Museum epigenomics: characterizing cytosine methylation in historic museum specimens

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Abstract

Museum genomics is one of the most influential fields in modern collections-based research. Recently, these methods have been extended to study epigenetic markers in ancient DNA. Previous work has focused on well-preserved ancient tissues; here, we develop and test epigenomic methods for traditionally preserved natural history specimens (dried skulls) up to 76 years old. We used a combination of ddRAD and bisulfite treatment to characterize genome-wide patterns of cytosine methylation in two species of deer mice (*Peromyscus spp.*). We were able to describe methylation in specimens from all age groups, and global methylation did not vary due to specimen age. Locus methylation was bimodally distributed and was reduced in promoter regions and heightened in gene bodies, consistent with expectations for *in vivo* methylation in mammals. Global methylation estimates for the two species were within the expected range for mammals but at the lower end, suggesting that estimates may have been depressed due to the choice of reference genome. The quantity of data varied more across older specimens. For example, although most specimens in the oldest group (76 yo) yielded few cytosines, two specimens performed as well as modern specimens sequenced in the same library. Our work demonstrates the utility of historic specimens for methylation analyses, as with genomic analyses; however, such studies will need to accommodate the large variance among individuals in the quantity of data produced by historic samples, either by screening specimens for DNA quantity and quality prior to library construction or by expanding sampling to allow for a higher failure rate. We suggest that analyses requiring fewer specimens overall, such as differential methylation analyses, may be the most successful. Museum epigenomics datasets can be used to describe methylation in historic populations or shifts in methylation over time; here we present comparative data tracking methylation patterns in deer mice collected over the past 76 years over the course of an ecological range expansion.

Keywords: natural history collections, historic DNA, epigenomics, epigenetics, methylation, *Peromyscus*

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Museum collections worldwide house billions of specimens and are an invaluable resource for tracking how organisms change over time. One of the most influential fields in modern collections-based research is museum genomics, which enables studies of long term change in genetic variation and is transforming the way that museum specimens are used in research. Until recently, museum genomics research focused exclusively on genetic sequences; however, a growing body of recent work in "paleoepigenetics" demonstrates that ancient DNA retains patterns of *in vivo* DNA methylation (Orlando and Cooper 2014; Gokhman et al. 2016), a well-studied epigenetic mechanism associated with transcriptional regulation and modulation of gene expression (Jones 2012). The implications of this discovery are compelling; methylation markers in museum specimens could elucidate patterns of gene expression in past populations, opening up a number of new directions for collections-based research. In addition, the ability to document how epigenetic effects change over time may help clarify the role of epigenetic processes in adaptation and evolution.

Around a dozen paleoepigenetic studies have been published to date (Briggs et al. 2010; Llamas et al. 2012; Gokhman et al. 2014; Pedersen et al. 2014; Smith et al. 2014, 2015; Orlando and Cooper 2014; Seguin-Orlando et al. 2015; Gokhman et al. 2016; Hanghøj et al. 2016; Gokhman et al. 2017; Murphy and Benítez-Burraco 2018). To our knowledge, all previous studies have focused on ancient DNA from paleontological and archaeological specimens rather than "historic DNA" from museum specimens collected by naturalists in the modern era, which range from decades old to a few centuries old. Historic specimens are more abundant and broadly available across taxa than ancient specimens, and can therefore be used for a greater diversity of study questions. Though researchers now routinely collect tissue vouchers for genomic analyses, traditional preparations such as dried skins and bones still comprise the majority of existing vertebrate collections and represent some of the oldest and rarest specimens. Somewhat counterintuitively, such historic tissues are not necessarily more amenable to genomic work than ancient (*i.e.*, paleo) tissues. Historic specimens have the advantage of being much "younger" than paleontological specimens, reducing the amount of time for *post mortem* DNA damage to accumulate. Such specimens are also likely to be more pristine, harboring less exogenous DNA, and have been stored in (hopefully) optimal conditions. However, high quality ancient specimens such as tissues obtained from permafrost are often remarkably well-preserved and may actually be less degraded than historic bones and skins despite their age. DNA degradation such as fragmentation and nucleotide damage (notably hydrolytic deamination) is the primary challenge for ancient and historic DNA studies, making DNA harder to extract and amplify, increasing contamination risk, and producing sequence errors due to base pair misincorporations (Willerslev and Cooper 2005). Nevertheless, the field of museum genomics is thriving, and new protocols and analytical methods continue to broaden and strengthen collections-based genomic analyses. Llamas et al. (2012) remark that the main challenge in ancient methylation protocols is extracting amplifiable nuclear DNA, which is now feasible even for low quality historic specimens such as bones and dried skins (*e.g.*, Irestedt et al. 2006; Bi et al. 2013).

In this study, we describe DNA methylation in skull specimens from deer mice (*Peromyscus spp.*) sampled over the course of an ecological range expansion. Methylation-mediated epigenetic effects have been hypothesized to promote rapid adaptation and buffer reduced genetic diversity in colonization events such as biological invasions or range expansions (reviewed in Hawes et al. 2018). Various mechanisms have been proposed; for example, increased epiallelic diversity (Chwedorzewska and Bednarek 2012; Richards et al. 2012; Schrey et al. 2012; Liebl et al. 2013) or reduced global methylation (Ardura et al. 2017, 2018) could promote random phenotypic variation, or pathway-specific differential methylation could induce adaptive phenotypic plasticity (Hawes et al. 2018). Here we sampled specimens up to 76 years old from two species: a species undergoing a northward

range expansion (*Peromyscus leucopus*) and a congeneric species (*Peromyscus maniculatus*) that has been historically present throughout the *P. leucopus* expansion area (Myers et al. 2005, 2009). This species pair provides an opportunity to describe spatiotemporal patterns in methylation variation across a range expansion gradient in a colonist species while controlling for local environmental effects using an ecologically similar, sympatric non-colonist species. We generate genome-wide reduced representation methylomes at base-pair resolution using a combination of double digest restriction site-associated DNA sequencing (ddRAD) and bisulfite treatment. We quantify the amount of data produced by specimens of different ages and compare observed patterns of genome-wide methylation with expected patterns for mammalian methylation. Specifically, we describe the distribution of methylation across loci, which we expect to show a bimodal distribution with peaks at 0% and 100%, and we compare methylation patterns in putative promoter versus gene body regions, which we expect to show reduced methylation and increased methylation, respectively (Jones 2012). We conclude with a discussion of the challenges of working with historic samples, in particular loss of data, and the sampling designs and epigenetic analyses that can accommodate these challenges. We also highlight how epigenetic datasets, including the dataset produced in this study, can be used in future work to infer gene expression in past populations and characterize change over time in epigenetic effects.

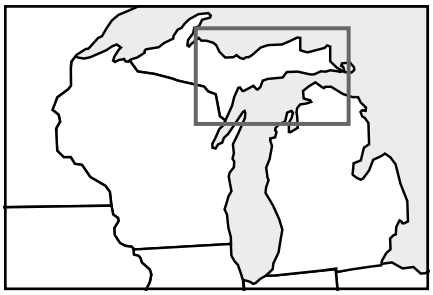
Methods

Specimens and sampling design

We sampled 150 specimens total: 77 white-footed mice (*Peromyscus leucopus novaboracensis*) and 73 woodland deer mice (*Peromyscus maniculatus gracilis*). All specimens were traditional museum skull preparations (dried skulls stored at room temperature) from adult mice collected between 1940 and 2016. When possible, we balanced sampling between the sexes. Modern skulls (2013-2016) were provided by the Dantzer Lab at the University of Michigan and the Hoffman Lab at Miami University. Older skulls (1940-2003) were provided by the University of Michigan Museum of Zoology. Detailed specimen information is included in Supplementary Table S1.

We compared *P. leucopus*, which is experiencing a northward range expansion into the Upper Peninsula (UP) of Michigan, with the congener *P. maniculatus*, which has a more northerly distribution and has been historically present throughout the UP (Figure 1a). The distributions of the two species and the progression of the range expansion were described based on regular small mammal trapping surveys conducted across the UP beginning in 1940 (see Long 1996; Myers et al. 2005, 2009 for details). Briefly, *P. leucopus* were absent in the UP prior to 1981 except in the southernmost county (Menominee), where they have been present since trapping efforts began (Figure 1c). Since 1981, they have been expanding northeastward across the peninsula at an estimated rate of 15 km/year (Myers et al. 2009). We sampled specimens of both species from three time periods (1940, 2002-2003, and 2013-2016) and three localities (the 1940 edge, the 2002 edge, and the 2016 edge) across the range expansion to generate a balanced design for describing spatial and temporal trends in methylation (Figure 1b). Both species occupy similar ecological niches, sharing predators, diets, and diseases (Wolff et al. 1985). The two species do not readily hybridize, although rare hybridization events may occur (Leo and Millien 2017).

(a)



(b)

Year sampled	**Locality** 1940 edge *P. leu* / *P. man*	2002 edge *P. leu* / *P. man*	2016 edge *P. leu* / *P. man*
1940	13 / 8		
2002	14 / 13	5 / 8	
2016	13 / 14	16 / 16	16 / 13

(c)

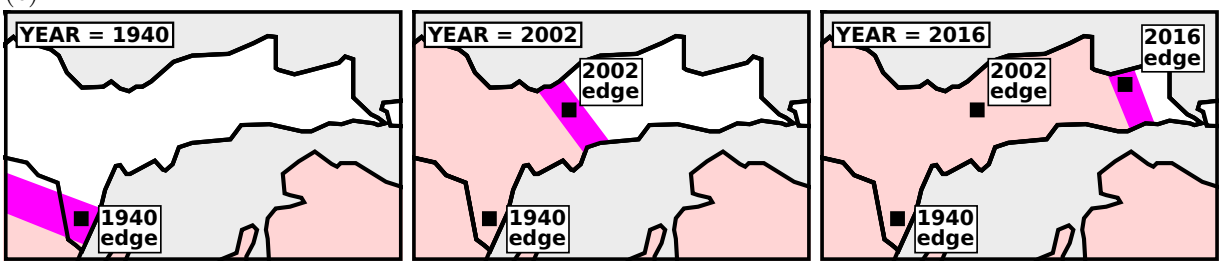


Figure 1: Sampling design. **(a)** Map of Michigan and surrounding areas. The gray box indicates the region shown in (c). **(b)** Number of mice sampled per population. The first value is the sample size for *P. leucopus* (*P. leu*) and the second value is the sample size for *P. maniculatus* (*P. man*). **(c)** Sampled populations, indicated by black squares, in 1940, 2002, and 2016. The pink region indicates the area occupied by *P. leucopus*. The bright pink band indicates the range edge.

Tissue sampling and DNA extraction

All pre-amplification steps were performed in the ancient DNA facility in the Genomic Diversity Lab at the University of Michigan following standard protocols for working with historic DNA. Briefly, all work was performed under a hood in a dedicated laboratory for processing historic specimens and followed stringent anti-contamination protocols, including dedicated reagents, unidirectional flow of equipment and personnel, filtered pipette tips, and additional negative controls. We sampled tissue from traditional skull preparations (dried skulls stored at room temperature). To minimize damage to the skulls, we sampled microturbinates (small nasal bones) by inserting a sterile micropick into the nasal cavity to dislodge 5 - 12 mg of tissue (Wisely et al. 2004; Taylor and Hoffman 2010). Prior to DNA extraction, the bone fragments were placed into thick-walled 2 ml microcentrifuge tubes with four 2.4 mm stainless steel beads and processed in a FastPrep tissue homogenizer (MP Biomedicals) for 1 min at 6.0 m/s. All 2013-2016 specimens and some 2002-2003 specimens were extracted using a Qiagen DNeasy Blood and Tissue Kit with modifications for working with museum specimens. To increase yield, the rest of the specimens were extracted using a phenol-chloroform protocol. Detailed extraction protocols are described in the Supplementary Methods (also see Iudica et al. 2001; Mullen and Hoekstra 2008; Rowe et al. 2011).

Library preparation

The samples were prepared for sequencing using a combination of double digest restriction site-associated DNA sequencing (ddRAD) and bisulfite treatment (see Supplementary Methods, also see Trucchi et al. 2016; van Gurp et al. 2016 for similar approaches). Samples were individually barcoded using a combinatorial indexing system (10 unique barcodes on the forward adapter and 10 unique indices on the reverse PCR primer) and processed into multiplexed libraries. Specimens were assigned to the libraries based on the amount of DNA that could be extracted or specimen availability. We prepared three libraries with different starting concentrations of DNA - one high DNA concentration library of 75 younger specimens (0-3 years old (yo)), one medium DNA concentration library of 39 younger and older specimens (0-76 yo), and one low DNA concentration library of 39 older specimens (13-76 yo). Two specimens were sequenced in both the medium and low concentration libraries.

We followed the ddRAD protocol outlined in Peterson et al. (2012) with added steps for bisulfite treatment. Briefly, we digested each sample with the restriction enzymes SphI-HF and MluCI for 1 hour at 37° C (New England Biolabs). We added a spike-in of digested unmethylated lambda phage DNA (Sigma Aldrich) to each sample at a concentration of 0.1% of the sample concentration; these phage reads were used to directly measure the bisulfite conversion rate for each individual sample. We ligated custom methylated barcoded Illumina adapters (Sigma Aldrich) onto the digested products and pooled samples into sublibraries. Size selection was performed on a Pippin Prep electrophoresis platform (Sage Biosciences), with 376-412 bp and 325-425 bp fragments selected in the high and lower concentration libraries, respectively (a wider range was chosen for the latter to ensure that the samples exceeded the recommended minimum mass of DNA for the Pippin Prep cassette). Bisulfite conversion was performed on the size selected sublibraries using a Promega MethylEdge Bisulfite Conversion Kit, which converted unmethylated cytosines to uracils, and amplified by PCR using KAPA HiFi HotStart Uracil+ MasterMix, which replaced uracils with thymines in the amplified product. Due to low DNA concentration in the final libraries for sequencing, the low concentration and medium concentration libraries were combined and sequenced on the same lane. The high

concentration library was sequenced in one lane for 100 bp paired-end reads and the medium / low concentration library in a separate lane for 125 bp paired-end reads on an Illumina HiSeq 2500 (San Diego, CA).

Illumina data processing

The raw sequence reads were demultiplexed and filtered for quality and adapter contamination using the *process_radtags* script of *Stacks* v.1.45 (Catchen et al. 2013). We allowed one mismatch in the adapter sequence and a barcode distance of one (--adapter_mm 1 --barcode_dist 1). All reads were trimmed to a length of 96 bp. The restriction site check was disabled because bisulfite treatment can change the sequence at the restriction site (--disable_rad_check). The resulting paired-end reads for each individual were aligned to the *Peromyscus maniculatus bairdii* genome (NCBI ID: GCA_-000500345.1) using the bisulfite aligner *Bismark* v.0.18.1 (Krueger and Andrews 2011). *Bismark* was run with default settings except for the mismatch criteria (-N 1) and gap penalties. Gap penalties (--score_min) were adjusted to account for differences between the target reads and the reference genome: species-level differences for *P. leucopus* (*P. leucopus novaboracensis* aligned to *P. maniculatus bairdii*; --score_min L,0,-0.6) and subspecies-level differences for *P. maniculatus* (*P. maniculatus gracilis* aligned to *P. maniculatus bairdii*; --score_min L,0,-0.4). An analysis was also run with the default settings for both species and returned the same global methylation trends, but fewer loci; therefore, the results from the less stringent criteria are reported here. *Bowtie2* v.2.1.0 (Langmead and Salzberg 2012) was used as the core aligner. We also aligned the reads to the lambda phage genome (NCBI ID: NC_001416) using default alignment settings and used these reads to estimate the bisulfite conversion rate for each sample.

Methylation calling

We focused on CpG methylation; in eukaryotes methylation almost always occurs on a cytosine, and in mammals almost exclusively in the context of a CpG dinucleotide (Jones and Takai 2001). Because methylation is tissue-specific, it is necessary to standardize the tissue sampled. We chose to sample bone tissue from dried skulls, focusing on microturbinates to reduce specimen damage. Even within a tissue the methylation state of a given CpG position in the genome may vary between alleles or across cells, so methylation at a given position is typically expressed as a percentage ranging from fully methylated (methylated in 100% of sequences) to fully unmethylated (methylated in 0% of sequences). Within a tissue, most CpGs are either fully methylated or fully unmethylated (though partial methylation is not uncommon), resulting in a bimodal distribution across loci (Rakyan et al. 2004; Eckhardt et al. 2006).

We used *MethylExtract* v.1.9 (Barturen et al. 2013) to filter for putative bisulfite conversion failures and to perform methylation-calling. We generated a list of all CpGs in our data with the number of methylated and unmethylated reads and used lambda phage-aligned reads to estimate sample-specific bisulfite conversion rates. Significant methylation calls were determined by assigning p-values to each CpG based on binomial tests incorporating the raw read counts and the sample-specific bisulfite conversion rate and using the Benjamini-Hochberg step-up procedure to control the false discovery rate for multiple testing (implemented with the *MethylExtractBSPvalue* function). We specified an accepted error interval of 0.2 (the default value) and an FDR of 0.05. Only significant sites were used in downstream analyses. For specimens with fewer than 200 phage cytosines analyzed (only 8 of the 150 specimens) we used the minimum bisulfite conversion rate from other specimens

from the same ddRAD sublibrary, which should have the same conversion rate since they were pooled together in the same bisulfite conversion reaction.

Data analysis

All analyses were conducted in *R* v.3.5.1 (RCoreTeam 2018). We modeled total read counts and CpG counts using negative binomial regression implemented using the *glm.nb* function of the *R* package *mass* v.7.3-50 (Ripley et al. 2013). Separate models were used to compare aligned read counts across libraries (with library as a fixed effect) and to compare aligned read counts within libraries based on specimen age (with specimen age as a fixed effect). The total number of CpGs was modeled with specimen age as a fixed effect. We used Tukey tests for all pairwise comparisons, implemented using the *glht* function of the *R* package *multcomp* v.1.4-8 (Hothorn et al. 2014). We report Bonferroni corrected p values for all pairwise comparisons.

We modeled locus methylation globally and by genomic region. Following conservative guidelines (Ziller et al. 2015), sequences with a read depth less than 10X were excluded, as were bases with abnormally high coverage to account for PCR duplication (defined as bases in the top 99.9th percentile of read depth for each individual, following Hu et al. 2018). Because cytosine methylation shows high spatial correlation, data from CpGs occurring within 2000 bp of each other in the genome were pooled, and are hereafter referred to as a locus (Eckhardt et al. 2006). All analyses modeled the raw read counts of methylated and unmethylated cytosines at each locus; though it is possible to have partial methylation at a given CpG position, each individual read will be either methylated or unmethylated. Locus methylation was modeled using binomial generalized linear mixed models with a logit link function and fit with Laplace approximation, implemented using the *glmer* function of the *R* package *lme4* v.1.1-20 (Bates et al. 2014). Because many loci were sequenced for each individual, we included specimen identity as a random intercept term in all models. We also included an observation-level random effect in all models to account for overdispersion (Harrison 2014).

Global methylation levels were quantified to test for abnormal methylation calling in older specimens. We modeled methylation at each locus with specimen age and species as fixed effects and specimen identity as a random intercept term. Methylation by genomic region (*i.e.*, promoter, gene body, or other) was modeled with genomic region and species as fixed effects and specimen identity as a random intercept term, with genomic regions defined by sequence annotations downloaded from *Ensembl* (the pbairdii_gene_ensembl dataset) and following the classification method outlined in Pedersen et al. (2014). Briefly, putative promoter regions were defined as the region 500 bp upstream and 2000 bp downstream of the transcription start site (TSS) for the first exon in a gene, gene bodies were defined as the region from the end of the promoter (2000 bp downstream from the TSS) to the final transcription end position in the gene, and all loci not defined as promoters or gene bodies were labelled as other. *Ensembl* annotations were downloaded and processed using the *R* package *biomaRt* v.2.36.1 (Kinsella et al. 2011).

Results

Bisulfite conversion efficiency

The bisulfite conversion rates calculated from the lambda phage reads indicated almost complete conversion in all samples (sequencing statistics for each specimen are included in Supplementary Table S1). The 0.1% phage spike-in produced a sufficient number of cytosines (over 200) to estimate

conversion efficiency in all but eight samples; for those samples, the average conversion rate of the sublibrary was used for methylation calling as described in *Methods - Methylation calling*. Conversion rates ranged from 99.7% - 100% in the High concentration library (mean 99.8%), 98.7% - 100% in the Medium library (mean 99.6%), and 95.4% - 100% in the Low library (mean 99.0%).

Raw and aligned read counts

In general, the amount of data produced (aligned reads and total CpGs) was greater for specimens sequenced at a higher DNA concentration and for younger specimens. While the opposite trends were sometimes seen in raw reads (demultiplexed and cleaned, but not yet aligned), these trends disappeared after alignment to the reference genome and likely reflected exogenous DNA contamination (Table 1). The number of aligned reads per specimen was greater in the high concentration library relative to the medium concentration library ($2.899^{+}_{-}0.292$; $z = 9.93$, $p < 0.0001$) and the low concentration library ($2.539^{+}_{-}0.292$; $z = 8.69$, $p < 0.0001$); aligned read counts did not differ between specimens in the low and medium concentration libraries. Within a library, younger specimens yielded more aligned reads than older specimens; in the low concentration library 13-14 yo specimens yielded more reads than 76 yo specimens ($1.890^{+}_{-}0.583$; $z = 3.242$, $p = 0.001$), and in the medium concentration library 0-3 year old specimens yielded more reads than 13-14 yo specimens, though the effect was only marginally significant ($0.832^{+}_{-}0.349$; $z = 2.384$, $p = 0.041$). There was one notable exception to this trend. The two 76 yo specimens that were sequenced in the medium concentration library yielded more reads on average than 13-14 yo specimens and did not differ significantly from 0-3 yo or 13-14 yo specimens sequenced in the same library.

Table 1: Sequencing statistics for the three libraries: low DNA concentration, medium DNA concentration, and high DNA concentration. The first column indicates the amount of DNA per specimen used for library preparation (in nanograms). Data is grouped by species and year collected, and the number of specimens is indicated (N spec). Total read counts and average read counts per specimen are shown for demultiplexed reads (reads retained after demultiplexing and cleaning) and aligned reads (reads retained after alignment to the reference genome).

					Demultiplexed reads		Aligned reads	
Library	Species	Age	Year	N spec	Total	Avg/spec	Total	Avg/spec
Low conc. (40 ng)	*P. leucopus*	76	1940	13	2793629	214894.54	92101	7084.69
		13-14	2002-3	7	6612604	944657.71	510442	72920.29
	P. maniculatus	76	1940	8	5039497	629937.12	22237	2779.62
		13-14	2002-3	10	3560487	356048.70	102228	10222.80
Medium (150 ng)	*P. leucopus*	76	1940	2	4675593	2337796.50	61494	30747.00
		13-14	2002-3	12	50381785	4198482.08	343419	28618.25
	P. maniculatus	13-14	2002-3	11	26954398	2450399.82	83541	7594.64
		0-3	2013-6	13	22900629	1761586.85	554342	42641.69
High (350 ng)	*P. leucopus*	0-3	2013-6	45	41166302	914806.71	7872020	174933.78
	P. maniculatus	0-3	2013-6	30	78761119	2625370.63	18190217	606340.57

CpGs sequenced

The number of CpGs sequenced showed an inverse relationship with specimen age (Figure 2), with the youngest group yielding more CpGs than the 13-14 yo group ($2.480^{+}_{-}0.241$; $z = 10.289$, $p < 0.0001$) and the 76 yo group ($5.080^{+}_{-}0.327$; $z = 15.526$, $p < 0.0001$) and the 13-14 yo group yielding more CpGs than the 76 yo group ($2.600^{+}_{-}0.359$; $z = 7.244$, $p < 0.0001$). Among young specimens (0-3 yo), the maximum number of CpGs yielded by a single specimen was 252,604, roughly ten times more than the maximum for older specimens (31,807 CpGs for the 13-14 yo group, 17,634 CpGs for the 76 yo group). Older specimens (13-76 yo) showed greater variation in the number of CpGs sequenced, likely reflecting varying degrees of *post mortem* DNA damage. In particular, 76 yo specimens showed high variation in the quantity of data produced. Most 76 yo specimens (16 out of 20) yielded fewer than 1,000 CpGs, however, others performed comparably to younger specimens. As with the aligned reads, the two most robust 76 yo specimens did not differ significantly from 13-14 yo and 0-3 yo specimens sequenced in the same libraries.

Across all specimens, regardless of age, one third to one half of all CpGs were sequenced to a read depth of 5X or greater (Table 2; 0-3 yo: 48.9% of all CpGs; 13-14 yo: 31.5%; 76 yo: 34.8%). Roughly 20% of all CpGs were sequenced to a depth of 10X or greater (0-3 yo: 21.9%; 13-14 yo: 20.2%; 76 yo: 20.9%).

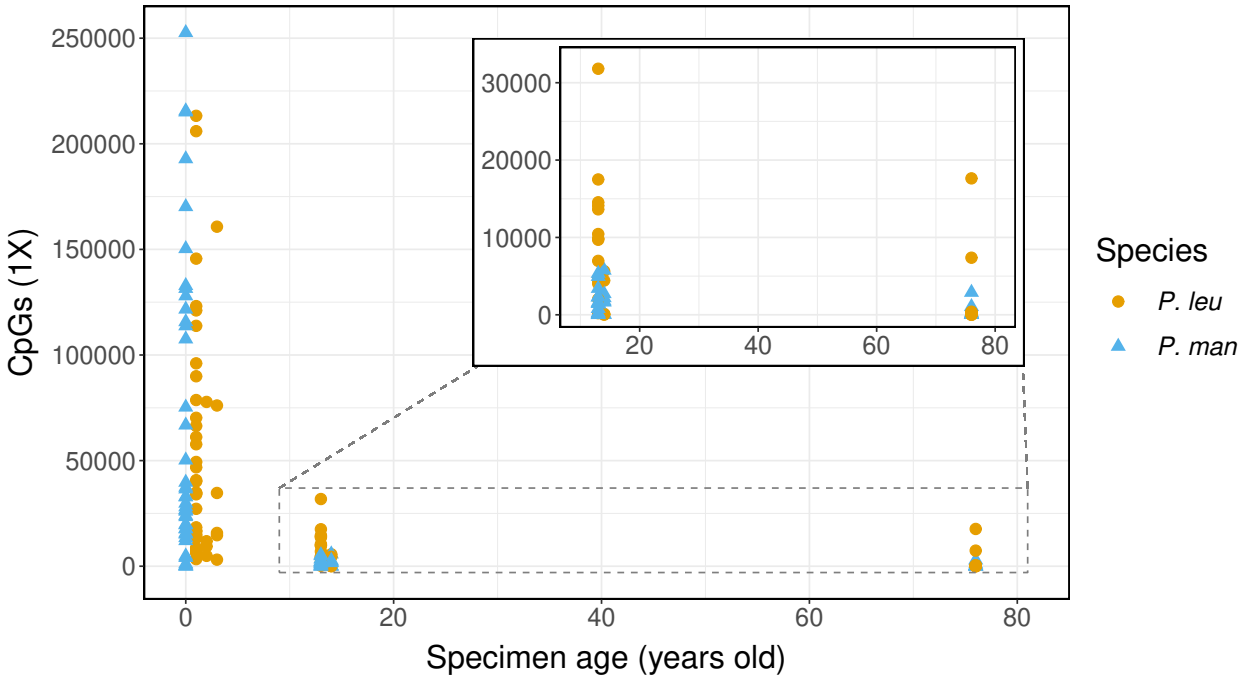


Figure 2: Total CpGs sequenced per specimen by specimen age (minimum depth = 1X). Orange circles indicate *P. leucopus* (*P. leu*) and blue triangles indicate *P. maniculatus* (*P. man*). Inset: Zoomed view of specimens 13 - 76 years old (area shown in the dashed box).

Table 2: Number of CpGs sequenced by species and specimen age (years old). The total number of CpGs sequenced and the average number of CpGs per specimen are shown for a minimum read depth of 1X (also shown in Figure 2), 5X, and 10X.

				CpGs (1X)		CpGs (5X)		CpGs (10X)	
Species	Age	Year	N spec	Total	Avg/spec	Total	Avg/spec	Total	Avg/spec
P. leucopus	76	1940	12	26889	2240.75	9571	870.09	5460	546.00
	13-14	2002-3	19	154257	8118.79	50663	2666.47	32008	1882.82
	0-3	2013-6	45	2315245	51449.89	941095	20913.22	271066	6611.37
P. maniculatus	76	1940	8	4561	570.12	1382	230.33	1115	185.83
	13-14	2002-3	21	38386	1827.90	10211	510.55	6898	363.05
	0-3	2013-6	43	2745570	63850.47	1534517	39346.59	837795	21481.92

Methylation by specimen age and genomic region

Estimated global methylation rates were higher in *P. maniculatus* than in *P. leucopus* ($0.453^{+}_{-}0.080$; $z = 5.703$, $p < 0.0001$; odds ratio (OR) = 1.573); average methylation over all loci was 62.1% and 54.0%, respectively. Global methylation did not vary due to specimen age or the interaction between specimen age and species (Figure 3a). Fully methylated (100%) and fully unmethylated (0%) loci were more common than partially methylated loci. The distribution of percent methylation over all loci also showed a bulge at 50% methylation - these loci either grouped CpGs with opposite methylation states or showed a 50/50 split between reads (Figure 3).

Methylation rates varied between different genomic regions following expected trends for mammalian genomes (Figure 3b). Relative to unassigned genomic regions (other), global methylation was lower in promoter regions ($-1.155^{+}_{-}0.022$; $z = -53.51$, $p < 0.0001$; OR = 0.315) and higher in gene bodies ($0.476^{+}_{-}0.008$; $z = 61.679$, $p < 0.0001$; OR = 1.610). In *P. leucopus*, average locus methylation was 8.8% lower in promoters and 6.5% higher in gene bodies; in *P. maniculatus*, methylation was 14.8% lower in promoters and 5.8% higher in gene bodies.

Discussion

The cytosine methylation patterns we recovered from dried skull specimens, including samples up to 76 years old, demonstrate the enormous resource contained in natural history collections, however, our work reveals needed adjustments for increasing the success of epigenetic studies using historic samples. In particular, sampling designs or analysis methods should account for high variability in the data produced by historic specimens, as discussed in more detail below.

Global and genomic region-specific methylation patterns

The observed patterns were mostly consistent with expectations for *in vivo* mammalian methylation. Locus methylation showed a bimodal distribution with peaks at 0% and 100%, as is expected for locus methylation within a tissue (Rakyan et al. 2004; Eckhardt et al. 2006). A small peak at 50% methylation mostly reflected loci that grouped together two or more CpGs with opposite methylation states, though roughly 10% of individual CpGs showed methylation levels around 50%, indicating that half of reads at that position were methylated and half were unmethylated. These sites may reflect loci associated with imprinted genes, which show parent-of-origin-specific expression regulated

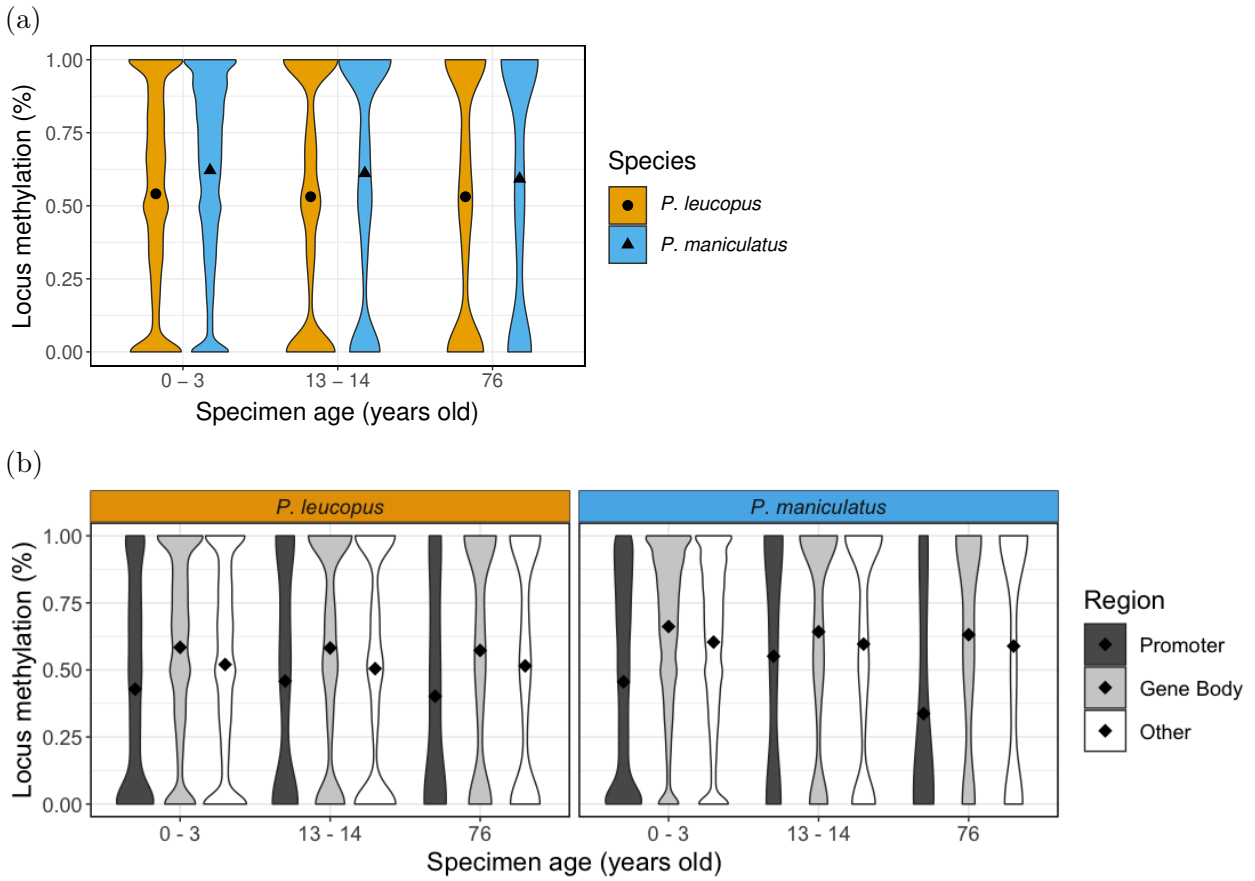


Figure 3: Violin plots of percent methylation across all loci. Mean values are overlaid. **(a)** Locus methylation across the entire genome by specimen age and species. **(b)** Locus methylation by genomic region. Promoters shown in dark gray, gene bodies in light gray, and all other regions in white. Left panel: Data for *P. leucopus*. Right panel: Data for *P. maniculatus*.

by methylation (Feinberg et al. 2002). Methylation of CpGs associated with imprinted genes is expected to be exactly 50% since one parental allele will be fully methylated and the other fully unmethylated. Methylation was also lower in promoter regions and higher in gene bodies, consistent with expectations for mammalian DNA (Jones 2012).

While the intraspecific patterns appear to be robust, it is unclear whether global methylation estimates in this study accurately reflect absolute methylation levels. Global estimates for both species were somewhat low; our estimates for global methylation of CpG sites were 62.1% for *P. maniculatus* and 54.0% for *P. leucopus*, while global CpG methylation for mammalian somatic cells is typically expected to range from 60% - 80% (Smith and Meissner 2013). It is possible that global methylation estimates were influenced by the choice of reference genome. The reference genome for *P. maniculatus bairdii* was used to align the two species analyzed in this study, *P. maniculatus gracilis* (a different subspecies relative to the reference) and *P. leucopus novaboracensis* (a different species). Our methylation calling program *MethylExtract* identifies putative SNPs and removes them when possible, however, any unfiltered C/T SNPs would be misinterpreted as methylation variation. Bases that transitioned from a C in the reference to a T in the study species would be recorded as fully unmethylated cytosines, resulting in depressed global methylation estimates. This would also

explain the difference in global methylation estimates between the two species; while it is feasible that *P. leucopus* has lower global methylation than *P. maniculatus*, an alternative explanation is that lower estimates resulted from a greater genetic distance between *P. leucopus* and the reference. For this reason, it is advisable to restrict analyses to within-species comparisons. Alternatively, aligning bisulfite sequences to a reference from the same species is likely to give a better estimate of true global methylation levels.

Variation in methylation as a function of specimen age

Methylation estimates did not vary due to specimen age, suggesting that methylation calling was not compromised in older specimens. In particular, we did not find a signal of *post mortem* hydrolytic deamination, which can produce erroneous sequences in ancient and historic specimens (Willerslev and Cooper 2005). Deamination causes the spontaneous conversion of cytosine into either uracil (in the case of unmethylated cytosine) or thymine (in the case of 5-methylcytosine). In museum genomics studies, this conversion results in erroneous C to T SNP calling; in bisulfite studies, deaminated cytosines could be misinterpreted as unmethylated cytosines and cause depressed methylation estimates for older specimens. It is not entirely surprising that we found no signal of deamination in this study; deamination is more problematic in ancient specimens compared to historic specimens (Burrell et al. 2015), and we followed special protocols to minimize the number of miscalled bases (namely, longer read end trimming and increased minimum read depth). Nevertheless, future studies should incorporate a similar test and in datasets in which estimated methylation decreases as specimen age increases, data should be further processed to account for deamination.

Variability in specimen yield

Our protocol worked well for high quality specimens, including older specimens; however, older specimens tended to be more "hit-or-miss." This disparity in specimen performance is typical of older historic specimens, which tend to show high variation in the quantity of recoverable DNA. Our results suggest that starting DNA concentration may a better predictor of performance than specimen age. Indeed, our two 76 yo specimens with the highest extracted DNA concentrations (over 9 ng/μL) performed as well as specimens in the 13-14 yo and 0-3 yo age groups. Our ddRAD protocol required us to dilute most samples to standardize concentration; both of our high quality 76 yo samples were diluted for library preparation, suggesting that they could potentially yield more CpGs if prepared at a higher concentration.

Increasing the success of epigenomic studies based on historic samples

Several steps of our bisulfite ddRAD protocol could be modified or replaced to increase yield from historic specimens. For example, the size selection window could be reduced; historic DNA tends to be fragmented, so selecting for smaller fragments may increase yield for historic libraries. Steps could also be taken to minimize DNA degradation during the bisulfite treatment; for example, shortening the bisulfite incubation time should reduce DNA damage, though it may also reduce conversion efficiency (Grunau 2002). Our protocol also cleaved the DNA with two restriction enzymes; historic DNA is often already fragmented, and enzyme digestion may have contributed to problems in amplification and sequencing associated with DNA fragmentation. Many genomic library preparation protocols have been described for increasing yield in damaged and fragmented historic DNA. For example, libraries can be prepared without digestion or sonication and sequenced

directly (Burrell et al. 2015). Enrichment methods seem to be particularly effective for sampling degraded historic and ancient DNA (Jones and Good 2016; Suchan et al. 2016). Seguin-Orlando et al. (2015) described methylation-based enrichment methods for ancient DNA which may be promising for museum epigenomic work. In general, most library preparation protocols could be used in conjunction with bisulfite treatment as long as bisulfite conversion is performed before the DNA is amplified (methyl groups are not replicated during PCR). Several ancient epigenomics studies have avoided bisulfite conversion altogether by reconstructing methylation maps from patterns of hydrolytic deamination (*e.g.*, Briggs et al. 2010; Gokhman et al. 2014; Pedersen et al. 2014; Hanghøj et al. 2016). This approach would not have been possible for our specimens, which did not show a strong deamination signal, however it may be an option for museum specimens that are highly degraded. In addition, several cheaper options are available for measuring methylation at fewer sites, such as MS-AFLP and targeted bisulfite sequencing; for example, Smith et al. (2015) used targeted bisulfite pyrosequencing to describe methylation at an imprinted site in ancient humans.

Museum epigenomics studies will need to accommodate the large variance in the quantity of data produced by individual historic specimens. Sampling designs should account for a high failure rate in older specimens, or if possible, specimens should be screened in advance of library preparation for DNA quantity and quality. We expect that most samples that can be used for genomic work can also be used for epigenomic work. Because high quality specimens are likely to be rare, analyses that require fewer individuals will probably be more successful. For example, differential methylation studies often sequence only one or a few individuals per treatment group. Differential methylation analysis is one of the most common approaches in methylation research and can be used to investigate pathway-specific regulatory mechanisms or conduct genome-wide screens for novel differentially methylated regions. Museum epigenomics datasets may be particularly well-suited to differential methylation analyses focusing on a small number of high quality historic specimens.

Applications of epigenomic data from historic specimens

Methylation is one of the best-studied epigenetic mechanisms and is associated with a range of processes, from development to disease response to phenotypic plasticity. One of the most intriguing directions for museum epigenomics research is the study of characteristics that don't fossilize, such as non-morphological traits or historical environmental conditions. For example, methylation variation modulates gene expression related to various behavioral (*e.g.*, Meaney and Szyf 2005) and physiological traits (*e.g.*, García-Carpizo et al. 2011). Murphy and Benítez-Burraco (2018) used methylation patterns to infer the expression of language processing genes in Neanderthals. Environmentally-induced methylation variation can reflect environmental conditions such as food availability (*e.g.*, Heijmans et al. 2008), climate (*e.g.*, Fu et al. 2010; Gugger et al. 2016), and exposure to disease or toxins (Robertson 2005; Baccarelli and Bollati 2009). Gokhman et al. (2017) demonstrated how methylation patterns can be used to study past environments by describing markers of prenatal nutrition in Denisovan and Neanderthal genomes. Ancient and historic epigenomic studies may allow us to explore aspects of past populations that are not reflected in a specimen's morphology or genetic sequence.

Museum epigenomics studies also provide the opportunity to directly measure how epigenetic effects change over time. Just as in museum genomic studies (Burrell et al. 2015), epigenomic studies can use collections to describe temporal changes in population-level variation. Such studies could help clarify a range of unresolved questions in ecological epigenetics, including the transgenerational stability of epigenetic marks, the timescales of induction of epigenetic effects, and the relationship

between epigenetic and genetic variation. It is still unclear what role, if any, non-genetic mechanisms such as epigenetic effects play in evolutionary processes (*e.g.*, Laland et al. 2014). Observing change over time in epigenetic effects may provide insights into their role in adaptation and evolution.

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Author Contributions

Conceived the experiment: TLR and BD. Designed the experiment: TLR, BD, and LLK. Contributed reagents: TLR, BD, and LLK. Performed the research and data analysis: TLR. Wrote the manuscript: TLR, BD, and LLK.