were successfully trimmed during demultiplexing and filtering (-u 5, -a ACCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNNNATC -m 62; Magoč and Salzberg, 2011). All trimmed files were quality assessed (fastqc/0.11.9; Andrews 2010) and visualized (multiqc/1.14; Ewels et al. 2016). Individuals required a minimum of 0.1 million reads and a GC content within 45–55% to be retained. The remaining samples were aligned to the indexed ('mem' and 'index'; bwa/0.7.17; Li and Durbin, 2009) domestic cat genome (DomCat_AnnotatedWholeGenome_9.0.fna, GenBank Assembly Accession: GCA_000181335.4; Buckley et al. 2020; Pontius et al. 2007). Aligned reads were then processed and quality assessed ('view', 'sort', 'flagstat'; samtools/1.9; Li et al. 2009, Li 2011). SNPs were then called ('ref_map.pl -X "populations: -p 1 -r 0.8 --min_maf 0.05'; stacks/2.3; Catchen et al.

2011, 2013) and filtered for depth, read quality, missingness, and minimum allele frequency (--minDP 10 --minGQ 30 --max-missing 0.5 --maf 0.05; VCFtools/0.1.13; Danecek et al. 2011). The depth and heterozygosity for remaining SNPs and individuals (--site-depth, --hardy, --depth, --het) were assessed and plotted. SNPs were then filtered by distance to avoid physical linkage, requiring a minimum of 10 000 bp between SNPs (--bp-space 10000; plink/1.9; Purcell et al. 2007; Chang et al. 2015).

To prioritize the retention of individuals while maintaining sufficient high-quality SNPs necessary for analysis, these data were filtered to a subset of high-quality unrelated individuals from which to select SNPs. Individuals were filtered to include only those with >50% of SNPs represented (missing-indv; VCFtools/0.1.13; Danecek et al. 2011) and individuals were filtered manually so that no pair of individuals had a pairwise kinship value >0.23 (--make-king-table; plink/2; Purcell et al. 2007; Chang et al. 2015). I filtered for relatedness to account for a disproportionate number of related individuals sequenced. SNPs were then filtered for severe deviations from Hardy-Weinberg equilibrium (HWE; --hwe 0.0000000001; VCFtools/0.1.13; Danecek et al. 2011). SNPs were then filtered for quality and depth as above but with stricter missingness (--minDP 10 --minGQ 30 --max-missing 0.1 --maf 0.05; VCFtools/0.1.13; Danecek et al. 2011). These SNPs were used across all individuals in the original filtered file (step immediately preceding physical linkage trimming; Figure 2). Individuals were again filtered based on missingness (--missing-indv; VCFtools/0.1.13; Danecek et al. 2011) and kinship as above (--make-king-table; plink/2; Purcell et al. 2007; Chang et al. 2015). Cut-off points described above for heterozygosity, depth, and kinship were partially informed by field standards as well as by assessing the data. These data were assessed by kinship plots (plink/2; Purcell et al. 2007; Chang et al. 2015) and by comparing the read depth of individuals and SNPs to their respective heterozygosity values (--site-depth, --hardy, --het; VCFtools/0.1.13; Danecek et al. 2011).

## Spatially Agnostic Population Structure

Spatially agnostic techniques in this research describe analyses that do not use spatial or geographic information. Three spatially agnostic population structure techniques were used, including principal component analysis (PCA; Chang et al. 2015; Purcell et al. 2007), discriminant analysis of principal components (DAPC; Jombart 2008; Jombart and Ahmed

2011), and an iterative Bayesian clustering analysis (STRUCTURE; Pritchard et al. 2000). PCA and *de novo* DAPC use similar maths; however, PCA prioritizes differences between individuals and *de novo* DAPC prioritizes differences between groups (Chang et al. 2015; Jombart 2008; Jombart and Ahmed 2011; Purcell et al. 2007). In comparison, STRUCTURE uses allele frequencies to predict populations (Pritchard et al. 2000). The PCA was run (plink/1.9; --freq --allow-extra-chr –pca; Purcell et al. 2007; Chang et al. 2015) allowing for 20 principal components (PCs) and plotted (ggplot2/3.3.2; Wickham, 2016). The DAPC was run using *de novo* clustering to predict subpopulations. I used the K-1 PCs technique (Cullingham et al. 2023; Thia 2022), where K= 5 as this was the maximum number of K tested across analyses in this research ('find.clusters', n.iter = 100, n.start = 100; adegenet/2.1.7; Jombart 2008; Jombart and Ahmed 2011). The optimal number of distinct genetic groups (i.e. subpopulations or K) was chosen using 'find.grp$stat' and plotted using the K=1 method with the DAPC predicted K value ('dapc'; n.pca = 1, n.da = 2; Cullingham et al. 2023; Thia 2022). Further, I assessed for the disproportionate contribution of particular SNPs via variable contribution analysis ('loadingplot', $var.contr, axis = 2, thres = 0.07, lab.jitter = 1). STRUCTURE inputs were produced using plink/1.9 (Purcell et al. 2007; Chang et al. 2015) and manually edited for use in Structure/2.3.4 (Pritchard et al. 2000). K 1-5 was run 10 times each with 500 000 repetitions and 50 000 burn-in, all allowing for admixture. Optimal K was chosen using the consensus of best fit K across delta K and LnP(K) plots produced using StructureSelector (Evanno et al. 2005; Kopelman et al 2015; Li and Liu 2018). In cases of disagreement, the smaller K was chosen (Pritchard et al. 2000).

## Spatially Explicit Population Structure

In addition to the spatially agnostic testing, three tests incorporating location information were conducted (i.e. spatially explicit), including isolation by distance (IBD; Dray and Dufour 2007; Jombart 2008; Jombart and Ahmed 2011) with a Mantel test, analysis of molecular variance (AMOVA; Dray and Dufour 2007; Jombart 2008; Jombart and Ahmed 2011; Kamvar et al. 2014; Kamvar et al. 2015; Paradis 2010), and an iterative Bayesian clustering analysis with *a priori* location information (STRUCTURE with locprior; Pritchard et al. 2000). IBD was run twice, the first compared individuals by plotting pairwise genetic distance to pairwise geographic distance between individuals and their sampling locations. The second analysis used the pairwise genetic distance developed from allele counts of each management unit against the geographic

distance between the centroids of management units. IBD analyses were run using the structure file format above and read, converted, run, assessed via Mantel test, and visualized (mantel.randtest, n = 1.0x10^6; adegenet/2.1.7; ade4/1.7-2; Dray and Dufour 2007; Jombart 2008; Jombart and Ahmed 2011). Five different AMOVAs (poppr.amova, proppr/2.9.3, ade4/1.7-2, adegenet/2.1.7, pegas/1.0-1) were run using four different environmental and spatial attributes of the management units (Dray and Dufour 2007; Jombart 2008; Jombart and Ahmed 2011; Kamvar et al. 2014; Kamvar et al. 2015; Paradis 2010). While population structure was not expected, potential variability on the landscape could arise as a result of ecological region, clinal patterns (north-central-south, and east-west), and/or proximity to the Rocky mountains (i.e. mountains, inner-outer; Figure 3). Specifically, these geographic variables were tested to address learned behaviour and adaptation to environment of sampling location (ecological region), migration from out-of-province populations (north-central-south i.e. United States of America, and east-west i.e. British Columbia), and potential refugial populations in the mountains (inner-outer). The attributes explaining the most between subpopulation variance (largest *phi* in pop-total) were then used for *a priori* location informed STRUCTURE analyses (Structure/2.3.4; Pritchard et al. 2000). As above, admixture was allowed (50 000 burnin, 500 000 repetitions) for K = 1–5, with each K being repeated 10 times. In these tests, however, spatial attribute information was included (locprior). The optimal K was chosen as described above by consensus of delta K and LnP(K) plots, or in the case of disagreement, the smallest K was chosen.

## Characterizing Predicted Subpopulations

All spatially derived and predicted subpopulations (including panmixia) were characterized using expected, observed total, subpopulation heterozygosity, and $F_{ST}$ where possible (basic.stats; hierfstat/0.5-11; Goudet 2005). Spatially agnostic population separations, specifically DAPC and STRUCTURE (with and without prior location information) were visualized on a projected map of the study area by management unit. Where admixture is present, the genetic group making up the largest fraction of an individual's genetic makeup was chosen, in the case of equivalent portions the individual was removed.

# RESULTS

## Sample Quality and Data Filtering

DNA extractions were attempted for 330 samples (146 hair, 184 tissue) of variable quality and quantity. Many samples contained a minimal amount of tissue/hair or were contaminated with dirt or tape. Of these 330 samples, 187 samples (52 hair samples and 120 tissue samples) yielded sufficient quantity and quality of DNA to proceed with library construction and sequencing. Thirteen samples were duplicated across sequencing runs as a means of assessing between-run consistency, resulting in sequence data for a total of 200 samples.

During trimming, sorting, and aligning sequences to the reference cat genome, 6 of the sequenced samples were determined to have insufficient reads, and were thus removed from further analysis. SNP calling from the remaining 194 samples identified 1 812 candidate markers. After filtering these 1 812 candidate markers for SNP depth, quality, and missingness, 1 732 SNPs remained. 276 SNPs were within 10k base pairs of another SNP and were removed and 30 individuals were removed from subsequent analyses for missing more than 50% of these remaining SNPs. The read depth and heterozygosity estimates for SNPs and individuals were plotted (Figure 4A and Figure 4B). Individual read count by heterozygosity indicated most individuals had heterozygosity values from 0.30 to 0.40. SNPs by comparison consisted of < 25 000 reads and heterozygosity values from 0.10 to 0.65, with a scattering of outliers. Pairwise kinship among the remaining samples ranged from -0.19 to 0.50 (Figure 4C). Individuals were then filtered to ensure that no first-order relations remained (kinship > 0.23) which resulted in the removal of 55 individuals. SNPs were then filtered to remove those with read counts > 30 000 (remaining SNP depth < 25 000) and heterozygosity values > 0.65. The resulting data set included 109 individuals and 1 401 SNPs. Further filtering led to one SNP being removed for severe deviations from HWE ($1x10^{-10}$), and 498 SNPs removed for insufficient coverage across individuals.

To prioritize the retention of individuals, these remaining 902 SNPs were isolated from the original filtered SNP file containing 194 individuals and 1 732 SNPs (immediately prior to physical linkage trim). The missingness of SNPs in individuals was tested, leading to 23 individuals being removed. Kin were removed as described above, which led to removal of a further 52 individuals from the data set. This resulted in retention of a total of 119 individuals in

the final data set. Depth, heterozygosity of individuals, heterozygosity of SNPs and pairwise kinship were then calculated and plotted once more before further analysis. Individual heterozygosity versus depth was similar with no notable changes in heterozygosity attributable to filtering (Figure 4D). SNP heterozygosity plot shows the range of values is retained without outliers (Figure 4E). Distribution of pairwise kinship at this stage ranges from -0.26 to 0.23 with a peak at 0.00 (Figure 4F). The remaining 119 individuals (hair samples = 30, tissue samples = 89) comprised 50 females, 56 males, and 13 cougars of unknown sex originating from across the foothills (n = 88), boreal (n = 11), and mountain (n = 20) regions (Table 1). The management units from which these samples originated covered more than 80 000 $km^2$ of discontinuous land in Alberta, Canada (Figure 1).

## Spatially Agnostic Population Structure

The first two principal components of the PCA analysis explained 16.8% of the genetic variance, forming a large cluster of individuals (Figure 5A). All identified outliers comprised samples originating from the foothills (Figure 5A). The DAPC suggested K = 2 was the best fit by BIC plot with a small increase in BIC between consecutive Ks (Figure 5B and Figure 6). The first subpopulation (n = 107) formed a tall peak, while the second population (n = 12) formed a low but wide peak. No SNPs contributed disproportionately to these findings (Figure 7). The 12 individuals in the second subgroup had no overlap with PCA outliers. STRUCTURE suggested an entirely admixed population at all levels of K tested, but delta K and LnP(K) plots suggested that K = 3 as the best fit in these data (Figure 5C; Figure 8).

## Spatially Explicit Population Structure

IBD analyses did not yield any correlation between geographic and genetic distance (Figure 9). Specifically, a weak and not statistically significant correlation was found between individual pairwise genetic and geographic distance ($r = 0.23$, $p = 0.07$). This nonsignificant geographic trend dissipated completely when these data were pooled by management unit ($r = 0.06$, $p = 0.46$). AMOVAs across five spatial attributes tested (Table 3) ascribed most of the variance to within individuals (i.e., samples, approximately 99%). The remaining variance was ascribed to between-population differences (0.83% to 3.19%) and within-population differences (-1.83% to -10.53%). The magnitude of data explained (*phi*) was low across all subpopulations

and tests (approximately = 0.00). However, across the attributes that were tested, the attribute with the largest between subpopulations (between pop) differences were management unit, north-central-south delineations, and inner-outer (*phi* = 0.03, 0.01, and 0.01, respectively). STRUCTURE will not allow for more *a priori* subpopulations than total K tested (i.e. 5), as such management unit distinctions were not used. North-central-south, and inner-outer distinctions were used to inform the second round of STRUCTURE analyses. When given *a priori* north-central-south information, admixture was found across all Ks tested; however, delta K and LnP(K) suggested that either K = 3 or K = 4 would be the best fit for these data, and so K = 3 was chosen (Figure 10). The K = 3 plot was highly admixed with only a slight difference in the distribution of the third genetic group (purple) between the southern group and the rest. When STRUCTURE was given inner-outer *a priori* information, a similar degree of admixture was clear across all Ks tested, again delta K suggested K = 3 and LnP(K) suggest K = 4 and therefore K = 3 chosen (Figure 10). Again, a slight difference in the distribution of the third genetic group between the inner and outer groups, but both subpopulations remained highly admixed (Figure 10).

## Characterizing Predicted Subpopulations

Each of the tested and predicted subpopulations from both the spatially agnostic and explicit analyses were characterized, and gave similar results. Observed heterozygosity ranged from 0.32 to 0.35, with corrected expected heterozygosity being higher across all subpopoulations ranging from 0.40 to 0.53 (Table 4). DAPC had the largest difference between observed and expected heterozygosity (Ho = 0.32, $H_T$' = 0.53), with STRUCTURE informed by north-central-south information having the smallest difference (Ho = 0.35, $H_T$' = 0.40). Corrected $F_{ST}$ values were low across all categories (0.00 – 0.02) except for the DAPC-predicted subpopulations, AMOVA tested Inner-Outer groups, and the basic STRUCTURE analysis (0.15, 0.15 and 0.23, respectively). The spatially agnostic (DAPC, STRUCTURE) predicted subpopulations were then mapped to the landscape by the management unit of origin (Figure 5). For STRUCTURE outputs, individuals were assigned a genetic group based on the largest fraction of the individuals genetic group based on the largest fraction of the individuals genetic makeup as determined by the analysis. All predicted subpopulations were highly admixed.

# DISCUSSION

Using 902 SNPs to genetically characterize 119 cougars sampled across a broad geographic range spanning much of Alberta, we identified no population genetic structure or isolation by distance. While the number of SNPs used for my analyses is less than many ddRAD-Seq studies report, the extensive filtering and quality control that I conducted on the initial SNP data set ensured that the SNPs that I used in this study were of high quality (O'Leary et al. 2018). Taking into consideration the quality of sample that was available for this study, I prioritized SNP quality and retention of individuals to develop the final data set used for population genetic analyses. This approach was facilitated by use of a heterologous reference genome, and not focusing on SNP quantity (O'Leary et al. 2018). All the tests that I conducted yielded a consistent pattern of nonsignificant genetic differentiation among samples and subpopulations. In the case of the spatially agnostic tests, no clear environmental or geographic variables emerged (e.g., barriers) when these data were mapped. DAPC provided the most distinct subpopulations, but given the lack of geographical pattern in their distribution and small group size (n = 12) of the second predicted sub population, this is unlikely to be indicative of population structure. Observed heterozygosity was high across all subpopulations and across individuals, indicating high genetic diversity and movement in this population. However, lower than expected heterozygosity under Hardy-Weinberg equilibrium is expected, as an expanding population violates the basic assumptions of Hardy-Weinberg (Austerlitz et al. 1997). Violation of Hardy-Weinberg equilibrium could also indicate structure; however, even with the assistance of *a priori* location information in the spatially explicit analyses, putative subgroups showed little population differentiation. Interestingly, unlike population structure, I expected but did not find isolation by distance (Knopff et al. 2014). Specifically, no correlation between genetic and geographic distance was detected which is generally expected in an expanded population (Excoffier et al. 2009). However, in the absence of clear colonizing events paired with unlimited movement, populations can present as only growing in population size (Excoffier et al. 2009). Cougars are highly vagile, with reported cases of cougars moving 750 km in as little as 64 days (Elbroch et al 2009), and local examples of animals dispersing 135 km (Smereka et al. 2021). At a local scale, human-caused mortality can make up as much as 50% of a population within a given management unit (EP 2019; EP 2022). This degree of mortality can lead to high turnover as new territory becomes available. Consequently, the lack of IBD where IBD might otherwise

be expected can be attributed to this species' tendency for movement and local mortality dynamics. Collectively, these factors contribute to a panmictic population.

A population exhibiting both lack of IBD and population genetic structure is unique in the cougar literature. Many cougar populations are experiencing population decline, loss of genetic diversity, and inbreeding (Castilho et al. 2011; Ernest et al. 2014; Gallo et al. 2020, 2021; Huffmeyer et al. 2022; Miotto et al. 2011; Trumbo et al. 2019; Wultsch et al. 2023) or subpopulation structuring (Balkenhol et al. 2014; Gustafson et al. 2019; Hedrick and Fredrickson 2010; Saranholi et al. 2017). In all populations that have been studied to date, connectivity and human-caused mortality are noted as suspected contributors to species decline. While the landscape in Alberta is heavily modified, most areas have extensive networks of linear features, such as roads and seismic lines, that cougars are known to use (Smereka et al. 2021), and human-caused mortality, while high, is regulated to prioritize a large population (AESRD 2012). Additionally, prey availability is a noted explanatory factor when considering the presence of predators (Fisher and Ladle 2022). White-tailed deer (*Odocoileus virginianus*), a common prey item for cougars in Alberta (Smereka et al. 2021), have increased in abundance and have expanded their range expansion in Alberta and throughout Canada (Dawe and Boutin 2016; Latham et al. 2011).

The growth in cougar populations and range is not necessarily generalizable to other heterospecific predators. For example, black bears (*Ursus americanus*) and brown bears (*Ursus arctos*) in this area primarily consume plants (López-Alfaro et al. 2015; Raine and Kansas 1990). Wolves, do consume white-tailed deer (Fischer and Ladle 2022; Latham et al. 2011); these predators have also been reported to have limited population structure (Cullingham et al. 2016) and are hunted without quota limits in Alberta, in part due to public distrust. The cougar population structure in Alberta may be more similar to that of a mesocarnivore like coyotes (C*anis latrans*), which has a recent history of range expansion and displays panmictic structure in some populations (Bohling et al. 2017; Laliberte and Ripple 2004), in part attributed to high turnover from unsuccessful eradication programs (Heppenheimer et al. 2018; Kierepka et al. 2017; Williams et al. 2003). Specifically, coyote populations, once depopulated, can rebound quickly due to immigration from surrounding areas (Kierepka et al. 2017).

# CONCLUSION

In conclusion, likely because of high turnover, landscape connectivity, increased prey availability, and decreased human-caused mortality, cougars in Alberta have expanded in both range and population size in a panmictic manner. Given no barriers to expansion, this population can be expected to remain homogeneous and continue expanding where suitable habitat exists. Moving forward, managers will need to consider the potential ramifications of a predator species recolonizing areas from which they have long been extirpated. Local human populations will need to be educated on how to safely coexist with these species, and impacts on vulnerable prey species must be considered. Alternately, if continued range expansion is not acceptable to stakeholders and rightsholder, managers will need to consider the struggle noted from coyote depopulation efforts. Simply increasing alotted harvest may result in selective pressure as hunters have been found to select older male cats, presumably for their increased size (Clark et al. 2014). Consistent size-selective hunting pressure can negatively impact long-term genetic diversity, development, social structure, and behaviour (Allendorf et al. 2008; Cooley et al. 2009; Fenberg and Roy 2008). Further research is needed to continue monitoring this species as it expands, including sampling from new edges of the species range and from surrounding populations which may act as a source of new cats. Future research should consider employing a more targeted sampling design with specific attention to sample quality and storage.

# TABLES

**Table 1:** Origin and sex of cougar tissue samples successfully sequenced and retained for analysis by ecotype across Alberta, Canada.

| | Female | Male | Unknown | Grand Total |
|---|---|---|---|---|
| Boreal | 5 | 4 | 2 | 11 |
| Foothills | 38 | 44 | 6 | 88 |
| Mountain | 7 | 8 | 5 | 20 |
| Total | 50 | 56 | 13 | 119 |

**Table 2.** Sample type, concentration of DNA by spectrophotometry (Nanodrop) and fluorometry (Qubit) as well as a purity estimate (260/280 by Nanodrop) separated by stage. Pure DNA is approximately equal to 1.8.

| | Sample Type | Nanodrop Concentration (ng/µl) | 260/280 | Qubit Concentration (ng/µl) | Sample Count |
|---|---|---|---|---|---|
| Extracted | Hair | 19.46 | 1.85 | 11.07 | 143 |
| | Tissue | 30.44 | 1.88 | 19.55 | 187 |
| | All | 25.68 | 1.87 | 15.88 | 330 |
| Sequenced | Hair | 24.71 | 2.02 | 22.03 | 52 |
| | Tissue | 36.12 | 1.89 | 26.52 | 120 |
| | All | 32.38 | 1.93 | 25.04 | 187 |
| Final Individuals | Hair | 27.45 | 2.01 | 27.03 | 30 |
| | Tissue | 35.33 | 1.88 | 26.84 | 89 |
| | All | 33.35 | 1.91 | 26.89 | 119 |

**Table 3.** Analysis of Molecular Variance (AMOVA) between subpopulations, within subpopulations, and within individuals across different spatially explicit subpopulations of cougars from across Alberta, Canada. Subpopulations were determined based on the sampling location (management unit) and associated metadata including local ecology (ecotype) and region (north-central-south, inner-outer, and east-west).

| Sub-populations | Test | Df | Sum Sq | Mean Sq | Proportion of Variance | % Variance | *phi* |
|---|---|---|---|---|---|---|---|
| Management Unit | Between pop | 38 | 13893 | 365 | 10.36 | 3.19 | 0.03 |
| | Within pop | 80 | 24338 | 304 | -10.53 | -3.24 | -0.03 |
| | Within samples | 119 | 38708 | 325 | 325.28 | 100.05 | -0.00 |
| Ecotype | Between pop | 2 | 923 | 461 | 2.82 | 0.86 | 0.01 |
| | Within pop | 116 | 37308 | 322 | -1.83 | -0.56 | -0.01 |
| | Within samples | 119 | 38708 | 325 | 325.28 | 99.69 | -0.00 |
| North – Central – South | Between pop | 2 | 1222 | 611 | 4.00 | 1.23 | 0.01 |
| | Within pop | 116 | 37009 | 319 | -3.12 | -0.96 | -0.01 |
| | Within samples | 119 | 38708 | 325 | 325.28 | 99.73 | -0.00 |
| Inner – Outer | Between pop | 1 | 708 | 708 | 3.46 | 1.06 | 0.01 |
| | Within pop | 117 | 37524 | 321 | -2.28 | -0.70 | -0.01 |
| | Within samples | 119 | 38708 | 325 | 325.28 | 99.64 | 0.00 |
| East – West | Between pop | 1 | 629 | 629 | 2.72 | 0.83 | 0.01 |
| | Within pop | 117 | 37603 | 321 | -1.94 | -0.60 | -0.01 |
| | Within samples | 119 | 38708 | 325 | 325.28 | 99.76 | 0.00 |

**Table 4:** Observed and expected heterozygosity (subpopulation and total) and $F_{ST}$ estimates across all subpopulations of cougars tested across Alberta Canada. Where ' is present indicates that this value has been corrected for heterozygosity. Subpopulations are based on geographic information associated with sampling locations as well as predicted groups from genetic population structure analyses (spatially agnostic and explicit means).

| | $H_O$ | $H_S$ | $H_T$ | $H_T$' | $F_{ST}$ | $F_{ST}$' |
|---|---|---|---|---|---|---|
| All | 0.35 | – | – | – | – | – |
| Management Unit | 0.34 | 0.38 | 0.41 | 0.41 | 0.07 | 0.07 |
| Ecotype | 0.35 | 0.42 | 0.42 | 0.42 | 0.01 | 0.01 |
| North-Central-South | 0.35 | 0.40 | 0.40 | 0.41 | 0.01 | 0.01 |
| Inner-Outer | 0.35 | 0.40 | 0.41 | 0.41 | 0.08 | 0.15 |
| East-West | 0.35 | 0.40 | 0.41 | 0.41 | 0.00 | 0.01 |
| DAPC | 0.32 | 0.45 | 0.49 | 0.52 | 0.08 | 0.15 |
| STRUCTURE | 0.35 | 0.39 | 0.40 | 0.4 | 0.02 | 0.23 |
| Structure with locprior, North-Central-South | 0.35 | 0.40 | 0.40 | 0.4 | 0.01 | 0.01 |
| Structure with locprior, Inner-Outer | 0.35 | 0.39 | 0.40 | 0.4 | 0.02 | 0.02 |

# FIGURES

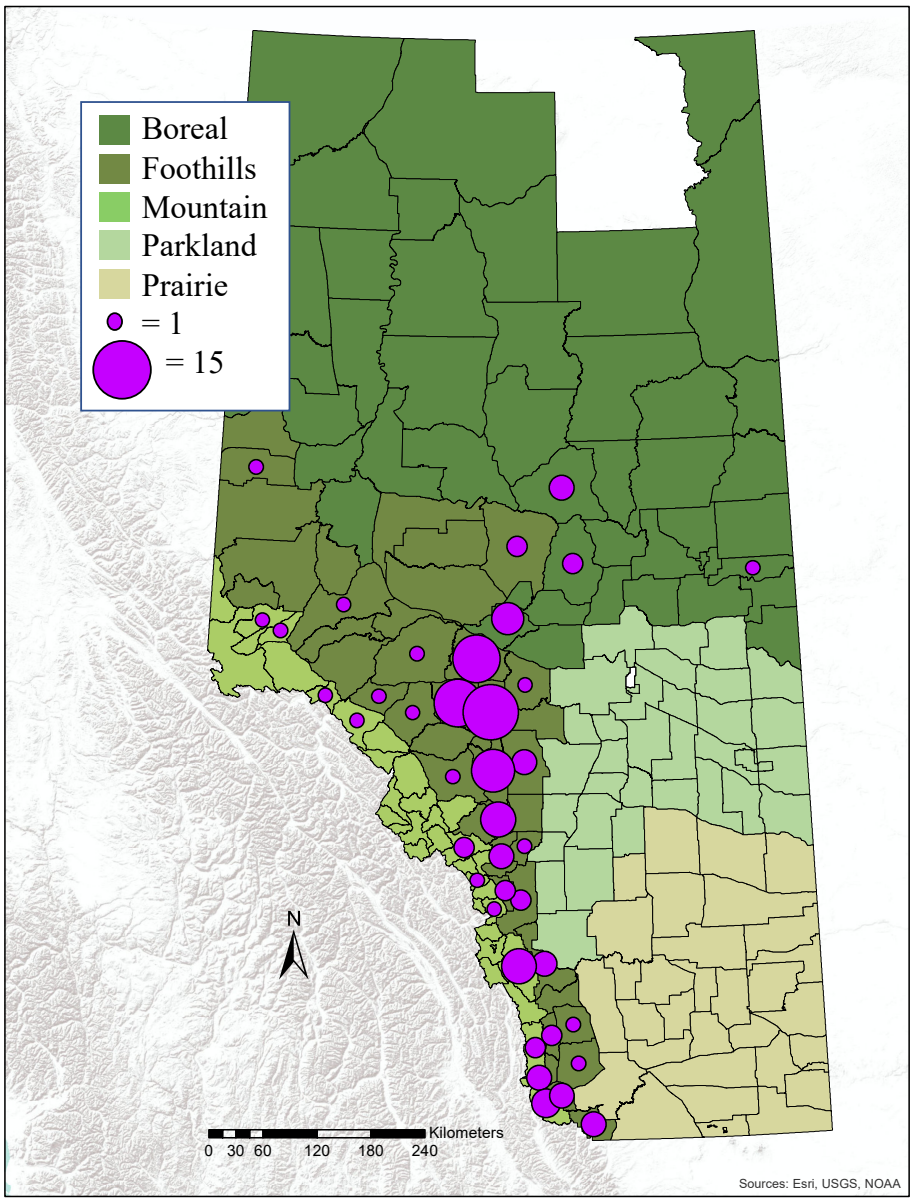


**Figure 1:** Sampling locations where circles are proportionate in size to the number of individuals sampled, mapped to management units in a western Canadian province (Alberta). Management units are colour coded by ecotype.

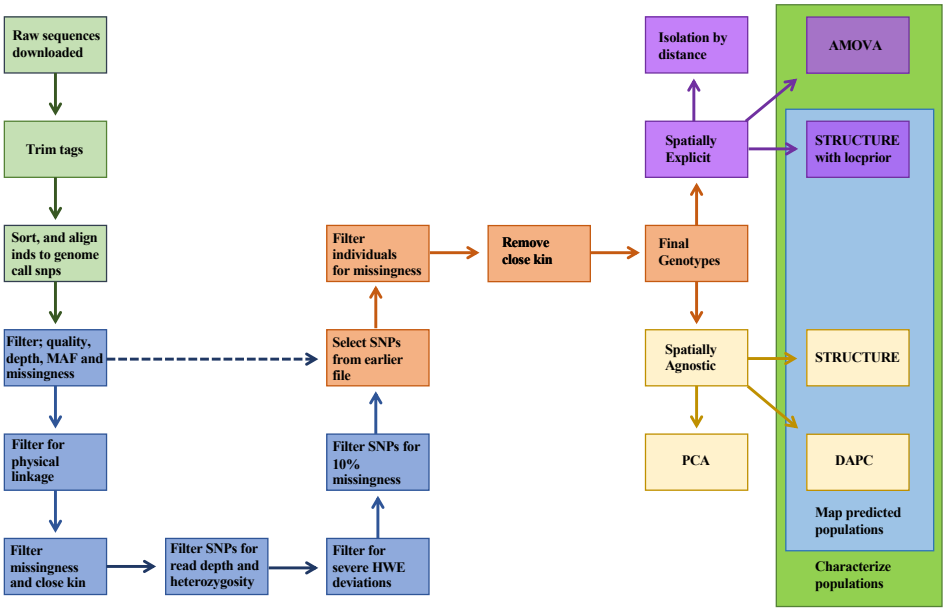


**Figure 2:** Data quality control, filtering and analysis pipeline, depicted from raw sequences to final analyses. Green boxes indicate pre-processing steps from original unprocessed sequence data, through processing (demultiplexing, trimming tags, aligning to the reference domestic cat genome, and indexing) through to SNP selection. Blue boxes indicate filtering for the highest quality individuals from which to pick SNPs. Specifically, the first two boxes show filtering for depth, quality, minor allele frequency (MAF), missingness across SNPs (0.5), and physical linkage. The remaining blue boxes show the selection process for high-quality individuals and SNPs where I filtered for missingness across individuals (0.5), close kin (> 0.23), SNP depth and heterozygosity as well as severe deviations from Hardy-Weinberg equilibrium (HWE; defined as $1.0x10^{-10}$). Finally, SNPs were filtered for 10% maximum missingness. Orange boxes represent pulling selected SNPs from pre-linkage filtered genotypes and repeating basic filters to prepare final genotypes for analysis (missingness across individuals allowing 0.5, and removal of kin). Yellow boxes indicate spatially agnostic population analyses, the first indicates converting genotypes into a structure file format for further analysis (DAPC, STRUCTURE, PCA). The first purple box indicates converting genotypes into a structure file format with location information and other geographic coordinate data needed for spatially explicit analyses (IBD, AMOVA, STRUCTURE with locprior). The light blue box indicates which final analyses produce putative subpopulations that were mapped. The greater green boxes indicate which tested and putative subpopulations were characterized by heterozygosity and $F_{ST}$.

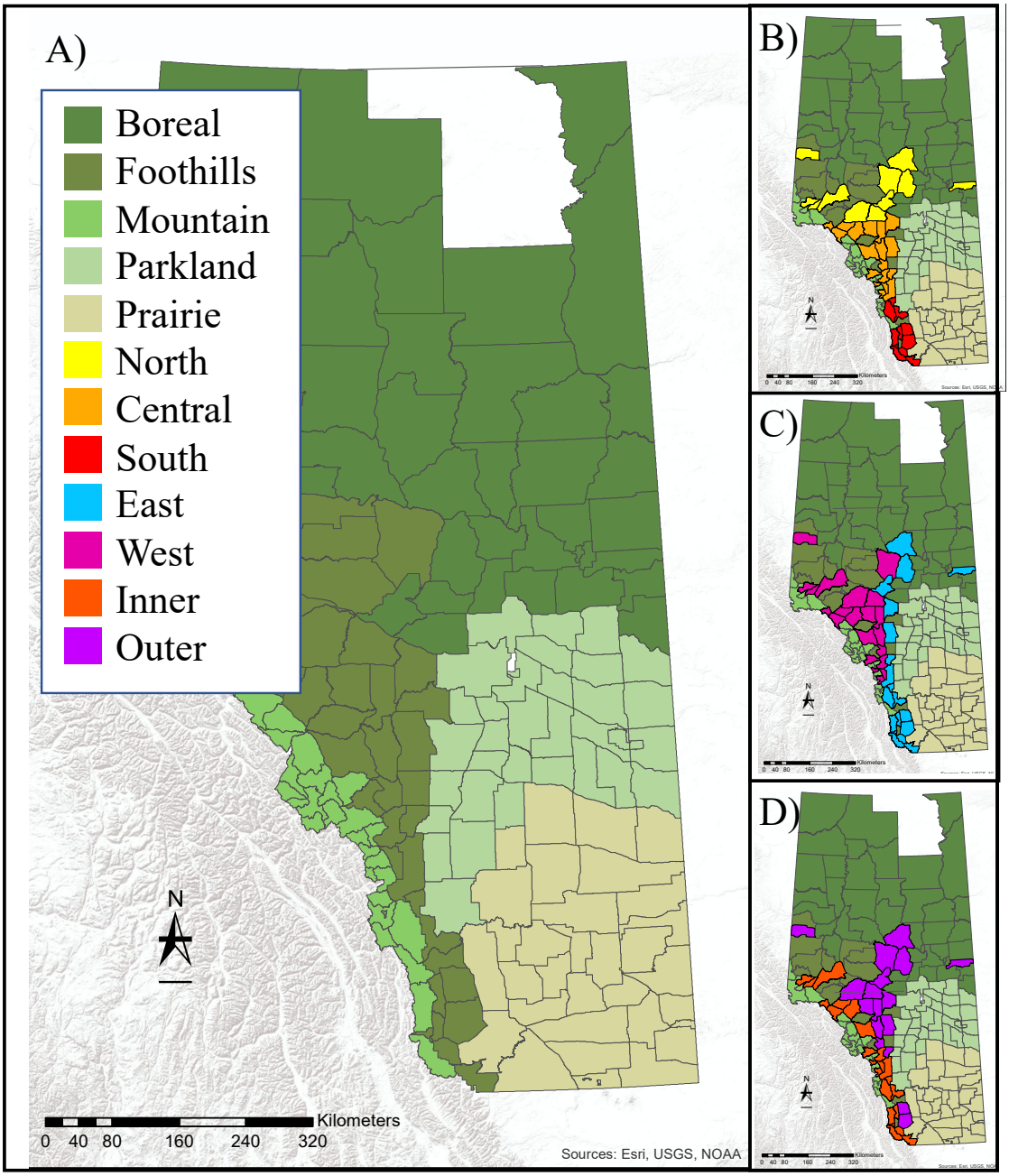


**Figure 3:** Distribution of wildlife units in a western Canadian province (Alberta) with ecologically and geographically informed subgroups. A) Includes provincial management units divided by provincially defined ecotype. B – D) Indicate wildlife management units where samples were collected and landscape attributes used as putative subgroups in subsequent analyses. B) depicts management units separated into north, central, and south, C) depicts east and west D) depicts inner (or mountain adjacent) and outer management units.

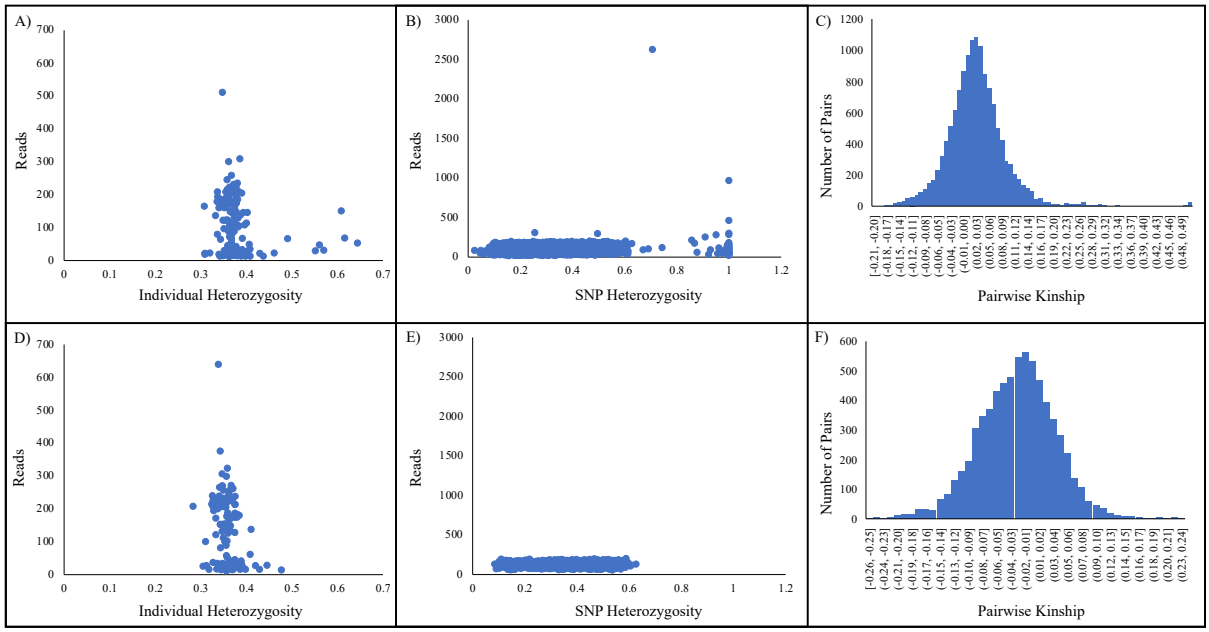


**Figure 4:** Heterozygosity, read counts, and pairwise kinship of individual cougars from across Alberta, Canada and SNPs pre and post-filtering. Prefiltering results include A) A scatter plot of individual heterozygosity (most are approximately = 0.35) and read count (most under 500). B) A scatter plot of SNP heterozygosity (ranging from, 0 to 1 with most between 0.1 and 0.6) and depth (most below 500) and C) a distribution plot of pairwise kinship across individuals which peaks at 0.02. Post-filtering results are plotted D-F in cougars from across Alberta, Canada. D) and E) show outliers with high and low heterozygosity were removed from analysis and F) shows peak is changed to -0.01.

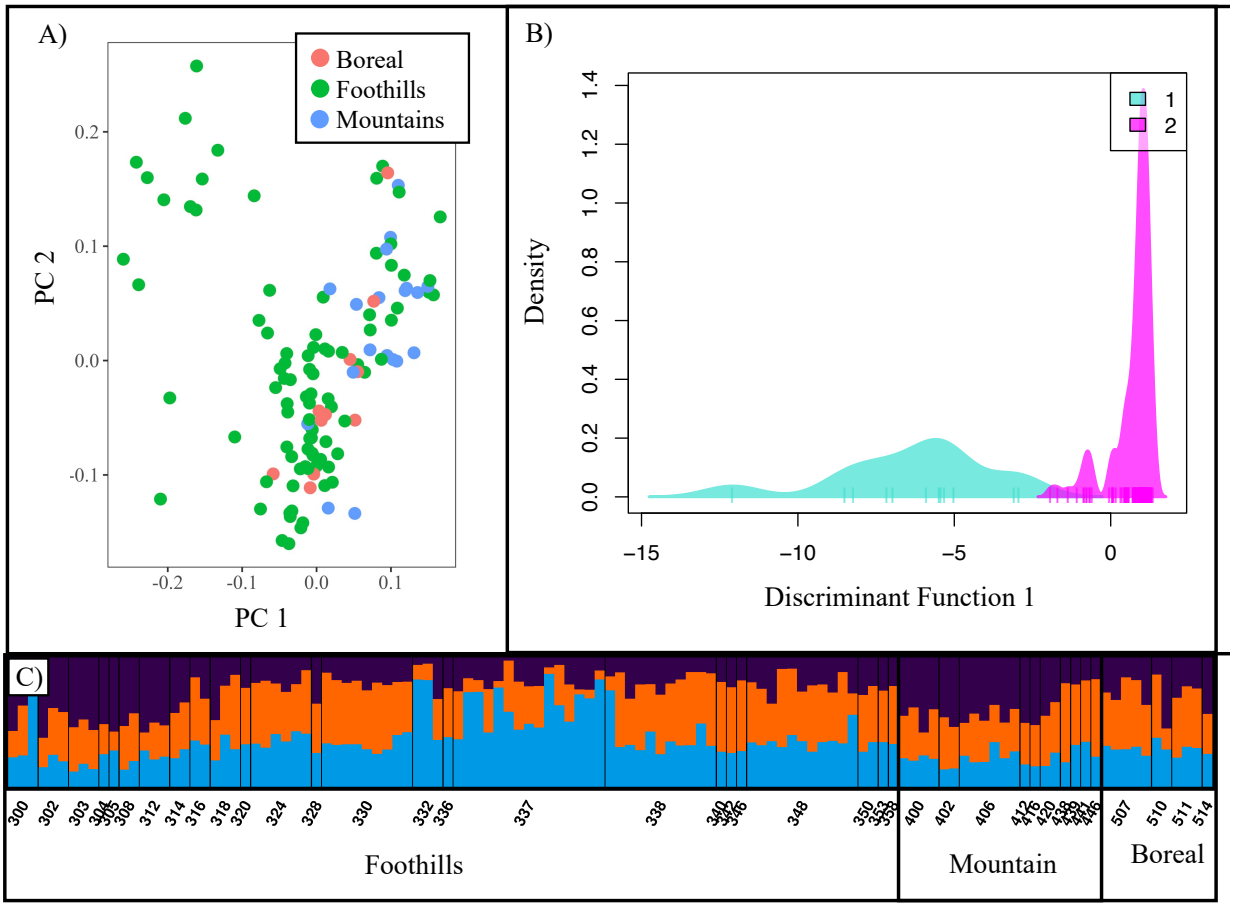


**Figure 5.** Spatially agnostic population structure analyses in 119 cougars from across Alberta, Canada. A) depicts Principal Component Analysis (PCA) of genotypes where each individual is coloured by ecological region. PC1 was found to explain 9.3% of the variation and PC2 explains 7.5% where green represents foothill samples (n = 88), pink represents boreal samples (n = 11), and blue samples represent mountain samples (n = 20) with 12 outliers from the foothills. B) is a Discriminant Analysis of Principal Components (DAPC) where K = 2 (blue n = 107, pink n = 12) with no SNPs contributing disproportionately (Figure 6). The twelve outliers identified in PCA and DAPC do not overlaps. C) depicts a bayesian probability of assignment with admixture (STRUCTURE) of K = 3 by delta K and LnP(K) plots (Figure 7), where each column is an individual, seperated by management unit and ecotype. The twelve individuals identified as distinct by PCA are represented as having a strong 'blue' genetic signature in the STRUCTURE analysis and the 12 identified by DAPC are highly admixed and distributed across the STRUCTURE plot.

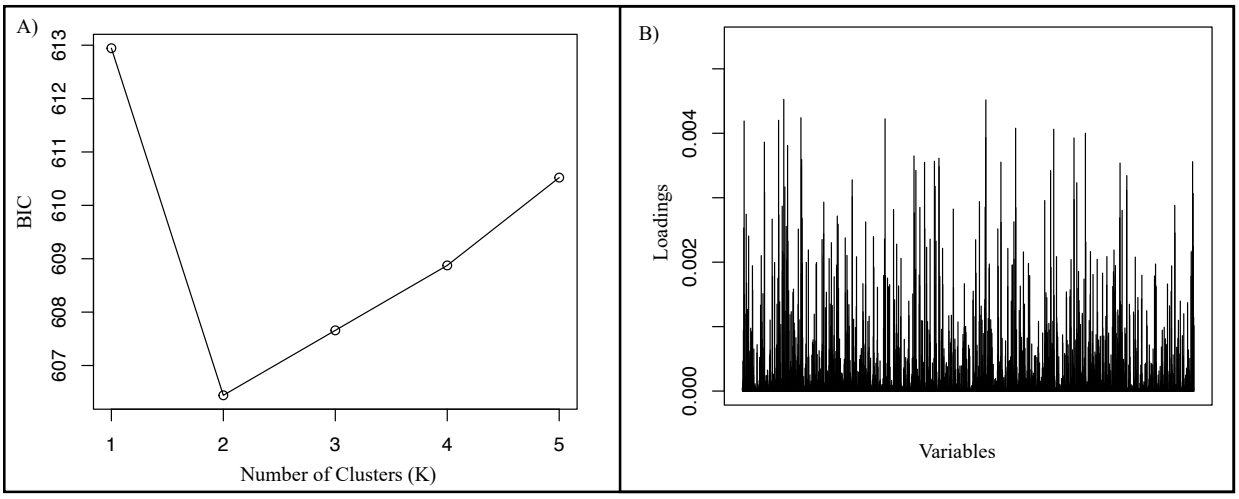


**Figure 6:** A *de novo* DAPC analysis of cougar samples from across Alberta, Canada. A) shows the BIC plot for Ks 1-5 with lowest BIC value for K =2 and B) shows the resultant variable contributions plot for the putative K = 2 division where no excess contribution (excess being defined as a loading value > 0.07) was found.

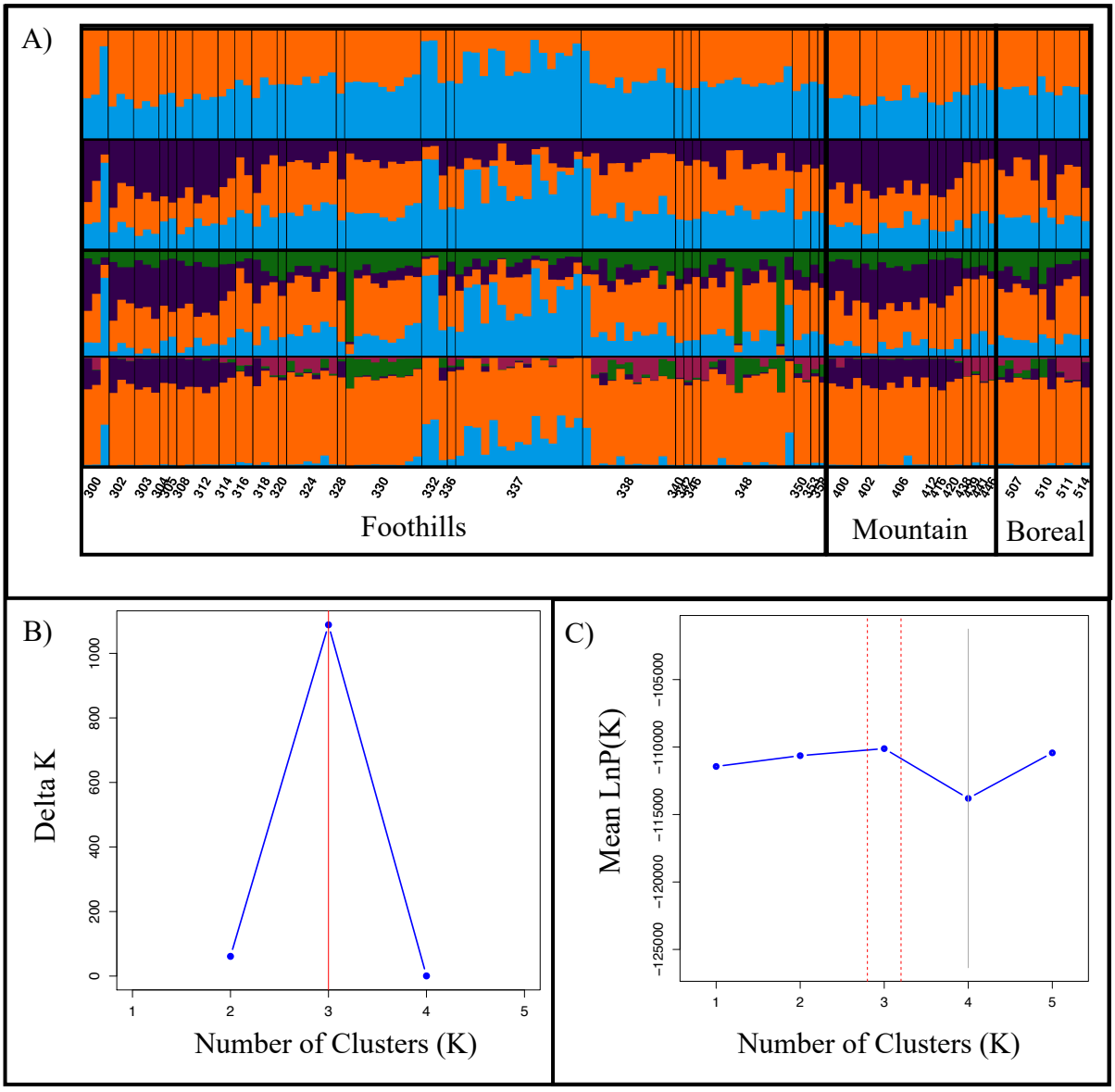


**Figure 7:** Bayesian probability of assignment allowing for admixture with no prior location information in STRUCTURE for cougars in Alberta, Canada. A) Bayesian structure plot where individual columns indicate an individual's admixture of genetic identities and black lines separate management units of origin across Ks 1–5. B) Delta K and C) LnP(K) plots for Bayesian probability of assignment analysis with admixture.

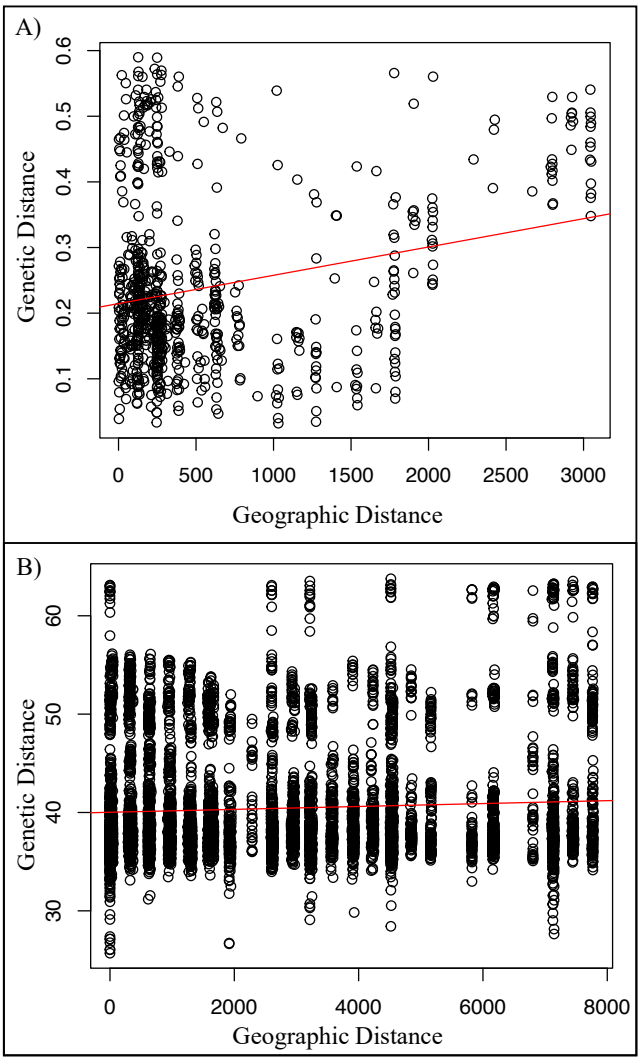


**Figure 8:** Mantel test of isolation by distance (IBD) of pairwise genetic and geographics data for cougars across Alberta, Canada. A) IBD plot where pairwise genetic distance between individuals was tested against geographic distance between their respective sampling locations where $r = 0.23$ and $p > 0.05$, B) is an IBD plot where the pairwise genetic distance between management units (based on allele counts of all individuals in each management unit) was tested against geographic distance between said management units where $r = 0.06$ and $p > 0.05$. Both A and B) used the geographic distance the centroids of each management unit where samples were collected.

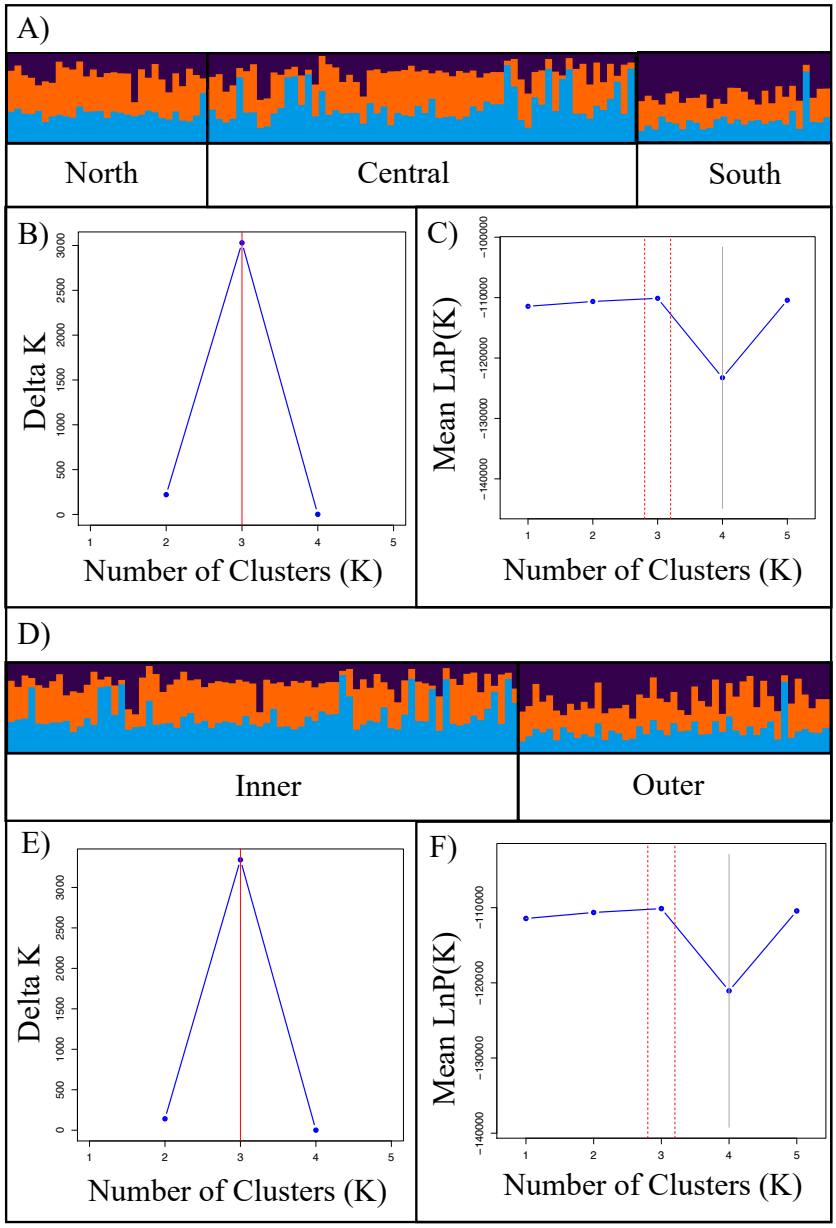


**Figure 9:** Two bayesian probability of assignment analyses allowing for admixture and using prior location (locprior) information in STRUCTURE for cougars in Alberta, Canada. A-C) uses north, central, and south location information based on the sampling location of samples. A) Shows STRUCTURE plot for K = 3 plot as this was the best fit in accordance with B) delta K and C) LnP(K) plots. D-F) uses inner and outer location information based on sampling location. D) Shows STRUCTURE plot for K = 3 plot as this was also the best fit K value in accordance with E) delta K and F) LnP(K) plots.

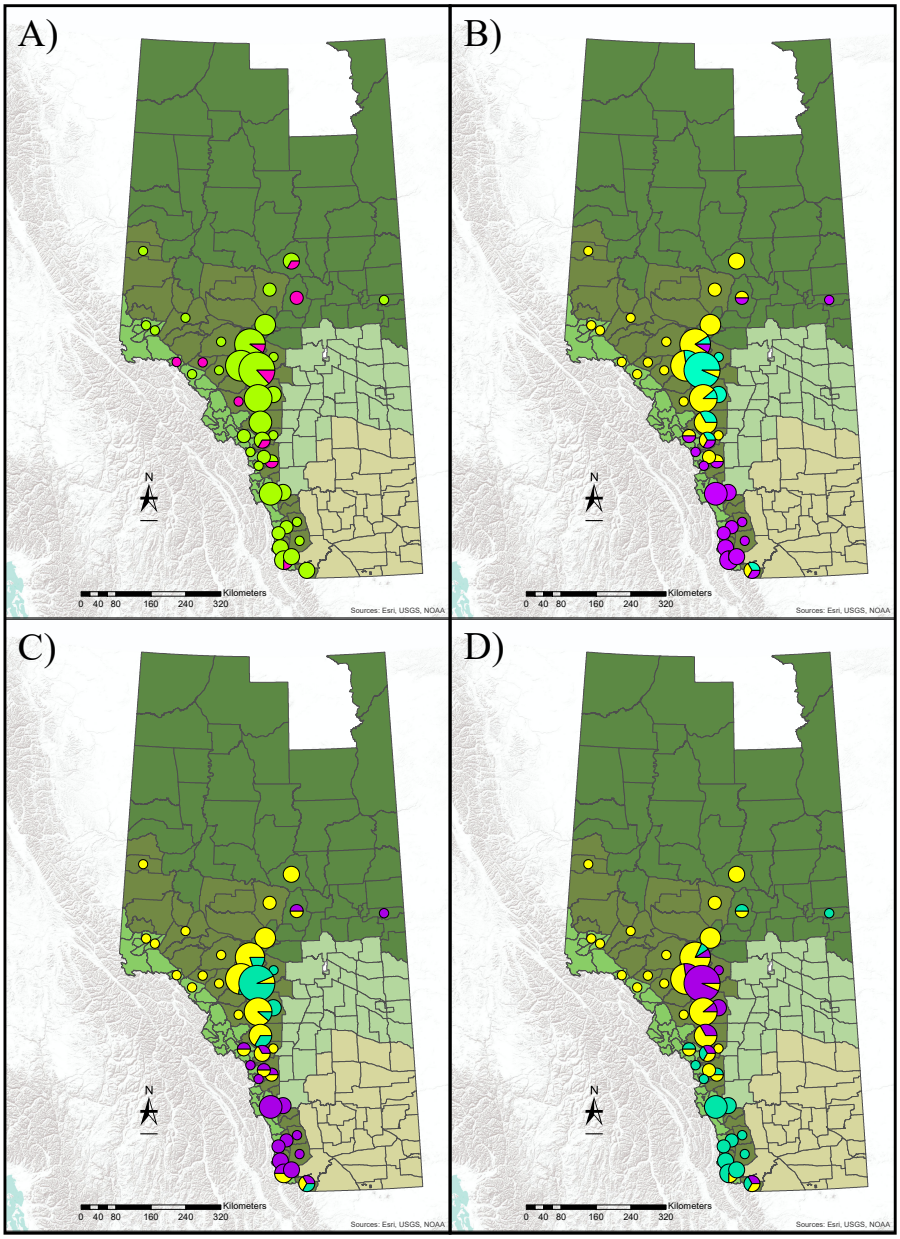


**Figure 10:** Putative genetic groups of cougars from Alberta (Canada) based on spatially agnostic and spatially explicit population genetic analyses. Distribution of putative subpopulations based on DAPC and STRUCTURE analyses. A) depicts putative DAPC (K = 2) subpopulations, B) depicts predicted STRUCTURE (K = 3) subpopulations without *a priori* location information. C) and D) depict STRUCTURE predicted subpopulations with *a priori* location information specifically C) used east-west (K = 3) information and D) used north-south (K = 3) location information.

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# APPENDICES

## Additional methods

**Appendix 1:** Modified DNeasy Blood and Tissue extraction protocol

Hair and tissue samples were added to a 1.5 mL tube to which 20 $\mu$l of Proteinase K and 300 $\mu$l of Buffer ATL were added. Samples were individually checked to ensure they were submerged before incubating for a minimum of 12 hrs at 56ºC or until tissue samples were visibly degraded. At this point, 300 $\mu$l of Buffer AL and 4$\mu$l of Linear Acrylamide were added before samples were vortexed. Samples were incubated for 10 minutes at 56ºC before 300 $\mu$l of 100% ethanol was added and vortexed again. The solution was pipetted into a spin column and centrifuged at 8 000 rpm for 1 minute. Flow through was discarded and 500 $\mu$l of Buffer AW1 was added. Samples were centrifuged (8 000 rpm for 1 minute) and flow through was discarded again. 500 $\mu$l of Buffer AW2 was added and again centrifuged (1 400 rpm for 3 min). Flow through was discarded once more and the spin column was transferred to a new collection tube. 200 $\mu$l Buffer AE was dispensed to the center of the spin column and incubated at room temperature for a minimum of 1 minute before centrifuging once more (8 000 rpm for 1 minute). Spin columns were discarded, and the solution (flow through) was transferred to its final storage tube and stored at -20ºC.

**A.2:** ddRAD-Seq library preparation protocol

DNA (10 $\mu$l of 20 ng/$\mu$l g) was digested with 10 $\mu$l of restriction master mix (2 $\mu$l of 10X Cutsmart reaction buffer, 0.4 $\mu$l Sbf1, 0.4 $\mu$l EcoR1, and 7.2 $\mu$l of water; New England Biolabs). These were then incubated at 37ºC for 2 hrs, then at 65ºC for 20 minutes, and held at 4ºC before progressing to ligation. Samples were ligated using 5 $\mu$l of working adaptor (16 individual 8 bp barcoded i5 indexes for Sbf1 cut site and a single adaptor for EcoR1 cut site) and 15 $\mu$l of ligation master mix (2 $\mu$l of 10X Cutsmart reaction buffer, 4 $\mu$l ATP at 10mM, 0.5 $\mu$l T4 DNA ligase at 400 000 U/mL, and 8.5 $\mu$l of water; New England Biolabs), for a total of 40 $\mu$l. Reactions were incubated for 80 minutes at 22ºC, 20 minutes at 65ºC, and held at 4ºC.

At this point, ligated samples were pooled (5 $\mu$l per individual) so that each pool contained one of each i5 primer (maximum of 16 individuals per pool and 80 $\mu$l). Each pool was cleaned using a QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA). Cleaned

and pooled reactions (10 $\mu$l) were then amplified with 5 $\mu$l of working adaptor (12 individual 8 bp barcoded i7 indexes for EcoR1 cut site) and 10 $\mu$l of amplification master mix (5 $\mu$l Phusion® High-Fidelity 5X Buffer, 2.5 $\mu$l 2mM dNTP, 1 $\mu$l of PCR1 primer (10uM), 1.25 $\mu$l of water, and 0.25 $\mu$l Phusion enzyme; New England Biolabs). Reactions were cycled heated to 98ºC for 30 seconds before cycling through 98ºC for 10 seconds, 54ºC for 20 seconds, 72ºC for 1 minute a total of 12 times. The reactions were then brought to 72ºC for 10 minutes and held at 4ºC. The final PCR product was pooled by i7 primer so that 7 $\mu$l of 12 reactions maximum were combined for a total of 84 $\mu$l and a maximum of 192 individuals in a final pool. The final pools were washed with Sera-Mag beads (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 30 minutes and washed with 80% ethanol twice. Samples were allowed to air dry completely before eluting out into 30 $\mu$l of EB.