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Genome-scale sampling suggests cryptic epigenetic structuring and insular divergence in Canada lynx

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Abstract

Determining the molecular signatures of adaptive differentiation is a fundamental component of evolutionary biology. A key challenge is to identify such signatures in wild organisms, particularly between populations of highly mobile species that undergo substantial gene flow. The Canada lynx (*Lynx canadensis*) is one species where mainland populations appear largely undifferentiated at traditional genetic markers, despite inhabiting diverse environments and displaying phenotypic variation. Here, we used high-throughput sequencing to investigate both neutral genetic structure and epigenetic differentiation across the distributional range of Canada lynx. Newfoundland lynx were identified as the most differentiated population at neutral genetic markers, with demographic modelling suggesting that divergence from the mainland occurred at the end of the last glaciation (20–33 KYA). In contrast, epigenetic structure revealed hidden levels of differentiation across the range coincident with environmental determinants including winter conditions, particularly in the peripheral Newfoundland and Alaskan populations. Several biological pathways related to morphology were differentially methylated between populations, suggesting that epigenetic modifications might explain morphological differences seen between geographically peripheral populations. Our results indicate that epigenetic modifications, specifically DNA methylation, are powerful markers to investigate population differentiation in wild and non-model systems.

KEYWORDS
DNA methylation, ecological epigenetics, population differentiation

1 | INTRODUCTION

Investigations into adaptive differentiation have relied on either quantifying phenotypic variation or contrasting genetic polymorphisms identified in an organism's DNA (Beaumont & Balding, 2004; Stinchcombe & Hoekstra, 2008). Environmental conditions are powerful drivers of population differentiation and phenotypic divergence, where relationships can be ascertained by correlating allele frequencies to environmental and morphological variation, which is consistent with local adaptation (Shafer & Wolf, 2013). Detecting adaptive differentiation in species that experience high rates of gene flow, however, can be challenging to detect due to the homogenization of genomic regions that are neutral or under weak selection (Feder, Egan, & Nosil, 2012). Moreover, an aspect of molecular variation that is undetected by standard genetic sequencing involves direct modifications to the structure of DNA. Epigenetic modifications like DNA methylation are influenced by environmental conditions and affect gene expression, and thus could be indicative of early divergence due to local adaptation (Greenspoon & Spencer, 2018; He et al., 2018; Vogt, 2017).

DNA methylation has been implicated in mediating ecologically relevant traits in response to varied environmental conditions

(Artemov et al., 2017; Baerwald et al., 2016; Lea, Altmann, Alberts, & Tung, 2016), primarily due to its regulatory relationship with gene expression, particularly around CpG islands that are dense clusters of cytosine-guanine dinucleotides (Fujita et al., 2003; Lorincz, Dickerson, Schmitt, & Groudine, 2004; Maunakea et al., 2010). If heritable epigenetic modifications are imparted by specific environments, and these modifications increase fitness regardless of underlying genetic sequence (Herman & Sultan, 2016), then these epigenetic loci have important long-term evolutionary consequences (Hu & Barrett, 2017). However, the relationship between epigenetic modifications and underlying genetic sequence is difficult to disentangle, where epigenetic modifications fall on a spectrum of complete dependency on a genetic variant, semi-dependency where genetic variation does not explain the entirety of the phenotypic variation, or autonomy where epigenetic modifications exist independently of genetic variation (Richards, 2006). Therefore, determining the extent of both genetic and epigenetic structuring across geographic and environmental space adds unprecedented power for assessing population-level variation and the molecular responses of local adaptation. Consequently, DNA methylation and neutral genetic markers could be used to characterize populations and investigate adaptive differentiation in species that exhibit low standing genetic variation or complete panmixia, despite extensive differences in available resources and conditions (Sentis et al., 2018).

Here, we assessed the population structure and environmental determinants of epigenetic variation in a free-ranging carnivore. Our study species, the Canada lynx (*Lynx canadensis*), is a midsized felid that is highly mobile with neutral genetic variation (i.e., microsatellites) that exhibits low levels of genetic differentiation across the mainland with divergent island populations in eastern Canada, although the time of divergence is unknown (Prentice et al., 2017; Row et al., 2012). Canada lynx populations are stratified more strongly according to climatic patterns than by geographic distance (Row et al., 2014), and thus provide an intriguing system for examining genetic and epigenetic structuring across a range of geographic and environmental conditions. We first used neutral genetic markers to retrace the historical demography of lynx across North America. We then generated methylation data to test the following predictions: (a) there would be distinct epigenetic divergence between geographically peripheral populations of Canada lynx due to hypothesized climatic subdivisions between populations (Row et al., 2012, 2014; Stenseth et al., 1999) and the environmentally-induced plasticity of DNA methylation; and (b) differential methylation would occur over genes related to morphology in the Newfoundland population, as this population exhibits smaller size (Khidas, Duhaime, & Huynh, 2013; Van Zyll De Jong, 1975) consistent with the "Island Rule" (Foster, 1964; Lomolino, 2005) and DNA methylation could mediate rapid phenotypic change if divergence with the mainland was recent (Gore et al., 2018).

2 | MATERIALS AND METHODS

2.1 | Study system

We collected 95 Canada lynx epidermal tissue samples from four populations across North America, including one insular population in Newfoundland (Figure 1; Table S1). The sampled populations have a wide geographical spread, with an average minimum distance between populations (Québec and Newfoundland) of 1,158 km and a maximum distance (Alaska and Newfoundland) of 5,520 km. Habitats around these populations present a dynamic range of environmental conditions, ranging from 32 to 432 mm of winter precipitation and a mean annual temperature range of −6.3°C to 4.7°C (Fick & Hijmans, 2017). Tissue was consistently taken from the same morphological location from each adult-sized pelt (i.e., front leg), although sex was not determined.

2.2 | Library preparation and high-throughput bisulphite sequencing

We adapted an existing genotyping-by-sequencing bisulphite sequencing workflow designed for multiplexed high-throughput sequencing to maximize sample size while sacrificing loci (van Gurp et al., 2016). Genomic DNA was isolated, quantified and standardized for 95 Canada lynx samples, digested with NsiI and AseI restriction enzymes overnight, and subsequently ligated with methylated

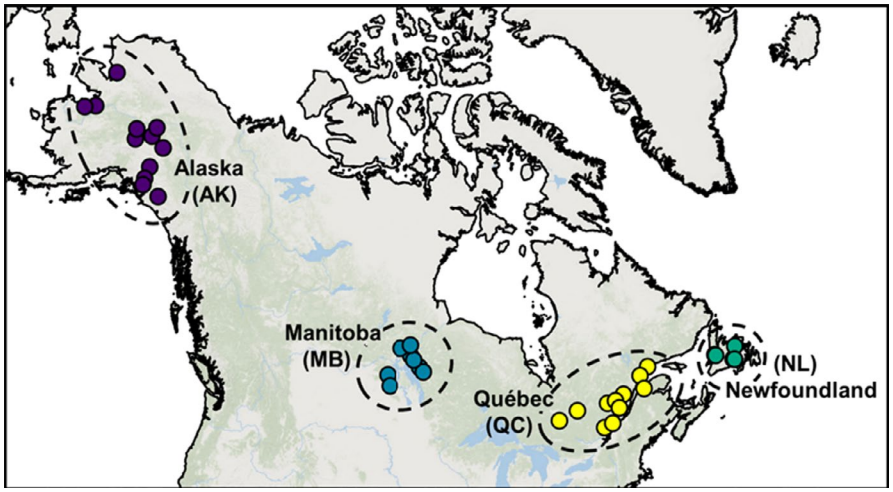

FIGURE 1 Distribution of 95 Canada lynx *(Lynx canadensis)* samples across North America, selected from four geographical areas which were compared at the genetic and epigenetic level

adapters (Table S2). An individual sample of completely nonmethylated lambda phage genomic DNA (200 ng; Sigma-Aldrich – D3654) with a unique barcode was included to assess bisulphite conversion efficiency (Table S3). Barcoded samples were combined into eight pools to ensure consistent reaction environments for the entire library using a QIAquick PCR Purification Kit (Qiagen) following manufacturer's instructions. We performed a SPRI size selection on each pool, repaired nicks between the 3′ fragment overhang and the 5′ nonphosphorylated adapter nucleotide, and performed bisulphite conversion on each pool using an EZ DNA Methylation-Lightning Kit (Zymo Research).

Pools were amplified in three separate PCRs to mitigate stochastic differences in amplification and were subsequently concentrated using a QIAquick PCR Purification Kit (Qiagen). The eight pools were quantified with Qubit 3.0 (Thermo Fisher Scientific) and appropriate amounts were added to a final super-pool for equal weighting. A final SPRI clean-up was performed to remove any adapter dimer. We checked final library concentration and fragment distribution with a Qubit 3.0 (ThermoFisher Scientific) and Hi-Sense Bioanalyzer 2100 chip (Agilent; Figure S1). Paired-end 125 bp sequencing was performed on a single lane on an Illumina HiSeq 2500 platform with updated software appropriate for BS-seq data sets (HCS v2.2.68/RTA v1.18.66.3) at the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Ontario, Canada).

2.3 | SNP calling, neutral genetic structure and historical demography

We assessed sequencing success and removed adapter and low-quality reads via FastQC (Andrews, 2010) and Cutadapt (Martin, 2011) implemented in TrimGalore! v0.4.4 (Krueger, 2012). Individuals were demultiplexed using python scripts (van Gurp et al., 2016). Paired-ends reads for Canada lynx samples were initially aligned to several genomes (*Felis catus*, *Homo sapiens*, Lambda Phage) using Bismark (Krueger & Andrews, 2011) to assess mapping efficiency and contamination (Table S1). Paired-end reads for downstream analyses were aligned to the domestic cat genome with Bismark, using Bowtie2 default settings and parameters seen in other studies (Kobayashi et al., 2016; Krueger & Andrews, 2011). We selected the cat genome due to our interest in functional annotations and the multiple revisions this genome has undergone resulting in a chromosome-scale assembly that is highly syntenic to the Canada lynx genome (Figure S2; NCBI: felcat9.0; GCA_000181335.4).

SNP calling from bisulphite-converted reads is amenable for defining population structure (Lea, Vilgalys, Durst, & Tung, 2017); thus we called SNPs from bisulphite converted reads using CGmapTools (Guo et al., 2017) that applied a coupled Bayesian wildcard algorithm with a conservative 0.01 error rate and a static 0.001 *p*-value for calling variant sites. Retained sites had at least a sequencing depth of five, were shared by at least 50% of individuals, were in Hardy-Weinberg equilibrium, and not shown to be under selection according to Bayescan (Foll & Gaggiotti, 2008; Figure S3). A pairwise Euclidean dissimilarity (distance) matrix was computed using the function *daisy* within the package *cluster* (Maechler, Rousseeuw, Struyf, Hubert, & Hornik, 2018) using R v3.4.2 (R Core Team, 2017). This dissimilarity matrix was then summarized in a principal coordinates analysis (PCoA) using the *dudi.pco* function in *adegenet* (Jombart, 2008). Missing data were imputed by mean allele at a population level and pairwise F_{ST} was calculated using VCFtools (Danecek et al., 2011). AMOVA between populations was computed using the *poppr.amova* function within *poppr* (Excoffier, Smouse, & Quattro, 1992; Kamvar, Tabima, & Grünwald, 2014; Figure S4). We determined if genetic structuring was consistent with patterns of isolation by distance using a distance-based redundancy analysis (db-RDA) with the first three axes of a PCoA on SNP data as response variables with the first axis of a PCoA on a Euclidean distance matrix of latitude and longitude as an explanatory variable.

Historical demography was assessed using the program ∂a∂i (Gutenkunst, Hernandez, Williamson, & Bustamante, 2009), which can be used to assess the fit between various demographic scenarios and the expected allele frequency spectrum according to diffusion equations; moreover it is suitable for our neutral SNP data (Shafer, Gattepaille, Stewart, & Wolf, 2015). For model selection, we used the demographic models outlined in Charles et al., 2018 (Figure S5, Table S4), within the model selection framework described in Portik et al. (2017) and compared to simulations generated using the process within Barratt et al., 2018. Further details on the methods involving our demographic inferences can be found in Supporting Information.

2.4 | Methylation calling

We generated a custom CpG island annotation track using hidden Markov models based on $CpG_{o/e}$ implemented in *makeCGI* (Wu, Caffo, Jaffee, Irizarry, & Feinberg, 2010). Only islands with a calculated posterior probability >99.5% were retained for analysis. Mapped and extracted methylated sites were then imported into Seqmonk (Andrews, 2007) using the generic text importer, and raw data were qualitatively visualized against the annotated domestic cat genome (felCat9.0). Methylated sites with a sequencing depth of at least five were extracted from aligned BAM files using *Bismark* (Krueger & Andrews, 2011). We analyzed DNA methylation over CpG islands and gene bodies by creating 5,000 bp sliding windows directly over and 25,000 bp upstream of gene bodies, combined with windows directly over CpG islands. Remaining, unannotated regions of the genome were then also divided into 5,000 bp windows and added to the analysis. Each window was assigned a methylated percentage score based on the overall ratio of methylated to non-methylated bases within the feature. A window size of 5,000 bp was chosen based on maximizing a number of windows with all 95 individuals and is similar to other studies (Guo et al., 2011). We filtered this window-set for regions that had at least one CpG and equal representation from each population due to low coverage of some individuals, although many windows contained more CpGs (Table S6). No overlap between SNPs and DMPs were identified (Figure 2).

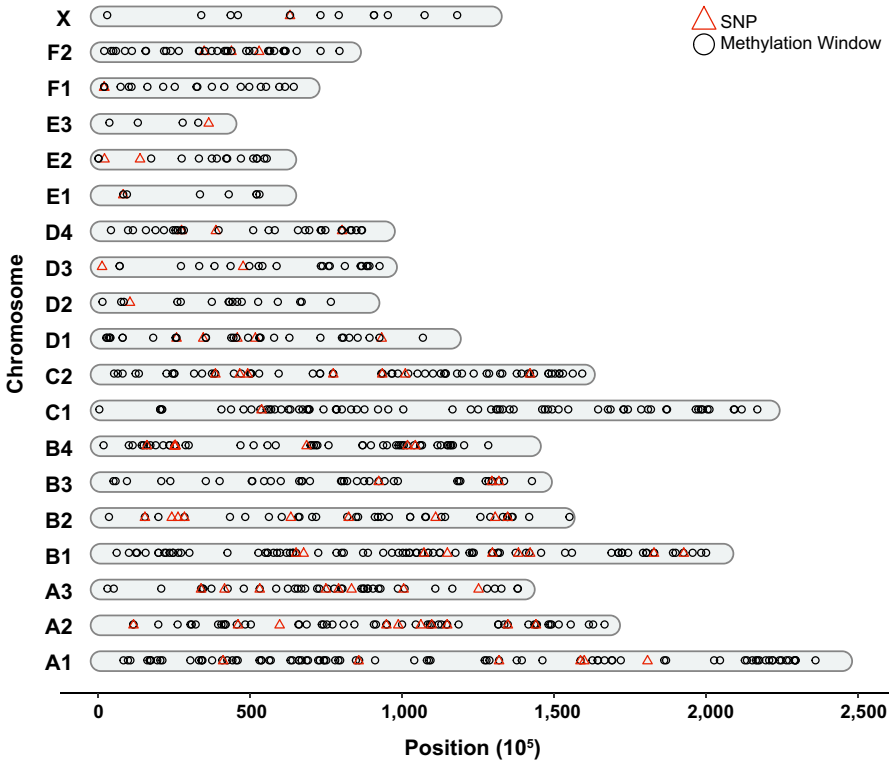

FIGURE 2 Sampling of the genome in this bisulphite sequencing study for 95 Canada lynx (*Lynx canadensis*), where filtered reads were mapped to the 19 chromosomes of the domestic cat

2.5 | Environmental determinants of epigenetic data

To determine if patterns of DNA methylation could be explained by macroscale winter conditions, geographic distance, or insular divergence, we performed a db-RDA on the summarized DNA methylation data. Meaningful axes explaining >30% of the cumulative variation in the data were used as response variables in db-RDA conducted in *vegan* (Dixon, 2003), using variables that putatively describe the environmental determinants of population structure in Canada lynx. Our covariates included a binary variable of insularity, which identified the Newfoundland population against mainland populations and was used to describe the largely impassable barrier of the Strait of Belle Isle between Newfoundland and mainland Labrador (Koen, Bowman, & Wilson, 2015). Although the strait is only 17.6 km at its narrowest (South, 1983) and is covered with an ice bridge for portions of the year, lynx avoid unforested habitats (Murray, Boutin, & O'Donoghue, 1994) and microsatellite analyses have identified only minimal rates of migration between Newfoundland and the mainland (Koen et al., 2015). A variable of geographic distance was included which was simply the first axis of a principal coordinates analysis (PCoA) on a Euclidean distance matrix of latitude and longitude (PCo1 = 99.7% of the variation, Figure S6). In addition to the geographic variables, we included a biotic variable of percent tree cover (DeFries, Hansen, Townshend, Janetos, & Loveland, 2000), a randomly generated numerical variable to assess the effect of noise, and a climate variable describing winter conditions. To create the climate variable, we performed a PCA on climate data to prevent multi-collinearity, which reduced annual temperature ranges, winter precipitation, and minimum coldest temperature to a single PCA axis (Fick & Hijmans, 2017) (PC1 = 85.6% of the variation, Figure S7). Linearity was confirmed between response and explanatory variables, and multi-collinearity between explanatory variables was assessed using the VIF and any variables >4 were removed (Table S7). Stepwise model selection using the function *ordistep* within *vegan* (Dixon, 2003) was performed to isolate the best overall model using a QR decomposition technique based on *p*-values (Table S8). To isolate the individual explanatory power of each variable, we performed partial distanced-based redundancy analyses (p-db-RDAs) on the variables that were identified as significant in the full db-RDA.

2.6 | Differentially methylated regions and gene ontology

We identified differentially methylated regions with functional biological correlates by performing beta-regressions on windows over CpG islands and gene bodies for all 95 individuals with percent methylation as the response variable and population as the explanatory variable, using a Bonferroni-corrected significance threshold of 3.19×10^{-5}. Beta regressions are appropriate for proportion or percentage data (Ferrari & Cribari-Neto, 2004), and we set an alpha threshold at conservative levels seen in similar studies (Le Luyer et al., 2017) ($p < 0.001$). Direct overlap between our differentially methylated regions and the felcat9.0 gene annotations were extracted using Seqmonk (Andrews, 2007). Samples were then categorized as "Mainland" or "Newfoundland" and population averages and standard error of the mean were calculated for visualization. We identified specific enrichment for biological functions of differentially methylated genes using the Panther-GO Gene Ontology Consortium database (http://geneontology.org/page/go-enrichment-analysis) with significance thresholds seen in other studies ($p < 0.05$; Le Luyer et al., 2017).

2.7 | Choice of reference genome and sensitivity analyses

We selected the chromosome-scale cat genome over the Iberian lynx (*Lynx pardinus*, Abascal et al., 2016) and the newly released Canada lynx (*L. canadensis*) genomes because they are fragmented scaffolds and not adequately annotated. To ensure this was not biasing our results we mapped a subset of reads to the lynx genome as well aligned the cat and lynx genome using E-MEM (Khiste & Ilie, 2015). We also performed sensitivity analyses to determine the impacts of missing data, temporal variation in sampling, batch effects, axis retention for db-RDAs, and feature-based divisions of analyses.

3 | RESULTS

Paired-end sequencing of our bisulphite-treated library generated a total of 210,773,612 filtered and demultiplexed reads. The distribution of methylated sites and SNPs are shown in Figure 2; which after filtering for shared positions and coverage left us with a total of 9,642 uniquely methylated polymorphic CpG positions in 705 windows and 85 neutral SNPs for characterizing population structure. Our choice of reference genome does not appear to have a significant impact due to similar mapping success and high synteny among felid genomes (see Figure S2, Table S1), and any undetermined downstream implications would affect all samples equally. The sensitivity analyses suggested experimental protocols and treatment of data did not impact overall inferences; more details can be found in Figures S8–S14.

3.1 | Historical demography and neutral genetic differentiation

We used the lowest AIC demographic model to estimate the time of divergence (τ_i) between the mainland and Newfoundland, suggesting a split during the last glacial maximum (τ_i = 26.5 ± 6.5 KYA; Figure 3, Table S5). The four likeliest demographic models all included a founder effect and had comparable levels of θ(3.1 ± 0.17), while the top two models both included asymmetric gene flow into Newfoundland (Table S13). This model indicated that Newfoundland likely experienced a founder effect with no contemporary admixture with the mainland, while overall diversity between populations was still relatively low (θ = 3.1; Figure S15). An additional one-dimensional site-frequency spectra (SFS) solely for Newfoundland corroborated our identification of a demographic bottleneck (Figure S16). An analysis of molecular variance identified extensive variation within samples (σ = 24.8) relative to overall variation between populations (σ = 0.46; Figure S4). Relative differences among all populations (ϕ = 0.02) was again negligible compared to the variation determined between Newfoundland and the mainland (ϕ = 0.09). Bayescan (Foll & Gaggiotti, 2008) confirmed neutrality of our loci (Figure S3) and a general pattern consistent with isolation by distance was observed R^2 = 0.31; $p \leq 0.001$. Population structure was relatively congruous among mainland populations (Figure 4), where the first axis largely described variation between the mainland and Newfoundland (PCo1 = 30.3%), while the second axis detected the more subtle differentiation between mainland populations (PCo2 = 6.1%).

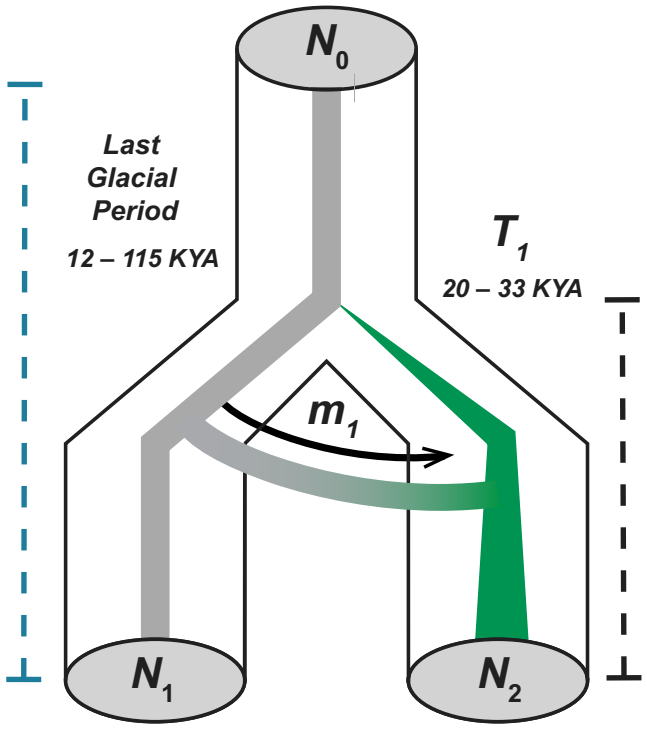

FIGURE 3 Schematic of the most likely demographic trajectory of mainland (N_1) and Newfoundland (N_2) Canada lynx (*Lynx canadensis*), as identified by the lowest AIC during model selection using the site frequency spectrum (SFS) determined using ∂a∂i. The model suggests divergence with early asymmetric migration into the Newfoundland population. The Newfoundland population exhibits signatures of a founder effect, with a recent increase in N_e and a lack of gene flow with the mainland

3.2 | Epigenetic structure and its environmental determinants

Epigenetic variation showed more population-specific distinctive trends compared to the SNP data set, where variation in DNA methylation clustered Alaska from the remaining mainland populations and indicated no subdivisions between the mid-continental populations (Figure 4). The first PCoA axis roughly correlates with a pattern of isolation by distance (PCo1 = 6.1%), while the second axis identified unique variation separating the geographically peripheral populations (Newfoundland and Alaska) from the mid-continental populations (PCo2 = 4.8%). We observed similar methylation patterns over CpG islands and gene bodies compared to unannotated regions of the genome and therefore decided to merge the two methylation data sets into a single set for all analyses (further details in Figure S13).

Epigenetic variation was significantly explained by geographic distance, winter climatic conditions, and the binary insularity variable (Figure 5). Tree cover (p = 0.77) added no explanatory power to the model (Table S8) and collinearity was low between all

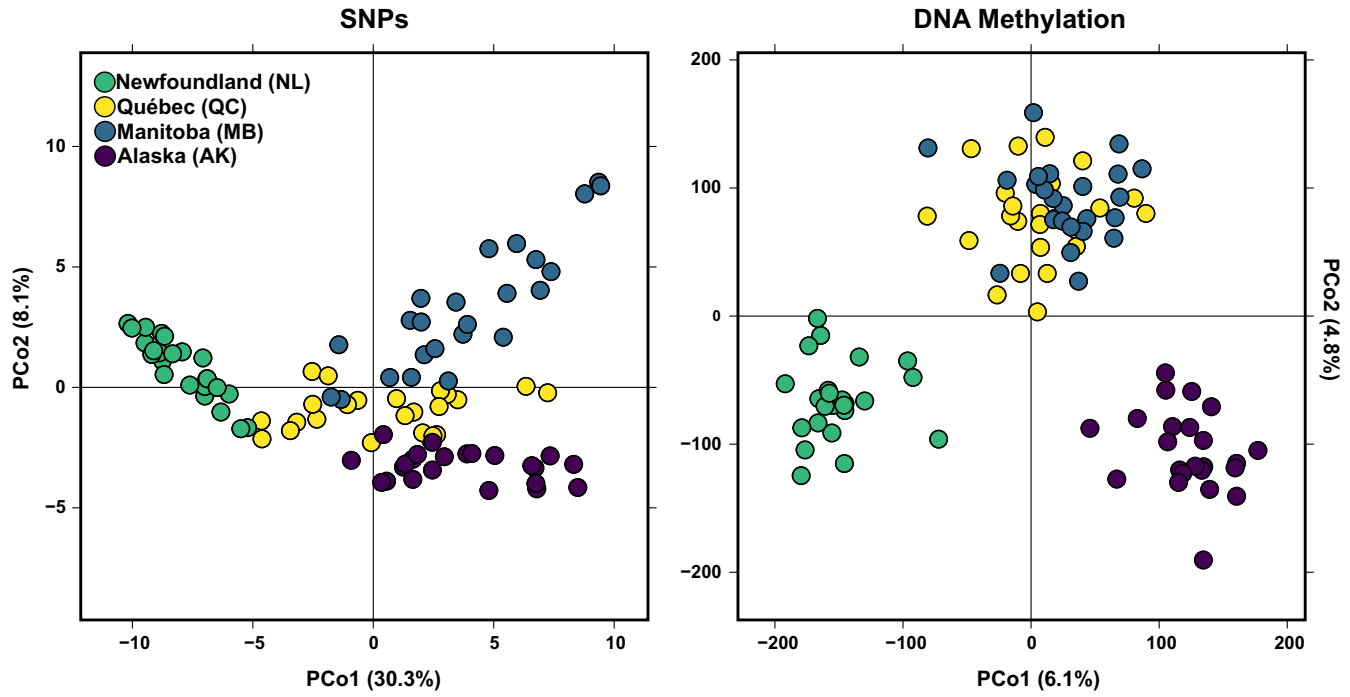

FIGURE 4 Principal coordinate ordination plots of genetic (left) and epigenetic (right) variation between Canada lynx (*Lynx canadensis*) populations indicate cryptic structure at epigenetic markers, with individuals as points and populations delineated by colour. All molecular data was summarized with a pairwise Euclidean dissimilarity matrix. Methylation data was summarized with 5,000 bp running windows across the genome (n = 705) and SNPs were called from bisulphite converted reads (n = 85)

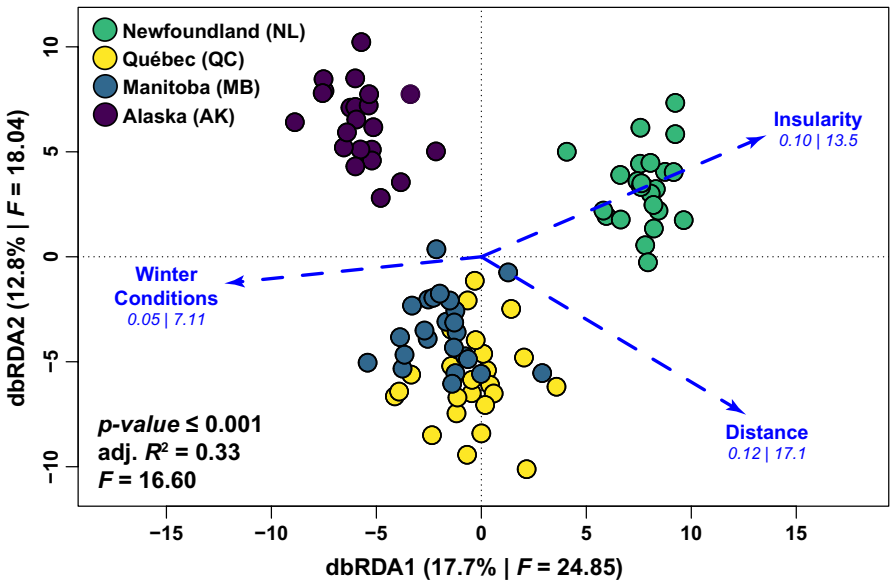

FIGURE 5 Distance-based redundancy analysis (db-RDA) ordination on DNA methylation indicate geographic distance, insularity, and winter conditions explain a portion of the observed epigenetic variation in Canada lynx (*Lynx canadensis*), where populations are delineated by colour. The axes of a principal coordinates analysis (PCoA) summarizing methylation data were used a response variable to determine biogeographical relationships. Values underneath each environmental vector indicate R^2 and F values obtained from partial db-RDAs

retained variables (Table S7). We examined the explanatory power of each variable independently using partial db-RDAs, which identified the most variation solely explained by geographic distance (pseudo – F = 17.1; R^2 = 0.12; $p \leq 0.001$), the least explained by winter conditions (pseudo – F = 7.1; R^2 = 0.05; $p \leq 0.001$), and an intermediate amount explained by the insularity variable describing the allegedly impassable barrier of the Strait of Belle Isle (pseudo – F = 13.5; R^2 = 0.10; $p \leq 0.001$) (Figure 5; Table S8). Less variation was explained by geographic distance in our SNP data (pseudo – F = 17.4; R^2 = 0.10; $p \leq 0.001$) than with DNA methylation data (Figure S17).

A total of 42 differentially methylated genes were identified across all populations, including 4 long noncoding RNAs. Gene ontology analysis identified cellular component morphogenesis (GO: 0032989, n = 4, p = 0.007) and embryonic development (GO: 0009790, n = 3, $p \leq 0.001$) as the most overrepresented biological pathways (Figure 6b;

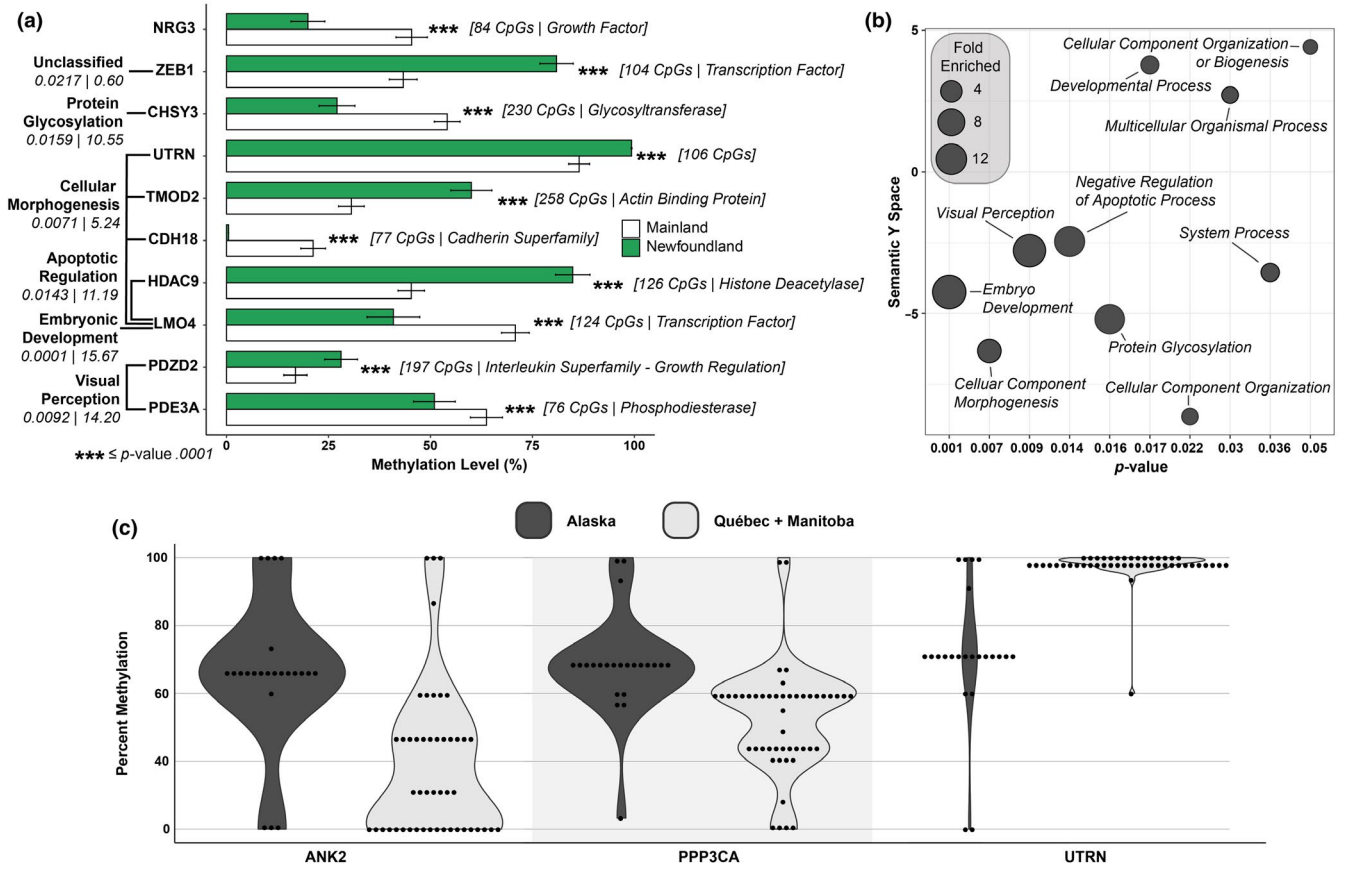

FIGURE 6 Summary of differentially methylated region analyses. (a) Enriched differentially methylated genes, highlighting differences between Newfoundland and Mainland Canada lynx (*Lynx canadensis*). Methylation levels are indicated with standard error of the mean, with all mainland populations lumped together. Enriched biological processes were identified using a GO-Panther analysis, with *p*-value and enrichment values indicated under each process (far left). The total number of CpGs per gene analyzed across populations and the GO-Panther gene-specific function is identified to the right of the bar plots. All differentially methylated regions were located directly over gene bodies. (b) GO term results for all 42 differentially methylated genes, with *p*-value along the *x*-axis and correlated terms clustered along the *y*-axis. Relative size indicates the fold enrichment for each term. (c) Distribution plots for methylation data between Alaska and the remaining mainland populations for three genes, with populations indicated by violins and individuals indicated by dots

Table S9). Three specific genes identified with the ontology analysis warranted closer examination due to their known direct regulation of body mass and morphology. The genes HDAC9, TMOD2, and ZEB1, were hypermethylated in Newfoundland compared to the mainland, with a difference in methylation of 39.6%, 29.4%, and 37.6%, respectively (Figure 6a). We also examined genes that were differentially methylated between individuals from Alaska compared to the remaining mainland (Québec and Manitoba), where three genes related to calcium ion binding (PPP3CA and UTRN) and protein kinase binding (ANK2) were differentially methylated (Figure 6c).

4 | DISCUSSION

4.1 | Newfoundland as a potential glacial refuge for Canada lynx

The historical demography of insular Canada lynx has been the focus of speculation, with estimates ranging from intraglacial colonization to introductions within the last century (Row et al., 2012). Provided that the Wisconsinan glaciation did not completely cover Newfoundland, it has been hypothesized that mammalian colonization could have occurred 18,000–20,000 years ago (South, 1983). Our demographic analyses and model selection with SNP data support a demographic history of initial colonization with early asymmetric admixture and subsequent isolation, where colonization likely occurred sometime during the last-glacial period (Figure 3); for example a year-round ice bridge is believed to have connected the mainland to Newfoundland and portions of the island were still thought to be ice-free (South, 1983). Population divergence time estimates are scaled by mutation rate and generation time, but in our case all reasonable metrics would support a historic colonization of 20–33 thousand years ago. And although we obtained a limited number of SNPs due to our sequencing effort and epiGBS library preparation protocol, they still provide adequate power appropriate for our analyses (Shafer et al., 2015) and our observed patterns are largely consistent with microsatellite data (Rueness et al., 2003). Therefore, the island lynx population appears to have been isolated for thousands of years, which appears long enough to have

generated distinct phenotypic differences from mainland lynx, notably in their diminished size (Khidas et al., 2013).

4.2 | DNA methylation improves detection of population structure

Despite the lack of genetic structure between mainland populations (Figure 4; Figure S4), we observed distinct patterns of DNA methylation that differentiate Alaska from mid- and east-continental populations. The homogeneity observed between individuals from Manitoba and Québec for both SNP and epigenetic data sets suggests low differentiation and high levels of gene flow, consistent with microsatellite data (Row et al., 2012). High connectivity across the Rockies is also suggested due to the low differentiation at genetic markers between Alaskan individuals and the remaining mainland, although these individuals are starkly separated at the epigenetic level. Our data cannot determine if this patterning is due to recurrent environmental induction or meiotically heritable adaptive plasticity, but does suggest that environmentally-mediated gene expression is occurring in Alaskan lynx. This result is biologically interesting, as phenotypic data suggests that Alaskan lynx are morphologically the largest compared to Albertan and insular lynx (Van Zyll De Jong, 1975), although only limited data are available. The three differentially methylated genes between Alaska individuals and the remaining mainland could be related to differences in ATP regulation and energy expenditure (Figure 6), although further examination of DNA methylation along promoter regions and explicit metabolic measurements would be required to substantiate this.

While the segregation of Newfoundland from the mainland was largely reflected across both genetic and epigenetic data sets, the methylated outliers suggest that epigenetic mechanisms are driving observed phenotypic differentiation. Provided that DNA methylation is affecting gene expression over these differentially methylated genes, epigenetic variation could explain morphological differences observed between mainland and island populations (Khidas et al., 2013; Van Zyll De Jong, 1975), as predicted by the Island Rule (Lomolino, 2005). Skull size (and hence body size) measurements in Canada lynx are hypothesized to be negatively correlated with population size (Yom-Tov, Yom-Tov, MacDonald, & Yom-Tov, 2007); we suggest that DNA methylation could be regulating phenotypic plasticity in body growth that maximizes a phenotypic optima to smaller sizes thereby regulating population dynamics as prey abundance decreases.

Three notable methylated genes in Newfoundland warrant further discussion. Genetic knockout of HDAC9 in laboratory experiments with mice (*Mus musculus)* have indicated diminished body mass (Chatterjee et al., 2014), and hypermethylation in the Newfoundland population suggests an analogous trend of repressed gene expression. Similarly, TMOD2 is a member of the tropomodulins family, which are associated with actin regulation where downregulation associates with a decrease in cell height during morphogenesis (Conley, Fritz-Six, Almenar-Queralt, & Fowler, 2001; Weber, Fischer, & Fowler, 2007). High levels of ZEB1 are also associated with larger body mass in mice (Saykally, Dogan, Cleary, & Sanders, 2009), and again, hypermethylation in Newfoundland suggests reduced expression of ZEB1. ZEB1's regulation of adiposity (Saykally et al., 2009) also suggests a potential role in phenotypically-relevant thermogenesis as a mechanism facilitating adaptation to variable climates, as DNA methylation is directly implicated in the regulation of brown adipose tissue (Sambeat, Gulyaeva, Dempersmier, & Sul, 2017). Together, these patterns suggest that epigenetic modifications play a role in the phenotypic differentiation observed between continental and insular populations. Follow-up assays to quantify genome-wide genetic variation and methylation-dependent expression are necessary to implicate DNA methylation in functional biological differences and control for genetic variation (Dubin et al., 2015; Tadut, Colomé-Tatché, & Johannes, 2016; Lea et al., 2018), and confirm consistent epigenetic patterning over gene promoters and enhancers. While we found minimal differences between our putatively regulatory and unannotated region methylation data sets, we predict that comparative analyses of methylation between promoter and unannotated regions could be used to assess relative variation explained by phenotypically-relevant environmental predictors and a step towards deconvoluting the evolutionary significance of certain loci (Figure S13).

Importantly, our demographic inferences suggesting a relatively recent (20–33 KYA) evolutionary establishment of Newfoundland Canada lynx supports DNA methylation as a marker to examine rapid evolutionary change. Evolutionary theory in model organisms has predicted that epimutations and methylome evolution often precede genomic changes (Smith, Martin, Nguyen, & Mendelson, 2016; Vidalis et al., 2016), which has been further substantiated with empirical evidence examining epigenetic changes over structural genomic variants in the absence of genetic variation (Ichikawa et al., 2017) and phenotypic responses in the absence of standing genetic variation (Sentis et al., 2018).

As evidence accumulates regarding the mechanisms underlying epigenetic inheritance (Yu, Wang, & Moazed, 2018) and the adaptive benefits of epialleles (He et al., 2018), DNA methylation has the potential to be worked into a framework of adaptive divergence (Greenspoon & Spencer, 2018; Vogt, 2017, 2018) and ultimately into the ecological speciation model (Feder et al., 2012). It is possible that the interplay of subtle nuclear divergence and DNA methylation could mediate local adaptation via hypermethylation of locally deleterious genes, although longitudinal common garden or translocation experiments are required to explore this (Dubin et al., 2015; Vilgalys, Rogers, Jolly, Mukherjee, & Tung, 2019). If phenotypically-plastic epigenetic marks are advantageous and heritable, then selection might act directly on the repression of these genomic locations, even in a homogenized genetic background, particularly if such genes are metabolically costly and provide no fitness benefit if activated. Such hypotheses align with theoretical models of epigenetically mediated adaptive plasticity, although further empirical investigations in wild populations are merited (Greenspoon & Spencer, 2018). Provided that epigenetic patterns are maintained across generations (Colomé-Tatché et al., 2012; Joo et al., 2018; Liu, Wang, Hu, Wang, & Zhang, 2018; Schmitz et al., 2013), DNA methylation could

be integrated into the working model of ecological speciation with gene flow by mediating gene expression and the optimal phenotype under local conditions (Greenspoon & Spencer, 2018).

SUMMARY

Overall, our results indicate that DNA methylation can reveal cryptic levels of population structure in genetically homogenous species while suggesting functional pathways that might shape phenotypic divergence between populations. Epigenetic modifications offer great utility for understanding the mechanisms intertwining ecology and evolution (Jeremias et al., 2018; Verhoeven, VonHoldt, & Sork, 2016), and we offer empirical results demonstrating the evolutionary insight that can be gained from population epigenetics, even in non-model organisms.

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AUTHOR CONTRIBUTION

J.M., D.L.M., and A.B.A.S. designed the study; J.M. performed research and analyzed data; J.M. wrote the manuscript with input from A.B.A.S. and D.L.M.

DATA ACCESSIBILITY

Raw sequence data FastQ files are available on the Sequence Read Archive (Accession: PRJNA509991). All bioinformatic and analytical code available on GitLab (https://gitlab.com/WiDGeT_TrentU/RADseq/).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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