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STUDYING PAST ECOSYSTEMS AND HUMAN BEHAVIORS USING ENVIRONMENTAL AND ANCIENT DNA

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By

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Abstract

STUDYING PAST ECOSYSTEMS AND HUMAN ACTIONS WITH ENVIRONMENTAL AND ANCIENT DNA

By

Sabrina Shirazi

Isolating and studying degraded DNA from preserved organismal remains and environmental samples allows new inferences about past ecosystem compositions, population dynamics, and, in the context of archaeological remains, human interactions with their environment. In this dissertation, I addressed how sequencing depth and stochasticity of metabarcoding PCR influences various measures of biodiversity. I found that sequencing depth and stochasticity between PCR replicates significantly influence estimates of alpha but not beta diversity. In my second chapter, I used eDNA isolated from permafrost cores spanning the last 50,000 years in the Klondike, Canada to characterize community composition and turnover of plant and mammalian communities. In this chapter, I characterized floral and faunal change over the last 50,000 years, with clear shifts from steppe to boreal forest habitat delineated with the presence and absence of arctic ground squirrels and woody plants. Finally, I isolated ancient DNA from archaeological moccasins to observe hunting patterns of Bison used by occupants of the Promontory Caves of Utah, an archaeological site occupied 1240-1290 AD. I found the majority (87%) of moccasins were constructed from female bison, supporting prior hypotheses of hunting strategies

targeting cow-calf herds at the end of fall preparing for overwintering. My dissertation highlights some of the many questions that degraded DNA present in soil, bone, and preserved hides can contribute towards answering.

Acknowledgements

The land on which I completed my Phd "is the unceded territory of the Awaswas-speaking Uypi Tribe. The Amah Mutsun Tribal Band, comprised of the descendants of indigenous people taken to missions Santa Cruz and San Juan Bautista during Spanish colonization of the Central Coast, is today working hard to restore traditional stewardship practices on these lands and heal from historical trauma (UCSC land acknowledgement statement)."

It is an impossible task to mention everyone, let alone give due at length acknowledgement to everyone, who supported me professionally, academically and emotionally throughout my PhD in a few pages here. I also wish I was not brain drained from writing this thesis when writing the acknowledgements as I surely am not justly giving each of you appreciative and comical enough mention. Each individual study I was involved with, both those included in this dissertation and not, were supported by some community of colleagues, collaborators, mentors, and citizen scientists who made the studies possible, stimulating, and rewarding. My communities- academic, social, and familial, were integral in supporting me through the emotional rollercoaster that was the last 5 years.

I would like to thank my primary advisor, Beth Shapiro, who brought me to UCSC and supported my education. I would also like to thank Rachel Meyer, who became an advisor, mentor, and friend one year into my graduate work. You both

dedicated an incredible amount of time, energy, and resources into my education that made it possible for me to join the Paleogenomics lab and complete my PhD.

The Paleogenomics lab was my daily community- whether it meant long days of meetings that made us delusional together, reliable taco truck lunch breaks, think tanks around the whiteboard, complaining about graduate life, and just generally looking at each other's beautiful faces all day everyday. A community like this one is very important for me in being happy in my workspace, and you not only gave me that but life-long friendships that I look forward to cherishing.

Among the long list of lab members that made the community great are a few that particularly influenced my career and life. Alisa Vershinina was there from the moment I interviewed with the lab and continued to serve as my lab mother, my personal witch, my guardian angel, and just an inspiring independent and hysterical person to spend time with. Please never forget about me because you are so cool. Nevé Baker, my desk mate and travel mate, I remember early on talking with you while camping about us being close ‘grad school friends’ to remember back to when we were done… little did we know we would become one. Shelby Dunn and Molly Cassatt, the shared desk intruders and creators of ‘mega desk’, I love you both for many reasons particularly including how fun you were around the lab. You made it a place I really looked forward to being at every day. Lourdes Gomez, I wish you were part of ‘mega desk’ longer… but if you were I think I really would never have gotten work done and finished. I’m sorry our 2020 plans were not met.

In addition to the Paleogenomics lab community, I also had the opportunity to be a part of the California-wide CALeDNA team. The CALeDNA research team became like a second lab family who I could always rely on for research questions, and research complaints. The CALeDNA team also includes hundreds of community scientists, many of whom I had the opportunity to work with. The community interest and desire to engage in our science was one of the most fulfilling parts of my graduate work. I have valued everything you have taught me and am thankful for the opportunities you gave me to teach you some things I know.

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Dedication

To my sisters- Ashley, Chanel, and Noa:

My best friends

and the subjects of my earliest scientific experiments.

This dedication is only a small token to express how integral each of you were in pushing and pulling me through the last 5 years...more than you know.

And to everyone working towards making academia a safe and accessible space for all... regardless of sex, gender, sexuality, race, socioeconomic status, and all other labels or histories that are used against us.

Introduction

Humans have been a driving force in environmental modification and climate change over the last 12,000 years, and likely longer (Ellis et al., 2021). Truly understanding the extent of human influence, and its cascading effects on the environment, requires an understanding of the biodiversity and ecosystem functioning of past ecosystems. This paleoecological knowledge can then in turn inform ongoing restoration efforts by providing an understanding of how systems react to natural and anthropogenic drivers (Wingard et al., 2017).

The complexity of ecosystem composition, population dynamics, and functioning makes studying even modern systems difficult (Evans, 2019). When considering paleoecosystems, the data we can gather are limited to geological, chemical, and biological indicators preserved in space and time. Despite this limitation, records recovered from sediment and ice cores, speleothems, tree rings, corals, and fossil assemblages among other physical and biological indicators together provide thorough reconstructions of past systems (Wingard et al., 2017). Within these records, preserved biomolecules such as isotopes, proteins, and DNA offer incredible amounts of information on community composition and system dynamics.

Preserved DNA is generally degraded into small fragments by physical and biotic agents, such as UV radiation and microbial activity (Kistler et al., 2017). We often refer to this degraded DNA as ancient DNA (aDNA). aDNA was first isolated

in 1984, with 229 base pairs sequenced from museum-housed tissue of the extinct quagga (Higuchi et al., 1984). Technological advancements now enable the isolation of genomic-level DNA from bones as old as 780 thousand years(horse- Orlando et al., 2013) to 1.2 million years (mammoth- van der Valk et al., 2021). aDNA preservation at these older dates is due to the protection of DNA from physical and biotic degradation in dry and cold conditions, such as caves and permafrost. aDNA can be isolated and studied from preserved bones, animal tissues, plant macrofossils, and even environmental samples.

Environmental samples are those that contain the shed DNA from several organisms, and we call this DNA environmental DNA (eDNA). Since all organisms in an environment- plants, animals, bacteria, and fungi- shed DNA, eDNA provides a single medium to survey biodiversity broadly across a site, unlike other methods of biomonitoring that may be limited to a single biotic group. The wider biodiversity captured by eDNA has allowed biomonitoring of specific taxa (ex.- Deiner et al., 2021; Mejia et al., 2021), and identifying biotic and abiotic drivers of environmental change (ex.- Erlandson et al., 2018; Deveautour et al., 2018) among many applications. The ease and low cost of sampling across large geographic scales has allowed larger spatial biodiversity surveys than would have been previously realistic (ex.- de Vargas et al., 2015; DiBattista et al., 2020; Lin et al., in press). eDNA exists within soil (ex.- Deveautour et al., 2018; Lin et al., in press), sediment (ex.- Willerslev et al., 2014; Ficetola et al., 2018), air (ex.- Johnson et al., 2019; Clare et al., 2021), water (ex.- Bista et al., 2017; Sigsgaard et al., 2019), and stalagmites

(Stahlschmidt et al., 2019). When this eDNA is deposited and preserved chronologically, ancient eDNA can be isolated. These ancient eDNA samples include ice cores, sediment cores, and stalagmites.

Though eDNA is a powerful tool for total ecosystem biomonitoring, methodological/technical biases can alter the composition and evenness we observe from the true distribution of DNA in an environmental sample. Technical biases can be introduced into eDNA experiments during field sampling, laboratory processing, and bioinformatics (Pedersen et al., 2015). These biases have the potential to influence resulting biodiversity profiles, and are difficult to decouple from true differences among organisms in DNA deposition rates and taphonomic processes that drive variation in long-term DNA survival (Taberlet et al., 2018). DNA isolation protocols, for example, are known to influence which organisms are detected in a sample (Deiner et al., 2018; Dopheide et al., 2019). For example, Deiner et al. (2018) reported a three-fold change in observed α diversity depending on filter material, pore size, and chemical extraction, including the absence of some taxa known to be present under some extraction conditions. Choice of polymerase for PCR can also influence biodiversity estimates. Nichols et al (2017) showed that the composition and relative abundance of taxa detected in the PCR amplicon pool changed during PCR amplification as some polymerases biased the amplicon pools toward sequences with a particular GC content. Differences in amplicon length, templates secondary structures, and base mismatches at the PCR primer binding site affect binding and copying efficiency during PCR (Fonesca et al., 2012; Elbrecht & Leese, 2015;

Krehenwinkel et al., 2017) and also affect binding and copying efficiency during PCR, skewing post-PCR taxon composition and relative abundance estimates (Pawluczyk et al., 2015). As the number of eDNA data sets grows, and along with that the possibility for comparative analysis across data sets, the need to understand and mitigate these many potential biases grows (Braukmann et al., 2019; Ruppert et al., 2019).

In my dissertation, I address one aspect of the methodological biases that influence eDNA studies (Chapter 1), and I then apply my understanding of optimal aDNA and eDNA assays to explore DNA preserved in permafrost and artifacts from caves to learn about past environments and human interactions with it (Chapter 2 and 3). Degraded DNA offers a unique opportunity for studying past ecosystems. Ongoing technological and methodological advancements will continue to improve the amount of authentic ancient and/or environmental DNA that can be isolated from ancient samples, as well as the conclusions that we can reliably make from it.

In chapter 1, I address the influence of PCR stochasticity and sequencing depth of PCR replicates on observed community composition and dissimilarity. Metabarcoding PCR is the most common approach to isolating DNA of interest from an eDNA sample, and is increasingly being used in aDNA analyses. Stochasticity in the amplification of molecules with PCR is expected (Robasky et al 2014; Leray & Knowlton, 2017), but the extent to which stochasticity influences measures of alpha and beta diversity from samples is not well understood. I performed 24 PCR replicates for each of two metabarcodes on six soil samples to observe variability in

estimated alpha and beta diversity, as well as the influence of sequencing depth and minimum read threshold on observed communities. I found both sequencing depth and stochasticity between PCR replicates significantly influence α but not β diversity. The results of this study inform researchers about methodological concerns over eDNA data, and the conclusions they can reliably draw. Chapter 1 is a collaboration with Dr. Beth Shapiro and Dr. Rahel Meyer. I designed and performed all experiments, performed the majority of analyses, and wrote the first draft manuscript. Beth Shapiro and Rachel Meyer aided in analysis and revision of the initial manuscript. At the time of submission, this manuscript is being revised for resubmission to *Ecology and Evolution*.

In chapter 2, I used eDNA metabarcoding to generate data from plants and animals that will form a component of a study of changing plant and animal communities in present-day Yukon, Canada. Beringia, a region that spans eastern Siberia to Western Canada and, during times of lower sea level, included a land bridge connecting Eurasia and North America, served as the major route of trans-continental migration of flora and fauna, including humans, throughout the Pleistocene. The cold and dry conditions of this geographic region facilitates the long-term preservation of DNA in both preserved bones and sediments, allowing dozens of studies using paleo indicators to reveal ecosystem transitions due to changing climates and community composition. The last 50,000 years are of particular interest as this time period spans the last glacial maximum, human arrival, the extinction of many megafauna, and the transition into the Holocene. Though we

have general understandings of the major transitions in floral and faunal communities that occurred over the last 50,000 years, we do not well understand the fine scale dynamics of environmental change that occurred at a local level, and which can more accurately inform ecosystem dynamics and interactions by looking at transition and extinction dates not averaged over a large spatial scale. To investigate changes in local community composition, I isolated plant and mammalian DNA from 33 permafrost cores collected in the Klondike region of Yukon that span the last 50,000 years. This study confirms previous observations of general community compositional trends, more clearly defines the timing of shifts in habitat types between boreal forest and a mammoth steppe, and illustrates the utility of eDNA in depicting local paleoenvironmental communities. This study will be included in a larger synthesis of the region that includes ancient DNA data generated by others for bison and horses as well as isotopic data generated from bones and sediment. My contribution will not be published separately, but is crucial to reconstructing the dynamics of Beringia's plant community in particular.

In chapter 3, I isolated ancient DNA from archaeological moccasins to examine hunting strategies practiced by Promontory people occupying the Promontory caves of Utah 1240-1290 AD. The hide used to construct these moccasins can be morphologically attributed (with some range of error) to Bison, but no further information can be gathered. By isolating ancient DNA from these moccasins, I was able to confirm the Bison identification of the hides and also determine the sex of the bison used. The abundance of bison remains found in the

caves make it clear that promontory people were specialists in big game hunting. By determining the sex of bison used to construct moccasins, I was able to further investigate the Promontory people's large game hunting specialty by illuminating their patterns of use of female bison. This project was a collaboration with Nasreen Broomandkhoshbacht, Jonas Oppenheimer, Jessica Z. Metcalfe, Wes Olson, Rob Found, Mike Heaton, Timothy Smith, John W. Ives, and Beth Shapiro. Broomandkhoshbacht aided in part of the sample processing, Oppenheimer aided in gathering data for the eight reference bison used in this study whose data was generated by Heaton and Smith, Olson and Found provided unpublished data on bison demographics from Elk Island National Park, Metcalfe aided in revision of the manuscript, and Ives provided background text for the Promontory caves and occupants as well as revisions to the manuscript. At the time of submission, this draft was nearly ready for submission to *Proceedings of the Royal Society B*.

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Chapter 1: Revisiting the effect of PCR replication and sequencing depth on biodiversity metrics in environmental DNA metabarcoding

Sabrina Shirazi, Rachel S. Meyer, Beth Shapiro

Abstract

Environmental DNA (eDNA) metabarcoding is an increasingly popular tool for measuring and cataloguing biodiversity. Because the environments and substrates in which DNA is preserved differ considerably, eDNA research often requires bespoke approaches to generating eDNA data. Here, we explore how two experimental choices in eDNA study design – the number of PCR replicates and the depth of sequencing of PCR replicates – influence the composition and consistency of taxa recoverable from eDNA extracts. We perform 24 PCR replicates from each of six soil samples using two of the most common metabarcodes for Fungi and Viridiplantae (ITS1 and ITS2), and sequence each replicate to an average depth of ~84,000 reads. We find that PCR replicates were consistent in composition and relative abundance of taxa assigned at least 3.22% of the reads for Plant ITS2 and 13.58% of Fungal ITS1, and that rare taxa are often unique to one or a few PCR replicates. Stochasticity in detection of rare taxa introduces significant variation in alpha but not beta diversity.Our results suggest that low sequencing depths (1,000 assigned reads) and few PCR replicates may be sufficient for many biological

applications, but DNA metabarcoding may never fully recover the true alpha diversity in a DNA extract.

Introduction

Environmental DNA (eDNA) metabarcoding of environmental samples is gaining traction as a biomonitoring tool (e.g. Deiner et al., 2021; Mejia et al., 2021) and for testing hypotheses about biotic and abiotic drivers of changes in community composition (Erlandson et al., 2018; Deveautour et al., 2018). Metabarcoding is used to measure species richness and compositional turnover in environmental samples, and can be used to measure changes in biodiversity over time (Willerslev et al., 2014; Epp et al., 2015; Bálint et al., 2018) and across large geographic ranges (e.g. the sunlit ocean, de Vargas et al., 2015; human impact gradients, DiBattista et al., 2020). This work has led to development of new essential biodiversity variables (Jetz et al., 2019) and bioindicators of environmental change (Kissling et al., 2018; Lin et al., 2021). In addition, because metabarcoding can be performed simultaneously for multiple loci that target different taxonomic groups, the technique can be used in applied ecology and habitat management without *a priori* knowledge of community composition. Despite the potential of metabarcoding, however, variation in metabarcoding results among biological samples from the same location, and even among technical

replicates from the same DNA extract, continues to complicate eDNA experimental design at all stages of sample collection, processing, and data analysis (Beng and Corlett, 2020).

Variation among replicates of metabarcoding experiments arises due to a combination of biological and technical biases. Biological biases reflect differences among taxa in the probability of DNA preservation due, for example, to organism size, seasonality, and behavior (Beng and Corlett, 2020). Technical biases are introduced by experimental choices during field sampling, data generation, and bioinformatic analysis. For example, technical biases can be introduced if DNA isolation protocols (Deiner et al., 2018; Dopheide et al., 2019), PCR polymerases (Nichols et al., 2017) and metabarcoding primers (Clarke, Soubrier, Weyrich, & Cooper, 2014; Deagle et al., 2014; Alberdi et al., 2017) preferentially recover taxa with particular physiological traits or genetic sequences. Biases may also emerge if taxonomic profiles become skewed during PCR due to PCR runaway (Polz and Cavanaugh, 1998), tag jumping (Taberlet, Bonin, Zinger, & Coissac, 2018), and overamplification (McPherson & Moller, 2006), although the latter can be mitigated somewhat by using quantitative PCR (qPCR) to determine the most appropriate number of PCR cycles (Murray, Coghlan, & Bunce, 2015). Finally, biases can be introduced by the stochastic nature of PCR amplification (Leray & Knowlton 2017; Beentjes et al., 2019), such that taxa that are rare in the DNA extract may become

common in the post-amplification pool if amplified during an early PCR cycle (Nichols et al., 2017). As a consequence of these combinations of biases, replicate metabarcoding PCRs can provide significantly different taxonomic profiles.

Previous studies have improved understanding of why PCR replicates often have different taxonomic profiles. A goal of this previous work has been to make generalizable recommendations as to how best these potential biases can be avoided in eDNA research, but results have been mixed. Smith and Peay (2014), for example, reported that two of the most common measures of biodiversity – alpha and beta diversity – did not change with higher numbers of PCR replicates. However, this study sequenced pooled rather than individual replicates, such that fewer reads were sampled from each replicate as the number of replicates increased, which may affect recovery of rare taxa. In a landmark study, Ficetola et al. (2015) used species occupancy modeling to determine the most appropriate number of PCR replicates based on predicted taxon abundance. In contrast to Smith and Peay (2014), this study found that as many as eight replicates should be used if the probability of detection was not high. When they tested this hypothesis using biological samples, they confirmed that using more replicates increased observance of rare taxa, and recommended bespoke replication strategies based on biological information. However, their replicate design included PCRs from multiple extracts rather than a single extract and so did not explicitly address differences between true replicates.

Although Ficetola et al. (2015) found that higher numbers of replicates were often important in surveying biodiversity, few studies use high replication to date. Nonetheless, studies have continued to show the importance of replication in surveying diversity. Alberdi et al. (2017), Leray and Knowlten (2017), and Beentjes et al. (2019) all performed three replicate PCRs and found that alpha diversity increased as replicates were added, suggesting that replication recovers rarer taxa. To test this explicitly, Dopheide et al (2019) performed up to 10 replicate PCRs for each of four metabarcodes and estimated species accumulation curves as PCR replicates were added. They found that curves began to flatten only after this relatively higher level of replication, and predicted that species accumulation would plateau with 10-20 replicates.

The influence of replication for surveying alpha diversity within a site or extract is better understood than is its influence on beta diversity – a measure of dissimilarity between sites or samples. Smith and Peay (2014) observed no influence on beta diversity based on the number of pooled replicates when sequencing depth was held constant, for example, although their pooled sequencing strategy may have reduced the possibility that rare taxa would be observed. Beentjes (2019) and Hajibabaei et al (2019) did not pool replicates and therefore probably recovered more rare taxa given their read sampling depth, but also found little to no effect of replication on beta diversity. Instead, Beentjes (2019) found that including biological

replicates sampled across space and over time was more likely to affect beta diversity than was replicate PCR amplification, probably because increasing the number of biological replicates sampled taxa that were not present in a single environmental sample.

PCR replication is not the only experimental choice that can influence recovery of rare taxa and therefore measures of alpha and beta diversity. Sequencing read depth, or the number of mapped reads to which each PCR amplicon is sequenced, may also affect the probability that rare taxa are observed. To test explicitly the influence of sequencing depth, Smith and Peay (2014) calculated pseudo-beta diversity from resequenced pools of the same extract. They found that dissimilarity between replicates decreased with increased sequencing depth, and concluded that sampling depth was more important than replication when recovering biodiversity within a PCR amplicon pool. Alberdi et al. (2017) observed a similar trend in which, when comparing read depths of ~2,500 to ~25,000 reads per replicate, alpha diversity increased with sequencing depth. While these results indicate that surveyed biodiversity increases with sequencing depth, how sequencing depth influences beta diversity remains underexplored.

Here, we examine how two key experimental choices – number of PCR replicates and depth of sequencing for each replicate – affect the composition and consistency of metabarcoding experiments. Because metabarcoding can only recover

taxa that are present in a DNA extract and amplifiable by the selected primers, we are not addressing the effect of these experimental on recovering the complete *biological* diversity of a particular site. Instead, our goal is to provide new insights into the reliability and replicability of PCR to recover the diversity of *amplifiable* taxa. We prepare a total of six DNA extracts from three geographic locations with distinctive biodiversity profiles, and, following the conclusions of Dopheide et al (2019), perform 24 individually-barcoded replicate PCRs from each extract. We sequence each PCR replicate to a target depth of >50,000 reads, and calculate alpha and beta diversity of replicates. To explore differences in potential bias between taxonomic groups, we perform this experiment with two commonly used metabarcodes that capture different phylogenetic biodiversity: the *Internal Transcribed Spacer* (*ITS*) for Fungi (*ITS1*) and for Viridiplantae (land plants and algae; *ITS2*). We use standard statistical approaches to explore how PCR replication and read sampling depth influence metabarcoding-based biodiversity estimates, and address explicitly detection of rare taxa and inference of community composition.

Methods

Soil Collection

We collected two soil samples from three ecologically distinct locations for a total of six samples. Two were from St Paul Island, Alaska, USA (StP.1: 57.136074, -170.82537; StP.2: 57.10577, -170.10563) and four were from sites in California, USA: two from Fort Ord Natural Reserve in Marina (FO.1, an open sand dune: 36.68448, -121.77731; FO.2, a chaparral ecosystem: 36.68301, -121.78071), and two from Younger Lagoon in Santa Cruz (YL.1, the basin of a coastal lagoon: 36.950081, -122.066756; YL.2, a grassland coastal terrace: 36.949314, -122.063575).

We designed field sampling protocols to minimize risk of cross-contamination. At each site, we wore clean gloves and used a trowel sterilized between samples to collect soil from 2-6'' below the surface in 50mL falcon tubes.

DNA Extraction, Amplification, Sequencing, and Taxonomy Assignment

We processed each soil sample in the UCSC Paleogenomics Lab eDNA room where no PCR amplification occurs, following clean room protocols. We homogenized and removed large plant matter (leaves and roots) from each sample, and subsampled two 0.25g aliquots of sediment from each sample. We extracted DNA from each of the 12 samples using the Qiagen PowerSoil kit and protocol (Qiagen, Germantown MD, USA), including one negative extraction control without

soil. We pooled the duplicate extracts for each site to ensure that sufficient quantities of DNA extract were available for the replication experiments.

We performed metabarcoding on each of the six extracts using the *ITS* gene in plants (ITS2- Plant ITS or PITS) and fungi (ITS1- Fungal ITS or FITS). We chose these barcodes because (1) they are among the most commonly used plant (Ankenbrand et al., 2015) and fungal (Nilsson et al., 2018) metabarcodes in eDNA; and (2) unlike other common barcodes that can only identify taxa to higher taxonomic levels, these can identify taxa to genus and species and are therefore valuable for considering the impact of rare taxa on eDNA-based biodiversity estimates. For PITS, we used primers described by Yao et al. 2010 (ITS-S2F and ITS-S3R) and for FITS, we used primers from White et al. 1990 (ITS5- forward) and Epp et al. 2012 (5.8S_fungi - reverse). The expected amplicon length was 450-480 base pairs (bp) for PITS and 200-350 bp for FITS.

For each extract, we used qPCR to assess PCR inhibition and determine the appropriate number of PCR cycles for metabarcoding (Murray, Coghlan, & Bunce, 2015). We performed qPCR with the Qiagen Multiplex PCR Master Mix following manufacturers protocol with a spiked 1:2000 dilution of SYBR Green 1 Dye. In triplicate for each extract, we set up a serial dilution of 1:0, 1:1, and 1:3 extract to water proportions of the 2 µL DNA extract, and compared qPCR Ct values across the dilution series. We observed no inhibition and proceeded with undiluted extracts. We

determined the optimal number of PCR cycles for each extract and primer as the cycle after which the exponential amplification phase ended.

We followed a '2-step' protocol to build amplicon sequencing libraries (Nichols et al. 2017) using the same reagent set up as for qPCR with the appropriate number of cycles and without SYBR Green. For each extract, we performed 24 replicate PCRs with PITS and 24 PCR replicates with FITS. We amplified four PITS and four FITS PCR replicates from the extraction negative control (no sediment) and added two additional PCR negative controls (no extract) for each marker. We purified amplicon pools with SPRI beads (Beckman, Indianapolis, IN, USA), then indexed all PCR products individually using Kapa Hifi (Roche, Pleasanton, CA, USA), following 25uL manufacturer's protocol, to add eight bp dual indices, followed by a second SPRI bead clean. We used unique combinations of dual indices for each PCR replicate. We then quantified the concentration of DNA in the purified amplicon libraries with a Nanodrop (Thermo Scientific, Waltham, MA, USA) and pooled the libraries by equimolar ratios into PITS and FITS pools. We then quantified the pools with a Qubit fluorometer (Thermo Fisher, Waltham, MA, USA) and estimated average fragment sizes with a fragment analyzer.

To detect index swapping (incorrect index assignment between adjacent clusters; van der Valk et al., 2019) during sequencing, we amplified the PITS metabarcode from a DNA extract of spiral ginger **(***Costus pulverulentus*), which is

native to the neotropics and not found in California or Alaska. We generated three replicate PCR amplicon libraries from the spiral ginger extract following the 2-step protocol described above.

We pooled and sequenced 308 sediment and three spiral ginger libraries on an Illumina MiSeq v3 600 cycle kit for 2x300 bp reads. We targeted 100,000 reads per FITS library and 50,000 reads per PITS library, based on the anticipated higher taxonomic richness amplified by FITS and higher discard rate of FITS-amplified sequences due to the incompleteness of fungal taxonomy databases.

We used the first step of the *Anacapa Toolkit* (Curd et al, 2019) to perform quality control trimming and generate merged and unmerged forward and reverse amplicon sequence variants (ASVs). We then used the second step of the *Anacapa Toolkit* (Curd et al, 2019) to cluster ASV tables into taxonomy tables, which employs a Bayesian Least Common Ancestor approach (see full description of *Anacapa* in Supplemental Text 1; Gao et al., 2017). Taxonomy is assigned in the *Anacapa* pipeline with both a local and global bowtie2 alignment of ASV clusters to CRUX databases (CRUX database generation description found in Supplemental Text 1). We used taxonomic resolution at the lowest level assigned within our confidence threshold for all analyses.

Data Filtration and Analysis

We used the PCR and DNA extraction negative controls to detect and remove contaminants and the positive ginger control to infer the rate of index swapping. We converted taxonomy tables and the PCR replicate-associated metadata to *phyloseq* (v. 1.22.3; McMurdie and Holmes 2013) objects using *Ranacapa* (Kandlikar et al., 2018). We then used the R package *decontam* (v1.1.0; Davis et al., 2018) to remove identified contaminants using prevalence 0.1 between true samples and controls. To test for index hopping, we examined the species composition of spiral ginger extracts and looked for spiral ginger reads in our soil extracts.

To simulate PCR replicate diversity at different read depths, we randomly drew different numbers of reads (rarefied) from the decontaminated taxonomy tables for each DNA extract. We used the rarefy_even_depth() function of *phyloseq* to rarefy our data at depths of every thousand between 1,000 to 20,000 (ex. 1k, 2k, 3k…). As we increased rarefaction depth, some libraries that were sequenced less deeply dropped out of the analysis. Following rarefaction, we generated three data sets for each rarified library in which we applied minimum read thresholds of 2, 5, and 10. The taxon richness average of 25 rarefactions per PCR replicate were plotted using data filtered with a minimum read threshold of 5.

We tested false positives in PITS data by evaluating the likelihood that taxa detected in a DNA extract are known local taxa reported to the Global Biodiversity

Information Facility (GBIF.org). We used GBIF data grabs from https://doi.org/10.15468/dl.yptmrz for Younger Lagoon and Fort Ord extracts and used https://doi.org/10.15468/dl.7c8huv for St. Paul Island. We performed 1-tailed t-tests in R to compare these local survey taxa to PITS taxa.

We generated empirical and extrapolated taxon accumulation curves for datasets prior to estimating various Alpha diversity metrics using the R package *iNEXT* (Hsieh et al., 2016). We implemented *iNEXT* with q=0, datatype="abundance", knots=40, se=TRUE, conf=0.95, nboot=50 extrapolate to replicates to twice their true read sampling depth. We performed outlier tests on extrapolated observed richness by identifying points that fall outside values of 1.5 times the interquantile range. We calculated observed richness, the Shannon diversity index (Shannon 1948), and Simpson index (Simpson 1949) with the *vegan* package in R. While observed alpha diversity considers only taxon presence, the Shannon and Simpson's estimators consider both the relative abundance of taxa within a sample in addition to taxon presence. We then performed two-sided t-tests and chi-square tests in R *stat*.

We performed statistical tests for beta diversity using *MicrobiomeSeq* in R that draws on the *vegan* and *phyloseq* packages. We plotted taxon relative abundance barplots using the Canberra beta diversity measure (a measure within the Bray-Curtis

beta diversity family of estimators), and calculated local contribution to beta diversity (LCBD) to quantitatively examine differences in composition and relative abundance.

Results

Data summary and evaluation of potential contaminants and false positive taxa

We generated an average of 78,809 PITS sequences (range: 9,352-282,579; Table S1.1) and 88,987 FITS sequences (range: 15,409-382,888; Table S2) for each of our 288 amplicon libraries (24 PCR replicates for each of six extracts, two markers). Following adapter removal and quality trimming, we retained an average of 37,640 PITS reads (range: 6,148- 166,279; Table S1.1) and 63,436 FITS reads (range: 12,360-323,310; Table S1.2) per PCR replicate.

Based on the sequence composition of the three *Costus pulverulentus* samples, we found no evidence of index hopping between libraries during sequencing. After prevalence-based decontamination, which identified and removed two taxa from the FITS dataset (*Malassezia restricta* and *Stereum hirsutum)*, 1099 unique taxa were retained in the FITS results and 353 were retained in the PITS results (Tables S1.3-S1.4), with 278 of the FITS taxa and 50 of the PITS taxa represented by only a single read. We assumed single read taxa and other low abundance taxa (<10 reads) were potential false positives and filtered these out with

thresholds of 2, 5, and 10 reads in downstream analyses. We used traditional observation cross-validation and an analysis of congeneric species in our results (Supplemental Text 1.2; Table S1.8) and found that while some low frequency taxa may be false positives, they are not overrepresented as singleton observations compared to taxa cross-validated as likely true, and therefore are not expected to impact downstream results. Following decontamination, the majority of taxa were identified to the species level, although some were identified to higher taxonomic levels (PITS- 260 species, 74 genus, 19 family, 7 order, 2 class; FITS- 875 species, 178 genus, 35 family, 18 order, 8 class; see Tables S1.3 and S1.4).

The influence of read sampling depth on alpha diversity

Taxon accumulation curves (Fig. 1.1) show that for all DNA extracts and PCR replicates, the PITS curves surpass the inflection point where slope begins to decrease (asymptote) at a sampling depth under 5000 reads, but the inflection point is less apparent in the FITS data set. Fig. 1.2 shows that increasing the read sampling depth from 1,000 to 10,000 reads resulted in an average 1.8-fold increase in observed alpha diversity for PITS and 2.4-fold increase for FITS (Fig. 1.2, Table S1.5). Shannon and Simpson diversity did not significantly increase with read sampling depth for most extracts in the PITS data set, but did significantly increase with all FITS data sets (Table S1.5).

We found the extrapolated variance in richness among PCR replicates of a single DNA extract was high for both metabarcodes and that the degree of variation was not consistent across extracts from different habitats. Observed richness estimates were rarely normally distributed and variance was high, with up to five replicates from the same extract being outliers from the mean (Table S1.6). After outlier removal, PCR replicate richness at the extrapolated asymptote still exhibited multiple fold differences in PITS and standard deviations equivalent to up to 30% of the maximum richness of the group (Table 1.1). We found the highest fold differences in observed richness in the PITS data set from YL.1 (Fig. 1.1e), a site situated within a marine lagoon at a location that is regularly inundated with both marine water and stream runoff. We observed fewer outlier replicates in extrapolated richness for FITS, with only up to two outliers per group, but that variation was high, with standard deviations up to 21% of the maximum richness of the group (Table 1.1). We observed the highest fold differences in observed richness in FITS at YL.1 and FO.2.

PCR replicates under different read sampling depths and minimum read thresholds

To measure the presence of low abundance and possibly unique taxa, we calculated increases in alpha diversity as PCR replicates are added to a combined data set, bootstrapping the analysis 100 times and plotting the mean (Fig. 1.3). Intriguingly, we

did not observe a plateau in species richness even after all 24 PCR replicates were included, indicating that this relatively high number of PCR replicates was insufficient to fully sample the diversity of taxa within the DNA extract (Fig. 1.3). Generally, increased read sampling depth increased the number of PCR replicates needed to reach saturation, while increased minimum read threshold lowered the number of replicates required to reach saturation (Table 1.2), where we define 'saturated' as when the number of taxa increases by less than one on average when another PCR replicate is added. We observed substantial differences among extracts.

Most taxa were present either in only one PCR replicate or in all PCR replicates (Fig. 1.4). We found a significant correlation between a taxon's within-replicate sequence abundance and its frequency across replicates at all read depths and minimum read thresholds (Fig. 1.5). Taxa present in all PCR replicates in the 5,000 read dataset (Fig. 1.5) were at sequence frequency 0.2-36.7% in the PITS dataset (average 3.22%) and sequence frequency 0.36- 74.68% in the FITS dataset (average 13.58%). Increasing the minimum read threshold reduced the number of taxa detected in only a single PCR replicate, while decreasing the minimum read threshold increased the number of taxa detected in a single replicate (Figs S1.2 and S1.3).

Composition and relative abundance (RA) variation across PCR replicates

The most abundant families detected across PCR replicates with PITS and FITS were found consistently across replicates, but some DNA extracts behaved as outliers in both relative abundance and composition (Fig. 1.6), and several PCR replicates were outliers in their local contribution to beta diversity (Table S1.7). At a read sampling depth of 5,000 and with a five read minimum threshold, LCBD statistics identified 11 such outlier PCR replicates from the YL.1 extract and two from the FO.2 extract for the PITS results, and one outlier replicate from the FO.2 extract in the FITS results (Table S1.7).

To explore how read sampling depth and minimum read threshold influence LCBD outliers, we repeated these analyses at all three read sampling depths (1,000, 5,000, and 10,000 reads) with minimum read thresholds of two, five, or ten reads (Table S1.7), and performed Chi-squared tests for significant differences among groups. The number of PCR replicates identified as LCBD outliers increased significantly with higher read sampling depth in the FITS data set ($p= 3.861e-15$), but not in the PITS data set ($p=0.25$). We found no significant effect of minimum read threshold for either the PITS ($p=0.71$) or FITS ($p=0. 79$) data set, suggesting that low abundance taxa, which are most likely to be impacted by changing the minimum read threshold, are not causing outliers. For both the PITS and FITS data set, we found that

DNA extract itself affected the number of observed PCR outliers significantly (both p <2.2e-16).

Variation among PCR replicates in beta diversity distance matrices

We evaluated inter and intra extract-based estimates of beta diversity using the Jaccard metric, which weighs all observed taxa equally. At both 1,000 and 10,000 read sampling depths, we found that PCR replicates in both PITS and FITS results clustered by extract, and that extracts from the same geographic area clustered near each other in ordinal space (Fig. 1.7). None of the identified PCR replicate outliers reduced the ability to differentiate extracts based on the PCoA. While read sampling depth did not affect dispersion in the PCoA between PCR replicates for either PITS or FITS, increasing read sampling depth changed the position of some extracts relative to each other in the ordination (Fig. 1.7). For example, the two Fort Ord extracts had similar PITS taxon composition and could not be distinguished from each other at either read sampling depth. However, the two St. Paul extracts could be differentiated at both read sampling depths for PITS but only at the lower read sampling depth in FITS. We next calculated beta diversity using the Bray-Curtis estimator (Bray and Curtis, 1957), which considers relative abundance, and compared the resulting PCoA plots to those generated using the Jaccard estimator (Figs S1.1 and 1.7). The plots

were similar overall, with slightly closer clustering among replicates at the two St. Paul extracts in the PITS data set. The similarity between results using Jaccard and Bray-Curtis estimates suggests that low abundance taxa may not strongly influence beta diversity.

Discussion

Both read sampling depth and the number of PCR replicates significantly affected our measures of alpha diversity (Fig.1. 2)**.** We observed stochasticity among PCR replicates in which and how many low abundance taxa were recovered (Figs 1.4 and 1.5), as has been shown previously using both simulated and real data (Smith and Peay, 2014; Ficetola et al., 2015; Piggott 2016; Alberdi et al., 2017; Dopheide et al., 2019; Beentjes et al., 2019). When we increased read sampling depth from 1,000 to 10,000 reads, observed alpha diversity increased for both the PITS and FITS datasets (Figs 1.1, 1.2a, 1.2d, Table S1.5), and Shannon and Simpson diversity, which incorporate abundance as well as presence/absence data, increased significantly with all FITS data sets but not for most PITS data sets (Figs 1.2b, 1.2c, 1.2e 1.2f, Table S1.5). These results, together with those of other studies, confirms that alpha increases with PCR replication and sequencing depth regardless of metabarcode choice.

Our results both confirm and extend previous results exploring the impact of replication and sequencing depth on biodiversity estimates. Specifically, our high replication strategy has revealed that it may not be possible to make generalizable recommendations about either parameter. As expected, we also showed empirically that potential biodiversity in the local environment is an important consideration when designing replication and sequencing strategies. For example, the sequencing depth at which individual PCR replicates recovered the full diversity within that replicate varied by both sample and metabarcode (Fig. 1.1). This variability in taxon accumulation with sequencing depth may be in part due to variation in richness, evenness, and/or the efficiency with which taxa present in the DNA extract are amplifiable (Kelly et al., 2019). We observed the highest variation in alpha diversity both when comparing different sampling depths and between individual replicates at the same sampling depth at the Californian lagoon site (YL.1) (Figs 1.1e and 1.2) where water and wind carries and deposits DNA-containing materials from the surrounding environment. Additionally, while species accumulation curves for each site were still increasing after data from all 24 PCR replicates were added (Fig. 1.3), the timing of saturation of these curves, which we defined as an average increase (after averaging bootstrapped samples) of fewer than one taxon with an added PCR replicate, varied significantly by site, again with the lagoon site the slowest to approach saturation (Fig. 1.3; Table 1.2). In general, when we either increased the

minimum read threshold – the number of reads required for a taxon to be counted as present – or increased the sampling depth of each PCR replicate, fewer replicates were required to saturate the species accumulation curves (Table 1.2). Together, these results suggest that many unique taxa are rare in sequence abundance in each PCR (as observed in Figure 1.5).

False positive taxa, or taxa incorrectly assigned to a particular replicate, can inflate both alpha diversity and the number of replicates required to saturate species accumulation curves. One source of false positives is index hopping, in which sequences are associated with the wrong indices due to proximate clustering during sequencing (van der Valk et al., 2019). We found no evidence of index hopping among spiral ginger data, suggesting this was not a major source of noise in our data set. In addition, we cross-validated our PITS data table with traditional plant survey data from each site, and found no bias in the frequency with which a taxon was observed in PCR replicates compared to its detection with the survey (Supplemental Text 1.2; Table S1.8), suggesting that many low abundance taxa are not false positives. While spiking and cross validation offer some evidence of authenticity, identifying false positives remains a challenge in metabarcoding research (Ficetola et al., 2016). Other approaches to detect and remove false positives include establishing minimum read thresholds and/or confirming taxon presence in multiple replicates, but these approaches also remove true positives present at low frequency and impact

subsequent analyses (Taberlet et al., 2018; Tsuji et al., 2019). We found, for example, that increasing the minimum read threshold removed low abundance taxa, reducing alpha diversity and the number of replicates required to saturate the taxon accumulation curve. Finally, approaches that generate mock or simulated communities (Ficetola et al., 2015) may help differentiate true and false positives, although simulated communities necessarily oversimplify the distribution and evenness of biodiversity in natural systems.

While previous work has described the potential impact of rare taxa including false positives (e.g. Beentjes et al., 2019; Dopheide et al., 2019; Nichols et al., 2019), no consensus has emerged as to how many PCR replicates are necessary to characterize biodiversity within an eDNA extract. Ficetola et al. (2015) estimated from simulated data that eight replicates should be sufficient to detect low abundance taxa, but Dopheide et al. (2019) predicted 10-20 replicates may be required to detect the full biodiversity of some extracts. We observed considerable variation between sites and barcodes in the number of replicates necessary to reach saturation of species accumulation curves (Fig. 1.3; Table 1.2) and, despite using 24 replicates, most of our species accumulation curves do not saturate. One explanation for this difference between our and other studies is our use of qPCR to determine the appropriate number of cycles for each sample. Using qPCR in this way makes it less likely that our PCR amplicon pools are overamplified, and therefore more likely that we retain

rare taxa (Murray, Coghlan, & Bunce, 2015). Cumulatively, these results suggest that it may not be possible to exhaustively survey biodiversity using eDNA metabarcoding, in particular for taxa, sites, and metabarcodes with high species richness and large numbers of potentially rare taxa. However, researchers focusing on rare taxa may want to consider an approach like qPCR to optimize recovery of rare or poorly amplified taxa.

While recovery of low abundance taxa remains challenging, we find that high abundance taxa are consistently recovered, suggesting that low replication may be sufficient to address some biological questions. Despite some PCR replicates being LCBD outliers (Table S7), we find strong evidence of consistency among PCR replicates in community composition (Fig. 1.5) and relative abundance estimates (Figs 1.6 and S1.1). This consistency leads to stability across PCR replicates in measures of beta diversity. In addition, this consistency remains at relatively low sampling depths spanning 1,000 to 10,000 reads (Fig. 1.7). This pattern is as observed in previous studies with lower levels of replication (e.g. Beentjes et al., 2019; Hajibabaei et al., 2019), including those that, as we did, found increases in alpha diversity with increasing replication (Beentjes et al., 2019). One exception was at St Paul, Alaska, where our two sites became indistinguishable in PCoA space at higher sampling depths in the FITS data set, presumably because these sites were not dominated by one or a few common FITS taxa and higher sampling depth included

more relatively rare taxa that were common to both sites (Fig. 1.7). In fact, increasing sampling depth for FITS caused all samples to converge somewhat in PCoA space (Fig. 1.7). This convergence has several possible causes, including rare fungal biodiversity that is shared across samples, misalignments of ASVs to taxa found in other samples, and/or contamination not detected in the negative controls. The observed decrease in dissimilarity with increased sequencing depth is in contrast to Smith and Peay (2014) who observed increased dissimilarity with increased sequencing depth. As Smith and Peay targeted the same FITS gene amplified here, we hypothesize that the difference may be in part due to our use of qPCR to avoid biasing the final PCR amplicon pool and reducing overall biodiversity via overamplification, a problem that may have been exacerbated by their pooled sequencing strategy. Our results suggest that low sequencing depths (as low as 1,000 reads assigned to taxa) and only a single PCR replicate may be sufficient to identify common taxa and estimate beta diversity between sites.

Finally, we found that outlier PCRs were more commonly amplified when extracts/sites have high taxonomic diversity. Outlier PCRs were most common in our Younger Lagoon sites, where biodiversity was high, and least common in the Alaskan sites, where biodiversity is lower (Figs 1.1 and 1.2, and Table S1.7). When we increased read sampling depth, the frequency of PCR outliers also increased, but only for the FITS data sets (Table S1.6). This may reflect a combination of the higher

number of low abundance taxa recovered by the FITS metabarcode and the low identifiability of sequences amplified by this barcode compared to others due to database limitations. Changing the minimum read threshold, alternatively, did not significantly influence the prevalence of PCR outliers (Table S1.6), suggesting that the lowest abundance taxa are not determining outlier status. While further work will be necessary to understand the precise cause of outlier PCRs, outliers are only observable (and removable) if more than two PCR replicates are performed. This rationale is often used in experiments that perform three PCR replicates per sample (Taberlet et al., 2018), as this experimental design allows disambiguation between an outlier and non-outlier replicates.

Conclusion

Here, we investigated the impact of PCR replication, read sampling depth, and minimum read threshold on estimates of alpha and beta diversity from eDNA extracts. At each of our sites and with both metabarcodes, alpha diversity increased with sampling depth and number of PCR replicates, and decreased with higher minimum read thresholds. We find that 24 replicates, a number higher than the standard recommendations in the field, was too few to survey the complexity of taxa

that are amplifiable using either metabarcode, suggesting that the ubiquitous nature of rare taxa may make exhaustively surveying biodiversity from eDNA extracts impossible. Future research using simulated communities, which necessarily oversimplify communities present environmental samples but have improved power to discriminate false negatives and positives, will improve our understanding of the impact of rare taxa on measures of alpha diversity and PCR outliers.

While alpha diversity estimates are likely to remain problematic for eDNA research, we found that beta diversity, which is often used to compare sites and track trends in community composition, is stable at different levels of replication, sampling read depths, and minimum read thresholds, presumably because beta diversity estimates less influenced by rare taxa. Beta diversity estimates could be used to distinguish and compare sites and extracts with as few as three replicates, which is an experimental design that allows detection of potential outlier PCRs. We have only addressed the consistency and predictability with which amplifiable taxa are recovered; biological differences in organismal abundance and likelihood of DNA persistence and technological differences in the efficiency of amplification of any particular taxon will all affect the probability that taxa present in the environment will be recoverable via metabarcoding. These differences, too, will impact whether it is possible to reconstruct the complete biological diversity of a given site or sample.

Together, these results reiterate the importance of considering physical and ecological settings as well as the targeted taxa and metabarcode choice as part of experimental design (Anderson et al., 2012; Ficetola et al. 2015). Experimental parameters not investigated here also affect biodiversity estimates from eDNA samples, including DNA extraction method (Piggott 2016, Deiner et al., 2018; Dopheide et al. 2019), the amount of soil processed (Dopheide et al. 2019), and metabarcode choice (Alberdi et al. 2017; Duke & Burton, 2020). Nonetheless, this work contributes to understanding of the complexity of eDNA research and underscores the power of simplified experiments that hold some parameters constant while allowing others to vary to facilitate development of experimental strategies that maximize the impact of eDNA.

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Table 1.1 - Variation in extrapolated taxonomic richness among PCR replicates after outlier removal.

SITE	MIN	MAX	STDEV
Plant *ITS2* (PITS)			
FO.1	21.2	48.3	5.8
FO.2	2.0	68.3	18.6
YL.1	2.0	122.8	31.4
YL.2	3.0	49.8	10.5
StP.1	1.0	51.4	15.2
StP.2	5.0	56.2	12.9
Fungal *ITS1* (FITS)			
FO.1	46.4	111.2	14.9
FO.2	24.5	106.7	22.2
YL.1	48.6	156.1	27.9
YL.2	97.0	199.8	24.4
StP.1	30.7	78.4	11.6
StP.2	34.2	75.2	12.0

Table 1.2 - Number of PCR replicates required to reach saturation of taxon accumulation curve, defined as the point at which taxon accumulation curve (shown in Figure 1.5) increases by less than one taxon with the addition of another PCR replicate. '>X' denotes that greater than the maximum number of replicates X retained after rarefaction is needed to suffice this point.

Read Sampling Depth	Minimum read cutoff	FO.1	FO.2	YL.1	YL.2	StP.1	StP.2
Plant *ITS2* (PITS)							
5k	2	5	20	17	4	8	10
	5	3	17	15	1	8	9
	10	2	10	12	1	3	8
10k	2	9	17	>23	5	15	19
	5	4	13	19	3	10	18
	10	3	9	16	1	9	9
Fungal *ITS1* (FITS)							
5k	2	>23	>23	>23	>24	>20	19
	5	11	7	15	12	12	6
	10	9	2	6	9	4	2
10k	2	>13	>18	>17	>21	>10	>16
	5	>13	>18	>17	>21	>10	>16
	10	>13	4	12	9	7	3

Figure 1.1 - Rarefaction curves tracking observed number of taxa identified among PCR replicates as a function of read sampling depth for the PITS and FITS data sets. Plots are created from the average of 25 rarefactions at each round thousand sampling depth between 1,000 and 20,000 reads, and a minimum read threshold of 5. Each line represents one PCR replicate. Termination of a line prior to the 20,000 read sampling depth denotes missing data. RAR = Retained after rarefaction, referring to the number of PCR replicates retained in analysis following rarefaction to read sampling depths of 5,000 and 10,000 reads. All 24 replicates for each of the six DNA extracts and both primers had sufficient data at 1,000 reads to be included in analysis (RAR.1k = 24 for all DNA extracts and amplicons).

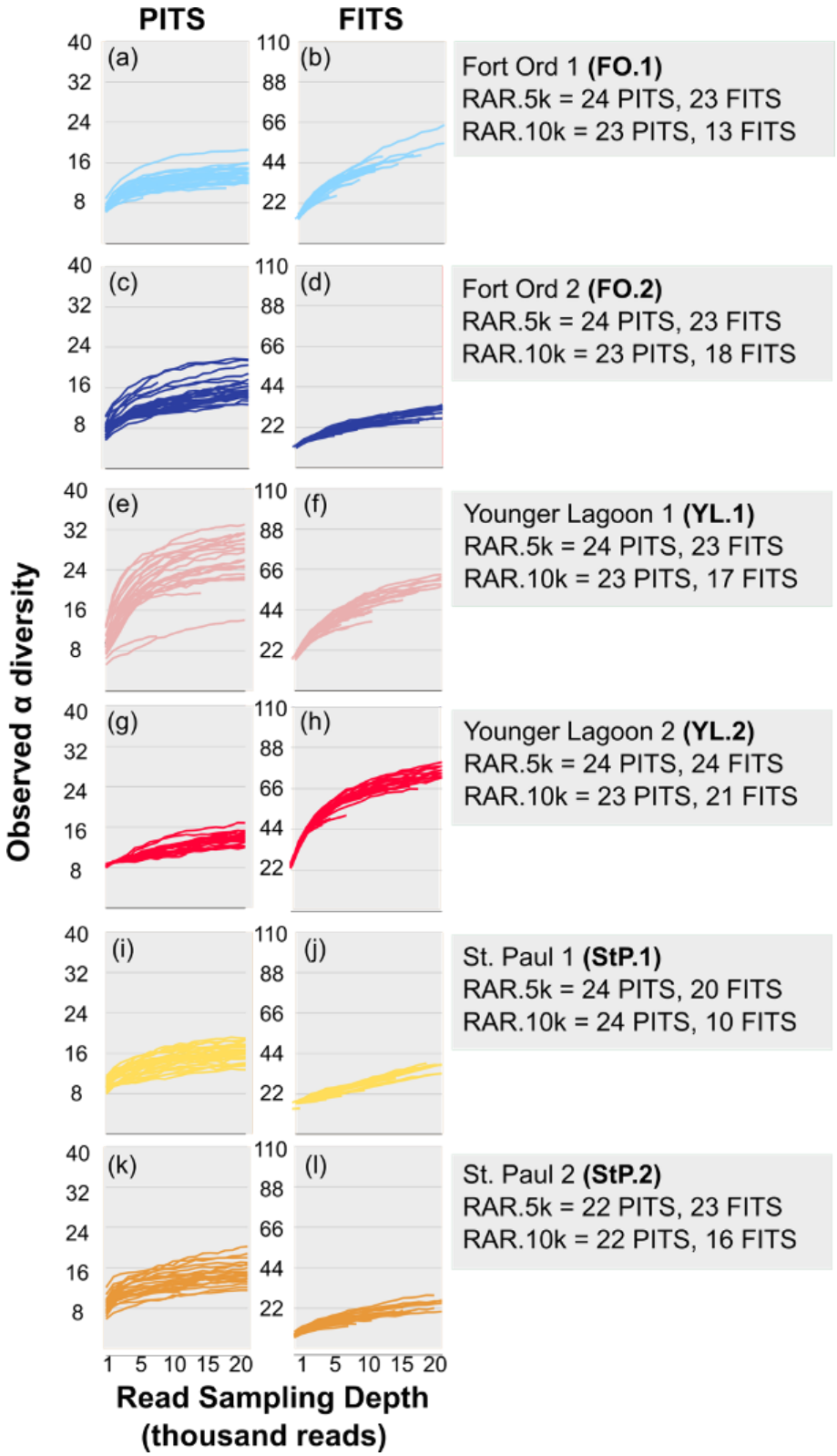

PITS
FITS
Observed α diversity
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
40
32
24
16
8
110
88
66
44
22
1 5 10 15 20
Read Sampling Depth
(thousand reads)
Fort Ord 1 (FO.1)
RAR.5k = 24 PITS, 23 FITS
RAR.10k = 23 PITS, 13 FITS
Fort Ord 2 (FO.2)
RAR.5k = 24 PITS, 23 FITS
RAR.10k = 23 PITS, 18 FITS
Younger Lagoon 1 (YL.1)
RAR.5k = 24 PITS, 23 FITS
RAR.10k = 23 PITS, 17 FITS
Younger Lagoon 2 (YL.2)
RAR.5k = 24 PITS, 24 FITS
RAR.10k = 23 PITS, 21 FITS
St. Paul 1 (StP.1)
RAR.5k = 24 PITS, 20 FITS
RAR.10k = 24 PITS, 10 FITS
St. Paul 2 (StP.2)
RAR.5k = 22 PITS, 23 FITS
RAR.10k = 22 PITS, 16 FITS

Figure 1.2 - A comparison of observed (a & d), Shannon (b & e), and Simpson (c & f) alpha diversity measured with read sampling depths of 1,000 (circles) and 10,000 (squares), and a minimum read threshold of five. Each dot represents a single PCR replicate.

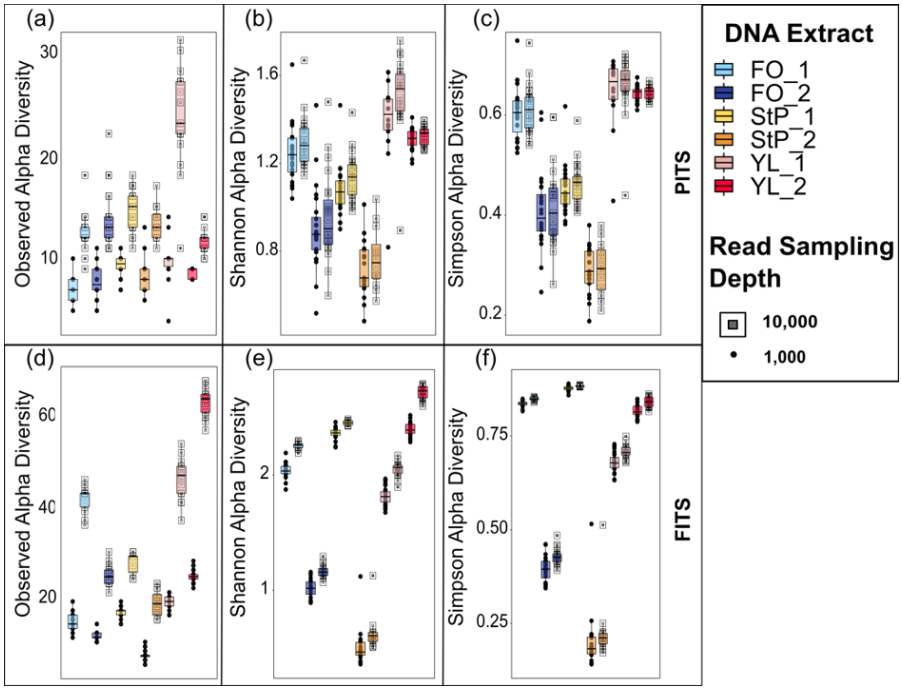


Figure 1.3 - Rarefaction curves describing the cumulative number of taxa detected with increasing number of PCR replicates, each sampled to a read depth of 5000 reads and using a minimum read threshold of five. We chose to plot the rarefaction depth at 5000 reads, as the exponential increase in taxon accumulation had begun to plateau by this read depth (Figure 1.1) and fewer PCR replicates had to be removed compared to higher read sampling depths. Each line reflects the average of 100 bootstraps in which the order at which individual replicates were added was shuffled.

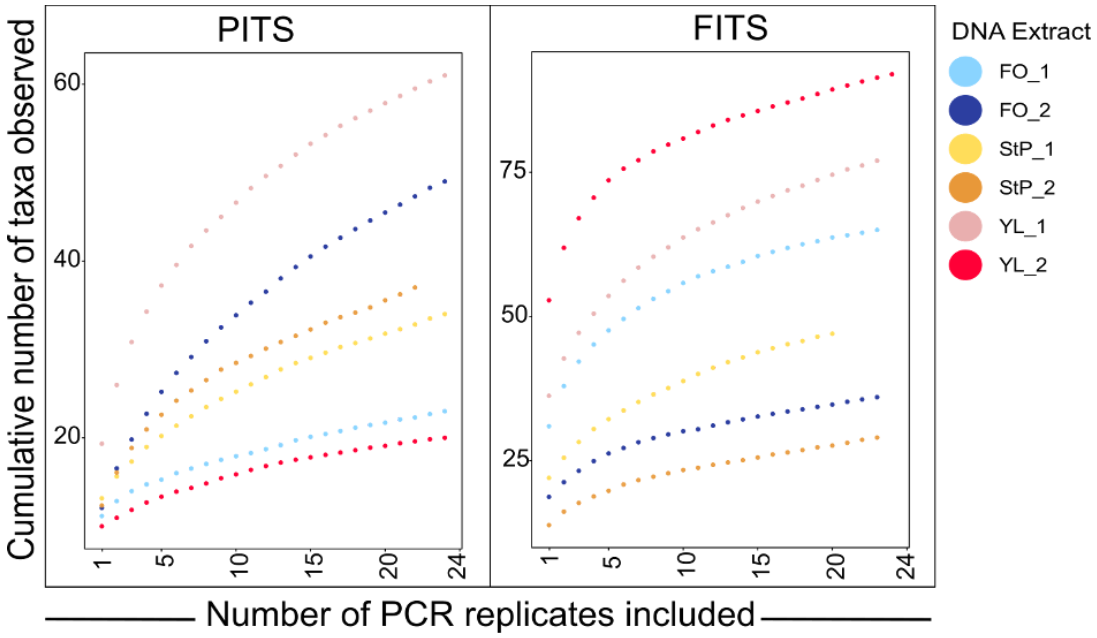


Figure 1.4 - Histograms describing the frequency of individual taxa detected across PCR replicates, each sampled to a read depth of 5000 reads and using a minimum read threshold of five, out of the total 24 replicates. The right-most bar in each plot is a count of taxa present in all replicates while the left-most bar is a count of taxa present in only one replicate.

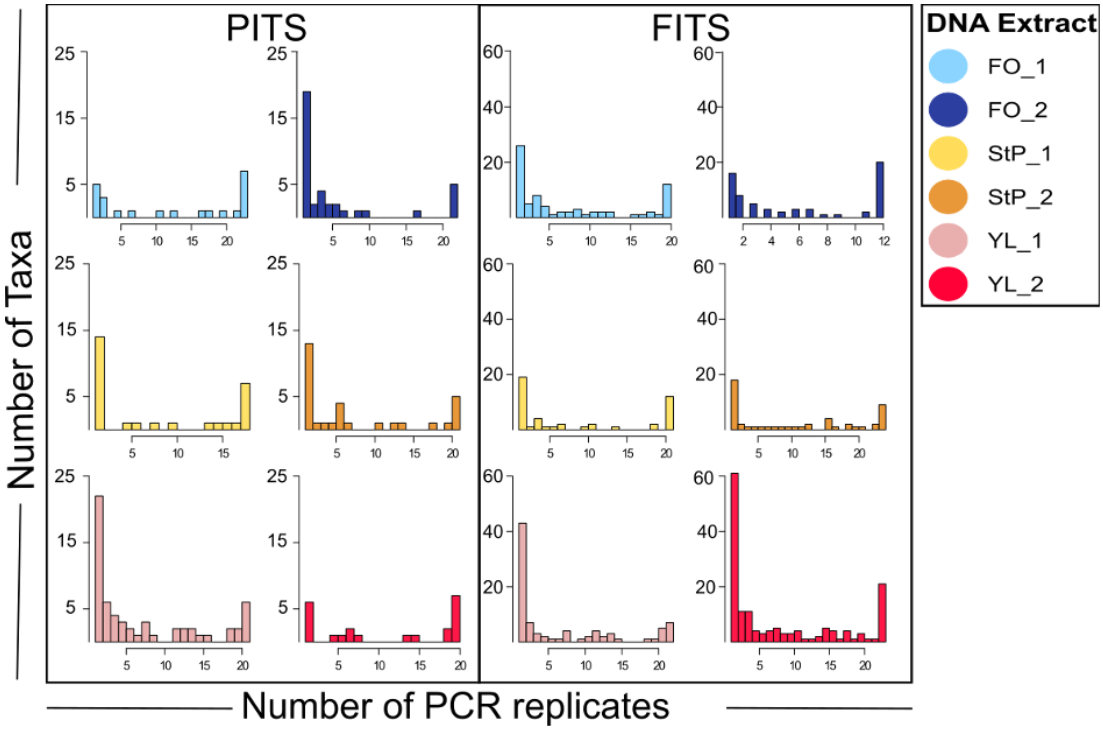


Figure 1.5 - Taxon accumulation curves by PCR replicate. Each data set comprises 5000 subsampled reads and incorporates a minimum read threshold of five. Sequence abundance is plotted as log-transformed counts of the number of reads per PCR assigned to a particular taxon, averaged across the PCRs in which that taxon is observed. We find a significant positive correlation between the number of PCR replicates in which a taxon is observed (fitted linear model results-- PITS: p<2e-16, T=24.73, adjusted r^2= 0.7324; FITS: p<2e-16, T=39.91, adjusted r^2=0.8219). Each dot represents an individual taxon and is colored according to DNA extract.

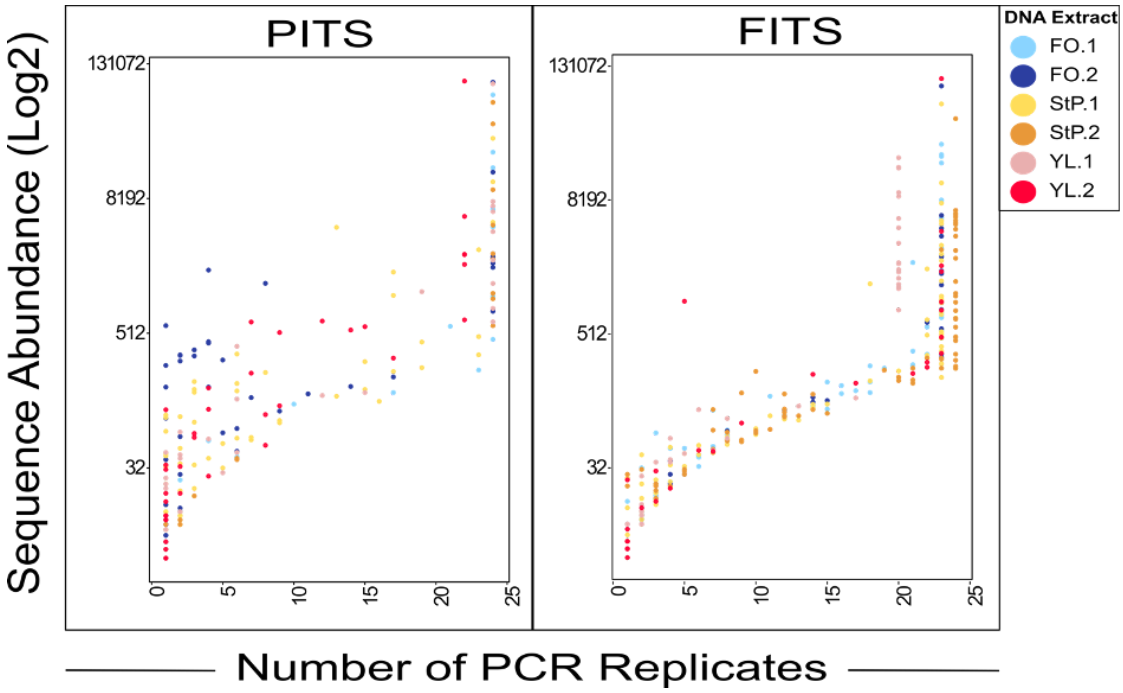


Figure 1.6 - Relative abundance of plant and fungal families detected with 5,000 reads and a five read minimum threshold. Each bar represents one PCR replicate. Only the 20 most abundant families are included here.

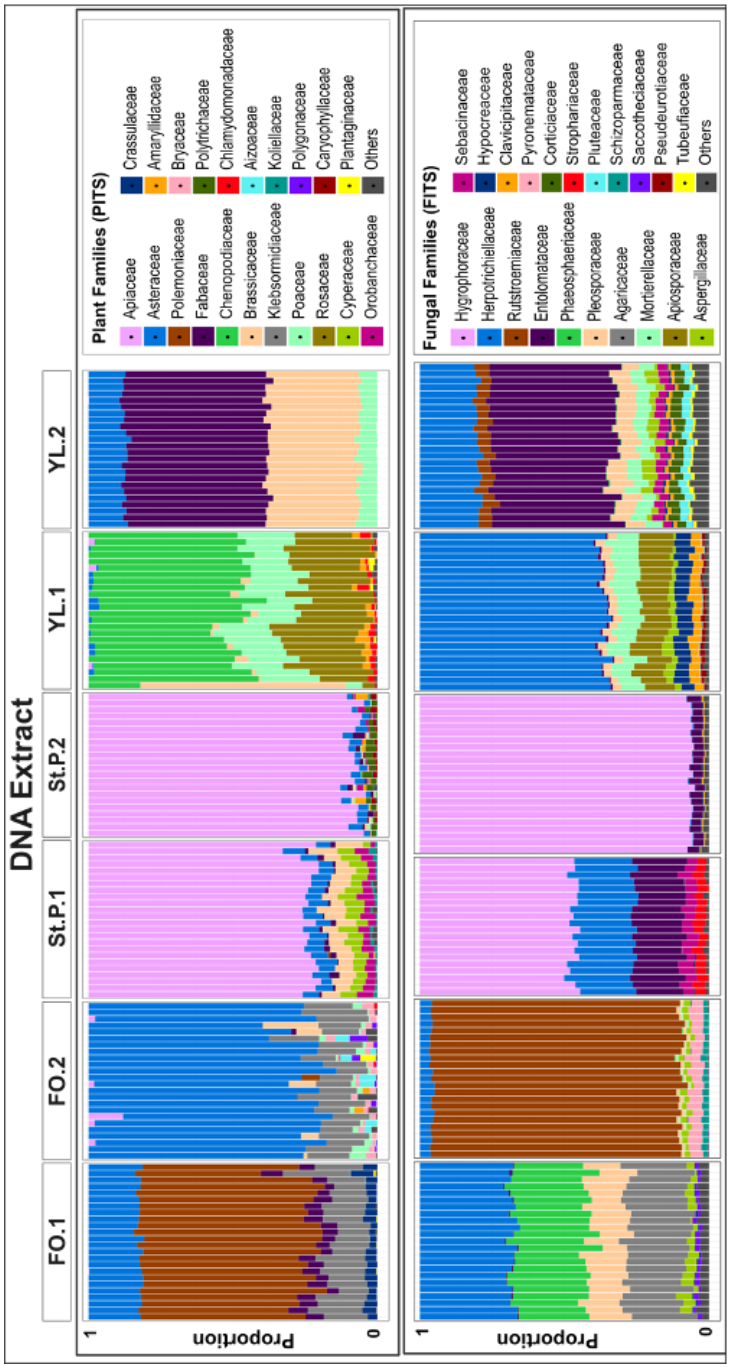
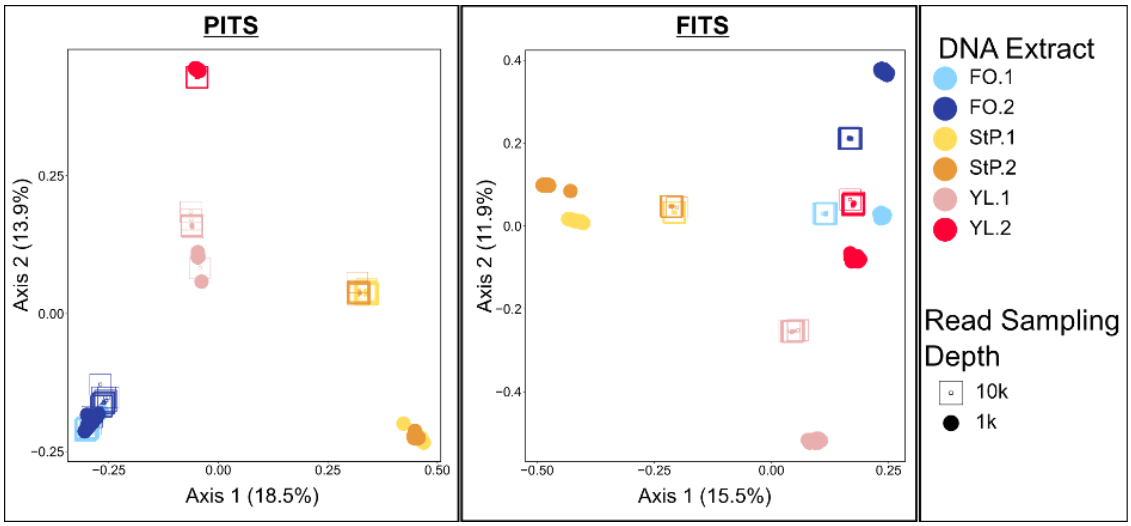


Figure 1.7 - PCoA on Jaccard beta diversity for PITS and FITS datasets with a five read minimum threshold. Each point represents one PCR replicate.



Chapter 2: Changes to the local plant and mammal communities of the Klondike region, Canada, over the last 50,000 years

Sabrina Shirazi, Molly Cassatt-Johnstone, Alisa Vershinina, Jonas Oppenheimer, Duane G. Froese, Beth Shapiro

Introduction

Beringia refers to the geographic land expanding between the Lena River in eastern Siberia and Mackenzie River in western Canada, inclduing a transient land bridge exposed during past glacial periods when sea level lowered. This region is a hotspot for paleoclimatic research, as it was a major passageway of cross-hemisphere, intercontinental migration of flora and fauna (Guthrie, 2001). In the last 50,000 years, Beringia underwent multiple climactic turnovers including the transitions into and out of the Last Glacial Maximum (LGM ~25-15 kya), the Bølling–Allerød, which was a time of rapid warming (~15-13kya), and the Younger Dryas, which was a brief cold period returning the climate to near glacial conditions, (~13-11.5kya), and the most recent warming event into the Holocene (~11.5kya-present). This time period is of even greater interest as it contains the mass extinction of Beringian megafauna across the Pleistocene-Holocene transition (Price et al., 2018, Mann et al., 2019), as well as the arrival of humans in North America (Guthrie, 2001). Beringian permafrost has preserved faunal and botanical remains, soil and water isotopes, and sedimentary ancient DNA (sedaDNA), all of which document these changes through space and time. Thus, permafrost provides an ideal study system for understanding

paleoclimate, communities, and species interactions in Pleistocene-Holocene Beringia.

During wet and warm interglacial periods, boreal forests dominated Beringia, while during cold, dry glacial periods, steppe tundra was the dominant vegetation (Guthrie, 1990; Zazula et al. 2003). The LGM (~25-15 kya), is of particular interest as the steppe habitat supported large populations of herbivorous megafauna that competed for resources, including mammoth, bison, and horse. Horses and mammoths survived on the steppe tundra through the LGM but soon after went extinct- the exact timing of which is regionally specific (Guthrie, 2003; Haile et al., 2009). Whether the eventual extinction of mammoths and horses was caused by changes in plant communities induced by climate change, or if the changes in plant communities were driven by megafauna grazing and trampling the land remains unknown (Zimov et al., 1995; Guthrie, 2001). To understand how this low productivity habitat was able to support populations of competing megafauna requires a true understanding of the floral composition of the steppe, and more exact timing estimates of floral change and faunal extinction. Although several previous studies have directly examined the floral communities using a range of biological proxies, they often disagree on the relative proportions of graminoids and forbs, and the presence of trees (Anderson and Brubaker, 1994; Guthrie, 2001;Bigelow et al., 2003; Kienast et al., 2005; Willerslev et al., 2014; Sadoway et al., 2014). These disagreements amongst previous studies are likely a result of the mosaic of

microhabitats, each with their own communities and turnover times, that made up the Beringian mammoth steppe ecosystem.

Guthrie (1984) first proposed the presence of microhabitats distributed across Beringia in a 'plaids' formation, where rapid climatic changes characteristic of the Pleistocene would cause deviations from normal 'striped' landscapes where gradual temperature gradients with latitude and altitude delineate general environmental structure. Since then, the mosaic of microhabitats across Beringia associated with drainage landscapes, aspect, and elevation were demonstrated with macrofossils (Goetcheus and Birks, 2001; Zazula et al., 2003 and 2006). This "plaid" distribution of landscapes was later hypothesized to have favored herbivorous megafauna, and that it was the eventual transition out of a 'plaids' landscape and into 'stripes' landscape that led to their extinction (Mann et al., 2019). To answer remaining questions about the causes and timing of turnover in Beringia, specifically during the LGM, we decided to focus on a single geographical area.

In order to focus on a single geographic area, we must consider the proxies that we use, and what exactly they can and cannot tell us. Pollen and macrofossil analyses, sedaDNA, and the study of rodent nests, are all common bioindicators used in studying Beringia's paleoclimates. While each of these indicators are valuable sources of information, they each come with specific biases that may alter observed communities and represent different scales of locality. Rodent middens and nests indicate an animal's presence and track the local vegetation from which that rodent would have foraged (Zazula et al., 2005, 2006b, 2007). Pollen analyses generally

recover a wider array of taxa, though the records tend to be biased towards plants producing high volumes of pollen (ex- graminoids and artemisia) and away from insect-pollinated taxa (Anderson et al., 2003). The migratory tendency of pollen with wind and water means pollen accumulations are not necessarily representative of a local environment (Joørgensen et al., 2012). SedaDNA allows a wider array of plant and animal records to be recovered via a single pathway, and at lower taxonomic levels than pollen, though biases in DNA deposition and processing can bias the organisms observed after DNA sequencing (Sønstebø et al., 2010; Ruppert et al., 2019; Mathieu et al., 2020). Macrofossils and sedaDNA represent the local environment, making them a better choice than pollen for examining local plant community composition (Joørgensen et al., 2012). The geographical range that a bioindicator represents is particularly important to consider as we begin to home in on particular geographical areas to understand the finer scale specifics of community turnover.

Here, we investigate the trends and timing of floral and faunal community turnover in the Klondike area of the Yukon, Canada over the last 50,000 years. We focus on determining when steppe tundra was present in a single slice of the mosaiced Beringian steppe, what plants and animals comprised the communities of the steppe tundra, what plants may have found refuge here, and when megafauna went locally extinct. We used sedaDNA as a proxy for these plants and animals, as sedaDNA data can be used to monitor turnover in both plant and mammal communities. By studying local community turnover, we observed similar general trends with more fine details

than previous studies which have often represented an additive community over wider geographical space.

Methods

Sample Collection

The Froese lab at the University of Alberta sent 33 permafrost soil plugs collected from the Klondike region of Yukon, Canada, to the UC Santa Cruz Paleogenomics Lab. Collection sites included Lower Hunker Creek (n=1), Upper Goldbottom Creek (n=16), Upper Hunker Creek (n=1), and Upper Quartz Creek (n=15), all within the Klondike (Table S1.1). These permafrost samples span from ~43,800 to 5,100 calibrated radiocarbon years before present (ka BP).

DNA extraction, amplification, and sequencing

We performed all work prior to amplification in the UC Santa Cruz Paleogenomics ancient DNA laboratory. The ancient DNA laboratory is located in an isolated PCR-free building and has an independent air filtering system and positive pressure. We wore sterile suits, gloves, face masks, hairnets, gloves and shoes while working in this lab to avoid contamination from outside sources. In order to further reduce contamination we bleached all surfaces daily and throughout any sample processing.

We completely thawed permafrost plugs mixed the sediment with sterile spatulas within the 50mL falcon tube they were collected in. We took four 500mg subsamples from each core to produce two DNA extractions per sample. We extracted DNA from permafrost following the protocol used in Seersholm et al. (2020). Following this protocol, two 500mg samples are combined following separate overnight digestions and before binding to a single MinElute column. We performed extractions in batches of 11 samples with one extraction negative control. Following extraction, for any heavily tinted or dark extract we cleaned those extracts with polyvinylpolypyrrolidone (PVPP) in columns following Arbeli and Fuentes (2007) in order to remove any PCR inhibitors.

To isolate DNA from plants and mammals, we chose to target the trnL plant and 16S mammal genes using primers that amplify small target regions, which is more efficient for ancient and degraded DNA. We used the trnL g and h primers introduced and tested on ancient samples in Taberlet et al., 2007 (g primer-GGGCAATCCTGAGCCAA, h primer- TTTGAGTCTCTGCACCTATC) which on average amplify 85bp fragments. We used 16S mammal primers introduced in Taylor et al. (1996) to isolate a 140 base pair amplicon (forward primer-CGGTTGGGGTGACCTCGGA, reverse primer-TGCTGTTATCCCTAGGGTAACT). Primers for both trnL and 16S included the Illumina TruSeq adapter to allow for use of the '2-step' amplification pipeline (Nichols et al., 2017).

We used qPCR to a) measure inhibition, b) calculate the appropriate number of PCR cycles for individual primers, and c) look for systematic trends in DNA preservation across our samples. We used the Qiagen Multiplex Master Mix, shown by Nichols et al. (2017) to introduce the least GC bias in PCR, for qPCR. qPCR reaction volumes included 12.5uL of the Qiagen MM, 2uL of each 2uM primer, 0.6uL of SYBR Green 1 Dye (diluted 1:2000), 5.9uL of water, and 2uL of our extracted DNA. For each extract, we performed nine total qPCR's for each primer-- a dilution series of 1:0, 1:1, and 1:3 (extract:water) amplified in triplicate. We performed 40 cycles of amplification with primer-specific annealing temperatures. We used the reaction with the lowest threshold cycle (C_t) value as informed by qPCR to determine the appropriate extract dilution to minimize the effects of inhibition. We calculated the appropriate cycle number by determining the point at which the amplification curve switched from an exponential to a linear increase. Using these qPCR results, we chose the extract per sample with less inhibitors and/or more DNA to move forward with. We plotted the C_t values observed with a 1:3 dilution from all samples, normalized the values to the highest observed value, and used a locally weighted regression to plot trends in DNA preservation through time.

We performed five metabarcoding PCR replicates for each extract and extraction negative, for both trnL and 16S-mammal primers. We used the sample-specific dilution chosen to minimize inhibition, and the cycle number chosen to avoid over-amplification. We introduced a PCR negative control for every 32 sample reactions. Following amplification, we used an 18% PEG SPRI bead solution

to clean all amplicon libraries. We then performed dual indexing PCR (iPCR) on each sample and negative control with Kappa Hifi. We again cleaned these sequencing libraries with SPRI beads.

We combined all libraries in two pools, one per primer set. We used the NanoDrop 8000 Spectrophotometer to calculate concentrations of all libraries, allowing for equimolar pooling between replicates and samples. We quantified each pool using a Qubit 4 fluorometer and estimated DNA fragment size distribution with an Agilent Fragment Analyzer 5200. We sequenced both pools at the UCSC Paleogenomics sequencing center Illumina NextSeq 550 runs of 2x75bp, targeting 50,000 reads per PCR replicate.

Data Processing and Analysis

We used the Anacapa pipeline (Curd et al. 2019) for general sequence quality control, trimming, and taxonomic assignment. We followed default settings aside from the additional end trimming where we changed -x and -y flag values to 5. We aligned Amplicon Sequence Variant (ASV) tables generated with the first quality control step of anacapa to CRUX formatted databases. The 16S mammal CRUX database was created following protocols presented in Curd et al (2019) to generate target databases from EMBL and BLAST. We used ASV clusters generated by *Anacapa* to search for *Haringtonhippus francisci* 16S gene (sequence-GAACAAAACAACCTCCGAGTGATTTAAATCTAGACTAACCAGTCAAAATATAGAATCACTTATTGATCCAAACTATTGATCAACGGAACA), a genus of horse

known in eastern Beringia towards the end of the Pleistocene (Heintzman et al., 2017), which was not in the 16Smammal CRUX database used. For trnL, we used the ArctBorBryo database, formatted as a CRUX library for processing through Anacapa. This ArctBorBryo database includes sequences from 815 Arctic plants (Sønstebø et al., 2010), 835 Boreal plants (Willerslev et al., 2014), and 455 bryophytes (Soininen et al., 2015) known across the arctic. We used the *decontam* R package (v1.1.0; Davis et al., 2018) to compare the ASV tables for extraction and PCR negative controls with soil plugs to remove any signs of lab-introduced contamination.

For the mammal data, we set a minimum read threshold of 5 reads assigned to a taxon within a single PCR replicate in order to accept that taxon as present. Additionally, we required an animal present in at least two PCR replicates to be considered a true positive. Given the low sequence attribution to various mammals, we did not rarefy the dataset. We removed all ASVs assigned to human, as with metabarcoding we do not see the native ends of DNA molecules and therefore cannot verify whether these sequences are a result of modern contamination. We removed any samples with less than 3 successfully amplified replicates from our analysis. For the plant data, we set a minimum read threshold of 10 reads, and rarefied our dataset to 15,000 reads to make comparisons of plant relative abundance among samples possible.

The 16S region amplified covers one SNP among bison that distinguish two clades of bison present in North America (Heintzman et al., 2016) (Clade 2-GAATAAAAAATCCTCCGAACGATTTTAAAGACTAGACCCACAAGTCAAATC

GCTCTATCGCTCATTGATCCAA[G]AAATTGATCAACGGAACA, Clade 1 and Siberian - GAATAAAAAATCCTCCGAACGATTTTAAAGACTAGACCCACAAGTCAAATC GCTCTATCGCTCATTGATCCAA[A]AAATTGATCAACGGAACA). Clades 1 and 2 differ by a single nucleotide SNP (A -> G). Though it is possible that sequencing or PCR error could introduce a single SNP, especially given it occurs in a poly A tail, an A to G transition would not be a result of known DNA degradation patterns (which are C->T and G->A, Dabney, Meyer, & Pääbo, 2013). Additionally, patterns of DNA degradation are known to occur towards the ends of DNA strands and this locus is within the middle of an amplified metabarcode that itself was somewhere within a DNA molecule in our permafrost extracts. We used the ASV file generated by *Anacapa* to search for these two known bison clades (Figure S2.2).

We used a combination of ggplot2 (v. 3.2.1) and the rioja package (v. 0.9-21) in R to create pollen plots of our plant and mammal data. Within rioja, we used the vegdist() function to calculate jaccard distance matrices (based on presence absence) from all plant data observed, Then we used the chclust() function to calculate connis clusters which take the distance matrix and breaks down the plant time series data into clusters based on dissimilarity.

Small mammal bone isolated from permafrost plug

While subsampling soil from MM12-134 in the ancient DNA lab, a small proximal half femur was found within the plug. In order to determine the species this

femur fragment originated from, we extracted DNA and prepared a single stranded shotgun library. We cleaned the bone with water and extracted DNA following the Dabney (2013) bone extraction protocol. We prepared a shotgun library following the Santa Cruz Reaction protocol (Kapp et al., 2021) (2010) protocol (Library ID MCJ063-1). We performed dual indexing PCR on this library with 50 uL AmpliTaq Gold 360 MM (Applied Biosystems cat. 4398881), 1 uL of 100uM forward and reverse index primers, and 48uL of our cleaned library in a 100uL total reaction amplified for 11 cycles. Following index PCR we cleaned the product with a 1.2X concentration of SPRI solution (Beckman, Indianapolis, IN, USA) beads, eluted in 20uL TET buffer. We quantified the library with a Qubit fluorometer (Thermo Fisher, Waltham, MA, USA), calculated the average fragment size with an Agilent Tapestation and sequenced the library, targeting 1 million reads on a Nextseq 2x75bp run.

After demultiplexing the raw data, we trimmed adapters, merged reads, and removed low quality reads with *SeqPrep2* (https://github.com/jeizenga/SeqPrep2). We removed duplicated and low complexity sequences with *prinseq* (v.0.20.4, Schmieder and Edwards, 2011). We used blastn (v.2.6.0) to align our data to the BLAST nucleotide database and visualized the resulting alignment with MEGAN (v. 6.19.8).

Arctic Ground Squirrel Nest Occurrence Data

In addition to the eDNA data generated here, we have gathered published data on the occurrence of arctic ground squirrel nests (AGSN) found in Klondike permafrost (see Table S2.2 for the complete list of references). The list of AGSN occurrences indicates a steppe environment (Zazula et al., 2005, 2006b, 2007) present in the Klondike 13,675 - 35,895 ka BP (n=39, Table S2.2).

Results and Discussion

qPCR and DNA preservation

We observed increased amounts of amplifiable DNA, indicated as a decrease in normalized C_t value, from 16-20 kya, during the latter half of the LGM(Figure S1.1). This observation of higher DNA presence during that last glacial period may be a result of increased DNA preservation due to stable cold temperatures. Though higher DNA preservation during the last glacial period is conceivable, our higher number of samples during the 16,000 to 20,000 year time zone could also explain this trend.

Mammals

We observed mammalian DNA with 16S mammal metabarcoding in 29 of the 33 soil plugs processed (Table S2.1). Two negative controls had low levels of non-human mammalian contamination and we removed a total six samples processed

in the batches associated with those negative controls (MM13-17, MM13-29, MM13-24, MM 13-28, MM 12-134, and MM 12-49). We also conservatively removed four samples from mammalian analysis due to lack of sufficient PCR replicates amplified (MM13-20 ⅖ replicates, MM13-40 ⅕ replicates, MM12-28 ⅕ replicates, MM12-42 ⅕ replicates). Following decontamination, a minimum requirement of 3 PCR replicates amplified, removal of human-aligned sequences, and a minimum read threshold of 5, 19 samples of the initial 33 remained in analysis; two samples dated to 5-6 kya, 16 samples from 12-25 kya, and one sample from 44 kya (Figure 2.1). The bulk of the samples (16) fall within the range observed with qPCR for increased DNA preservation. The PCR replicates included in analysis after filtering included 6-152,404 reads assigned to taxa (average 2838 reads, median 67 reads). We did not rarefy the 16Smammal data for any analyses.

After filtering, we identified eight taxonomic groups across all samples: snowshoe hare (*Lepus americanus*), bison (*Bison priscus*), wild boar (*Sus scrofa*), horse (*Equus sp.*), mammoth (*Mammuthus primigenius*), lemming (*Dicrostonyx sp.*), vole (*Microtus sp.*), and ground squirrel (*Urocitellus richardsonii*). Sus scrofa was not present in the Klondike and is a known common contaminant of PCR reagents (specifically from incomplete hydrolysis of dNTPs - Leonard et al., 2007), and so we removed *Sus scrofa* from the analysis. We found less than 5 reads aligned to *Haringtonhippus* for a given sample, which is not enough reads to be conservatively accepted as present. Further sequencing could potentially concretely identify more species, including *Haringtonhippus*.

We generated 1,252,453 paired end reads for the small mammal femur recovered from sample MM 12-134. 41,675 sequencing reads aligned to ground squirrel with BLAST, which was the most aligned reads to any mammal and confirms the ground squirrel origin of the femur (Figure S3). Bacterial sequences accounted for the majority of the remaining reads.

We found bison, mammoth, and horse at the highest frequency (Figure 2.1). Bison was the only mammal detected in our Holocene samples. We detected mammoth and horse consistently 26-12kya, though a sampling gap from 12-6kya makes it impossible to determine a local extinction date (Figure 2.1). Local extinction dates of horse and mammoth are of particular interest as they could represent longer-lasting refugia and may speak to the local floral environment. Previous sedaDNA records from the Klondike permafrost have signs of mammoth and horse recent as late as 9.7 kya (Murchie et al., 2020). Our sampling gap made it impossible to confirm such late presence, but our result confirms they were both still present at least 12kya.

We detected ground squirrels between 25 and 13 kya, which are a strong indicator of the local steppe environment (Zazula et al., 2005, 2006b, 2007) (Figure 2.1). Sample MM12-134 (35.9 kya) included hundreds of reads assigned to ground squirrel, but was removed from the final conservative analysis due to low levels of contamination in the negative control processed with the sample. This sample, MM 12-134, is the sample from which we recovered a ground squirrel proximal femur fragment, providing concrete evidence for the presence of ground squirrel in the

sample and supporting the hundreds of ground squirrel sequences recovered. Our sedaDNA ground squirrel observations between 13-35.8 kya (including MM 12-134) overlap almost exactly with records of ground squirrel nests recovered from the Klondike from 13.7- 35.9 kya (Figure 2.1). This indicates that ground squirrel DNA recovered from permafrost can be a reliable data point for recording the presence of a steppe environment. Though ground squirrel nests are clear evidence for a steppe tundra, and their composition provides a direct look into the local floral community, they are not reliably recovered (Zazula et al., 2006b, 2007).

In addition to ground squirrels, we detected lemmings, snowshoe hare, and voles (Figure 2.1). Snowshoe hare and lemming occurred only in a few samples and highlight the diversity of small mammals inhabiting the steppe. Hare are indicative of a boreal forest and are a signal of the transition from steppe to boreal forest habitat. We detected voles, which occurred 13,000-43,000 years BP, as consistently as ground squirrels, indicating that they too could be a reliable proxy for habitat differentiation. Vole species in the Yukon today each occupy niche habitats, including forest, tundra, and meadows (Galindo and Krebs, 1985). With the 16S metabarcode that we used, we could not distinguish species due to database limitations. The majority of phylogeny work completed on voles are based on the cytochrome b gene (Jaarola et al., 2004; Haring et al., 2011), with some recent genome-level research (Barbosa et al., 2018), and 16S sequences are not available for the various species known in the Klondike. Furthermore, it is not known if the vole species present in the Klondike thousands of years ago are of the same species present today. Just as ground squirrels are an

indicator of the steppe (Zazula et al., 2003, 2005, 2007), species-level identification of voles from permafrost has the potential to serve as an additional indicator of general local environmental composition. A sequencing project that targeted the 16S gene of all voles could allow future analysis of the vole species amplified here. The fossil record is biased against detecting small mammals since their bones are less likely to be preserved and be found (Turvey and Blackburn, 2011). Here, we showed sedaDNA is a reliable source for tracking small mammal communities.

Differentiation of bison clade 1 (which includes the Siberian bison) and clade 2 allowed us to investigate haplotype-level turnover in bison. We identified bison clades in all 19 samples, and found both clades 1 and 2 throughout the entire sampling period (Figure S2.2). This observation supports prior knowledge of the presence of both clades in the area (Heintzman et al., 2016; Froese et al., 2017). Rare cases like this, where SNPs within the barcode region are known to differentiate clades, can be used with eDNA metabarcoding to examine population level dynamics.

We recovered more sparse data, in terms of both diversity and successful amplification, from mammals than plants. This result is expected and is likely a result of both sparse distribution of mammals on the landscape compared to plants, and a larger marker region size that was used to metabarcode mammals compared to plants (Sadoway, 2014). These ancient sediments hold ancient degraded DNA that is often less than 100 base pairs in length, and the 16S-mammal amplicon is ~140 base pairs (compared to the trnL amplicon ~85 base pairs). Limitations by both DNA

preservation and a gap in our sampling period 12-6 kya have restricted the conclusions possible from these mammalian data (Table S2.1).

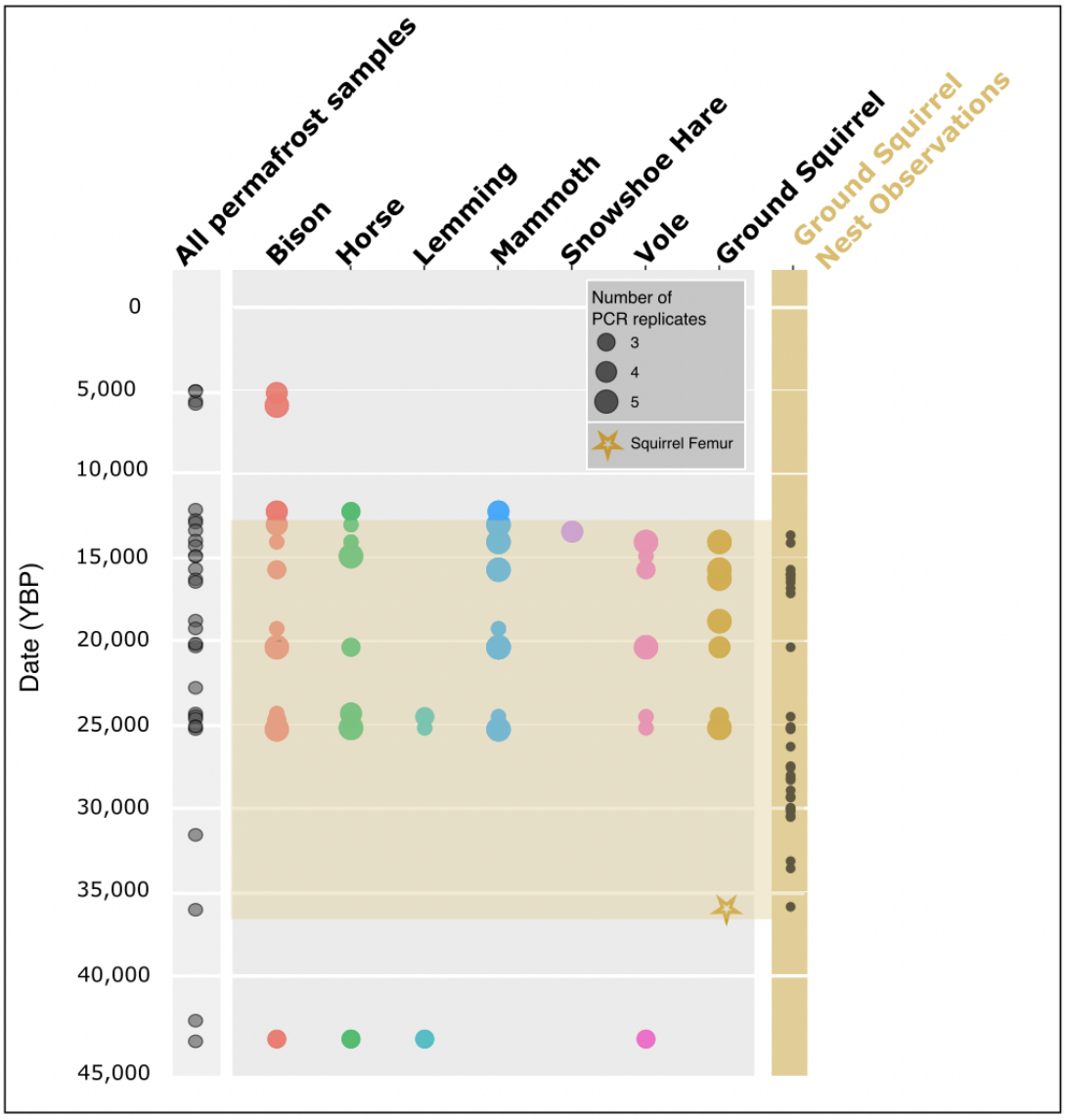


Figure 2.1: Presence of mammals observed in permafrost soil plugs detected with eDNA. The gold star denotes the sediment layer with a ground squirrel bone (soil plug MM12-134). Though ground squirrel was detected in all 5 PCR replicates for this soil sample, the sample was conservatively removed as the extraction negative

associated with it had some signs of contamination. The ground squirrel nest observations mark dated nests found in Yukon permafrost (yellow bar, Table S2.2).

Plants

We successfully amplified plant DNA from all 33 of the initial 33 soil plugs. 28 samples amplified with 5 PCR replicates, three with four PCR replicates, and two with one PCR replicate. Individual PCR replicates received 2,098-425,570 (average-135,670) reads assigned to taxa. We found no significant signs of contamination in the trnL data. We chose to rarefy our data to 15,000 reads to allow for comparisons of relative abundance of taxa between samples, and use a minimum read cutoff of 10 to negate inflation of biodiversity by low level contaminants and sequences resulting from PCR and sequencing error. Though we rarefied our data to compare relative abundance of different plant groups across samples, relative abundances represent the relative abundances of observed sequences. Both biological agents of DNA deposition and methodological aspects of DNA isolation and amplification can influence observed relative abundance, meaning relative abundance estimates from environmental DNA should not be assumed to represent the true relative abundance of species in nature at any given time. After rarefying our data, we included 31 of the initial 33 samples in analysis.

When grouped into the growth forms of forbs, graminoids, and woody plants (individual classifications found in Table S2.3), our rarefied dataset includes 44 forb, 9 graminoid, and 9 woody plant genera. We used the relative abundance of DNA sequences to categorize which growth form dominated the landscape at different time points. We observe woody plant dominated landscapes at 5-14 and 42-44 kya, and forb/graminoid dominance between 14-36 kya (Figure 2.2). The shift from a woody-dominated landscape to a forb/graminoid dominated landscape around 40kya and then back to a woody dominated landscape around 15kya confirms observations recorded from macrofossils and sedaDNA (ex- Mahony, 2015; Sadoway, 2014).

We observe a higher proportion of both forbs and graminoids 14-36kya, with a slight trend towards forbs in terms of both relative abundance (Figure 2.2) and diversity of genera (Figure 2.4). This time period aligns exactly with the time period predicted as a steppe with arctic ground squirrel nests (Figure 2.2; Zazula et al., 2005, 2006b, 2007). This prevalence of graminoids and forbs present in the steppe are of particular interest as they inform what food resources were available for several megafauna, some of which went extinct following the disappearance of the graminoids and forbs. Previous results using pollen and macrofossil have contradicting results, some claiming a forb-dominated landscape that supported the megafauna, and others graminoid (Guthrie 1990 and 2001; Kienast et al., 2005). SedaDNA studies by Willerslev et al. (2014) and Sadoway (2014), found a forb dominated landscape, with Sadoway et al. (2014) observing a higher prevalence of graminoids than Willerslev and co-authors (2014). Our data generally agrees with previous sedaDNA, pollen, and microfossil studies in trends of woody plants, forbs and graminoids, but paints a more detailed picture of the local community in teh Klondike. Five samples dated to 24-25kya, within this steppe time period, have high proportions of graminoids. Though this may signify some community turnover at the start of the LGM, samples filling in the gap 25-32kya for which we have no data would be required to resolve this. Regardless, we found that diverse groups of both forbs and graminoids throughout the LGM were available to the various large herbivores in the area competing for resources.

The higher proportion of forbs observed with sedaDNA by Sadoway (2014) and Willerslev et al. (2014) is likely due to the polymerase choice used in metabarcoding PCR. Sadoway (2014) and Willerslev et al. (2014) both used polymerase enzymes that are biased towards amplifying molecules with a GC content similar to that of forbs, meaning their PCR was biased towards amplifying forbs (Dabney and Meyer, 2012; Nichols et al., 2017). The polymerase used by Willerslev

et al. (2014), who estimated the highest proportion of forbs, was found by Nichols et al. (2017) to introduce the most GC bias of all polymerases tested in favor of amplifying forbs in their dataset. Sadoway (2014) used a polymerase found by Dabney and Meyer (2012) to be among the best in terms of minimizing GC bias among the tested polymerases, but still had bias towards the GC content present in forbs. Our enzyme choice was informed by Nichols et al. (2017) and chosen to minimize this known bias towards amplifying forbs. Though polymerase bias can be minimized (Dabney and Meyer, 2012; Nichols et al., 2017), approaches that use metabarcoding PCR to isolate sedaDNA may still be prone to over-amplification based on GC-richness. Other methods of DNA processing such as sequence capture (Murchie et al., 2020) may bypass this polymerase-bias and are recently becoming a focal point for sedaDNA, though the cost associated with sequence capture compared to metabarcoding is much higher.

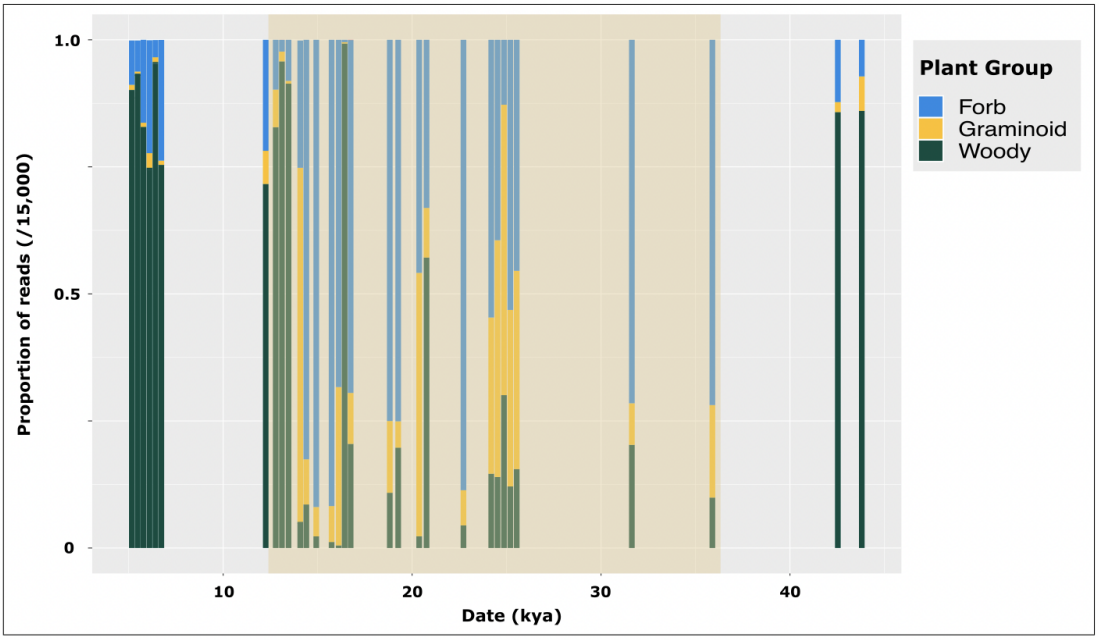


Figure 2.2: Relative abundance of forbs, graminoids, and woody plants in the trnL dataset rarefied to 15,000 reads, with a minimum read threshold of 10. The yellow zone denotes the presence of arctic ground squirrel nests which are a strong

indicator of a steppe.

We detected the presence of four tree species- Alder (*Alnus sp.*), Birch (*Betula sp.*), Spruce (*Picea sp.*), and Willow (*Salix sp.*) (Figure 2.3). Willow and Birch are both known as either tree or shrub form in Beringia. Throughout the entire time series, we found that Willow (Salix sp.) dominates the sequence signal and is found consistently between 43-5 kya (Figure 2.3). Willow dominance of woody plants in the sedaDNA record was also observed by Sadoway (2014) and could be a result of either true dominance of Willow over other woody plants, a higher rate of DNA deposition, or better amplification (as shown by Nichols et al., 2018 with some polymerases). Birch is detected in low frequency in our oldest sample, then disappears until 13 kya, and by 6 kya is as abundant as Willow. Alder first appeared at 13 kya and is consistently detected after 6 kya. Spruce is detected at low frequency starting at 26 kya and becoming more abundant 13, kya (Figure 2.3).

We detected spruce inconsistently 26-13, kya, and at greater abundance and at higher frequencies thereafter (Figure 2.3 and 2.4). Spruce dominates the current boreal forest in the Klondike, and is known to be present in the area before the LGM although it has remained unclear whether spruce were present through the LGM. In 1937, Hultén hypothesized ice-free areas of Beringia would have presented possible refugia for boreal trees throughout Pleistocene glaciations. It is also possible that spruce went locally extinct and modern populations are a result of later northern migrations of distributions (Ritchie, 1984; Ritchie and MacDonald, 1986). The possible refugia of spruce is a matter of ongoing debate, as some research supports this spruce refugia with low frequency pollen evidence (Colinvaux, 1964; Hopkins, 1972; Brubaker et al., 2005; Anderson and Brubaker, 1994) up to 24,500 14C yr BP (Zazula, 2006a), and some pollen evidence refutes it (Hopkins et al., 1981; Ritchie, 1984; Ritchie and MacDonald, 1986). The low frequency pollen data are attributed to

pollen migration, suggesting it was not actually locally present (Zazula, 2006a). Further, the absence of macrofossil evidence for spruce supports the claim that pollen data is a result of pollen migration (Ritchie, 1984; Anderson and Brubaker, 1994). However, genetic studies amplifying individual genes from the chloroplast of modern spruce trees in the Klondike have identified locally unique haplotypes, which provides evidence that spruce were likely present in the Klondike through the LGM in refugia, and modern populations are at least in part a continuation of Pleistocene populations (Anderson et al., 2006; Lafontaine et al., 2010). Spruce are known to reproduce asexually, via "layering" where lower branches touching the ground take root, especially in the northern limits of their range and under suboptimal conditions (Payette and Gagnon 1979; Lloyd et al., 2005). Therefore, inconsistent recovery of spruce in pollen studies yet the presence in our sedaDNA data here may indicate that spruce was present in low abundance refugia, reproducing asexually.

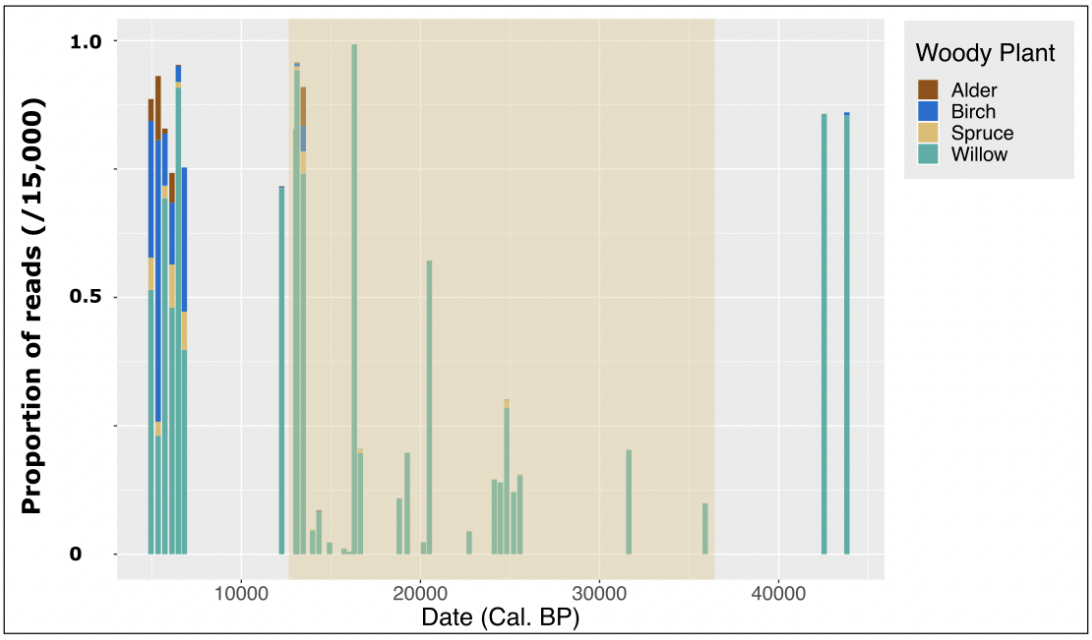


Figure 2.3: Breakdown of the woody plant tree composition demonstrated in Fig 2.2. Relative abundance of woody plants in trnL data rarefied to 15,000 reads with a 10 read minimum cutoff. The yellow bar denotes the presence of arctic ground squirrel nests which are a strong indicator of a steppe.

We found that willow, *Poaceae, Carex, Lupinus, Oxytropis, Papaver, Astragalus, Asteraceae, Rosaceae* are consistently present through the entirety of time series (figure 2.4, a full list of plants detected is available in Table S2.3). We observed a number of forbs restricted to the Pleistocene/early Holocene (Figure 2.4). Birch and spruce both occur during the LGM, and become more abundant across samples during the Bølling–Allerød warming period (Figure 2.4), which started in the Klondike 14,400 cal years BP (Irvine et al. (2012). We found *Ribes* sp, *Chamerion angustifolium, Alnus sp.* (Alder)*, Shepherdia canadensis,* and *Vaccinium vitis-idaea* all beginning to appear during this warming period (Figure 2.4). These genera would all thrive with warmer temperatures and increased water availability. Alder specifically is known to thrive in locations of disturbance or recent change.

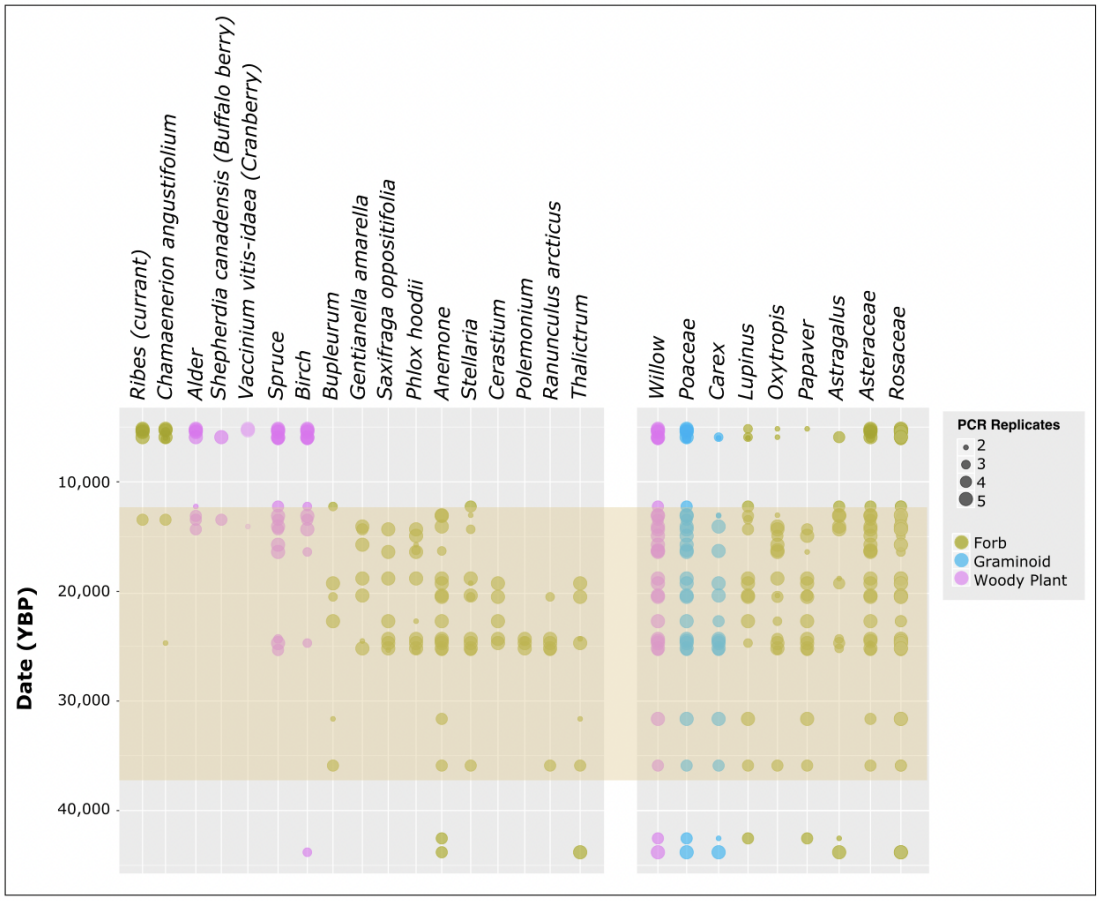


Figure 2.4: A subset of plants detected with the trnL data. The left panel shows plants restricted to given time zones, and the right - plants present consistently through time. The yellow bar denotes the presence of arctic ground squirrel nests which are a strong indicator of a steppe. This graph shows a subset of all plant species- including plants detected across most samples and plants seemingly associated with climatic changes.

Conclusion

Here, we gathered sedaDNA data for plants and mammals from 33 permafrost plugs collected from the Klondike spanning the last 50,000 years, and overlaid the data with the predicted presence of a steppe habitat as informed by the local presence of arctic ground squirrel nests. By focusing on the Klondike, and using an indicator

(sedaDNA) of local community composition, we examined turnover of plant and mammal communities and confirmed the timing of the steppe presence in the area. Though this area is the focus of many paleoecological studies, discrepancies between studies leave unanswered questions. We demonstrated expected general patterns and timing of plant and mammal community composition over the last 50,000 years as demonstrated with many other studies and proxies. We found the presence of Spruce refugia through the LGM, which has been a subject of ongoing debate (ex. Hultén, 1937; Ritchie, 1984; Ritchie and MacDonald, 1986; Anderson and Brubaker, 1994; Brubaker et al., 2005; Zazula, 2006a). Our consistent amplification of ground squirrels highlights sedaDNA as a reliable indicator of general habitat trends. Additionally, our observed consistent detection of voles highlights future potential routes in using small mammals for determining general landscape composition. Future sequencing of the 16S region, an amplicon small enough to be used with sedaDNA, for vole species present in the Klondike could open doors into examining species-specific composition of sites that may inform local habitat. This project was informed by previous eDNA studies that have directly addressed biases introduced with eDNA processing, allowing for more accurate representations of the DNA present in permafrost to be examined (Nichols et al., 2017; Ruppert et al., 2019; Mathieu et al., 2020). Our study was limited by sampling gaps that made it impossible to observe the timing of certain community turnovers, and future endeavors that target those regions would add to the data generated here and could further illuminate said turnovers. Ongoing research, including this study, that

addresses the environmental history of the Klondike region is continuously adding to our understanding of habitat turnover in response to climate change and the causes of extinction of the megafauna of North America.

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Chapter 3: Ancient DNA-based sex determination of bison hide moccasins provides evidence for selective hunting strategies by Promontory Cave occupants

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Abstract:

The thirteenth-century human occupants of Promontory Cave, Utah, distinguished themselves from surrounding Fremont populations by being highly successful large game hunting specialists, particularly of bison, in a region that has normally been peripheral for that species. Their success is evident from the abundance of faunal remains excavated from the caves, which has facilitated zooarchaeological study of bison hunting strategies. In addition to faunal remains, the dry cave conditions have preserved hundreds of moccasins worn by these bison hunting people. These moccasins are of particular interest because of the Canadian-Subarctic style in which they are made and for their potential to reveal more about hunting strategies of their wearers. Here, we use ancient DNA isolation techniques to determine the species and sex of animals used to construct 38 Promontory Cave moccasin and hide fragments, and to interpret this in the context of local hunting strategies. Of the 23 hide fragments from which we could recover DNA, all were bison and most (87%) were females. The strong female-bias in hide used to construct moccasins supports an overwintering model of large game hunting and the

targeting of cow-calf herds, further explaining the sophistication of large game hunting by Promontory Cave occupants.

Introduction

Analyses of communal bison kills have been a mainstay of Plains archaeological research, especially for interpreting Indigenous hunting strategies from archaeological bone assemblages (eg. Wilson, 1978; Johnson and Bement, 2009; Carlson and Bement, 2013). Age structures of archaeological bison assemblages have been characterized from tooth eruption sequences, occlusal wear patterns, cementum growth increments, foetal remains, and epiphyseal fusion (see Gifford-Gonzalez 2018 for a summary). Inferences regarding the sex structure of hunted bison have been drawn from osteometric indices that differentiate the larger male from the smaller female bison (eg. Hill et al., 2008; Driver and Maxwell, 2013). Knowing the age distribution and sex structure of animal remains in archaeological contexts can reveal nuances in the behaviors, social structures and natural histories of prey species as well as the cultural practices accompanying Indigenous hunting (Payne, 1973; Weinstock, 2000; Pečnerová et al., 2017; Gifford-Gonzalez, 2018; Royle et al., 2018).

Among the most common large mammals in archaeological bone assemblages of the Plains are bison. Analyses of sex structure in archaeological bison assemblages can be used, for example, to evaluate the extent to which Indigenous bison hunters were using selective hunting strategies, such as seasonal preference of sex for their

nutritional value (Speth, 1983). While it is well known that every part of the bison was subject to Indigenous use (e.g., Ewers 1958:14-15), osseous remains are more likely preserved than are more perishable tissues, such as hide. However, while bison are strongly sexually dimorphic, smaller males and larger females add a degree of imprecision to osteometric sex determination (e.g., Bedord 1974; Walde 2004). Sex determination can be influenced by the condition of the remains preserved (e.g. Speller and Yang, 2016), the element (bone) that is preserved (Buonasera et al., 2020), the age of the animal at death (Gifford-Gonzalez, 2018), geographic and temporal variation in animal morphology (Gifford-Gonzalez, 2018), and human intervention in animal development (e.g. Telldahl et al., 2012). Estimates of sex ratios from perishable remains therefore have potential to improve understanding of Indigenous hunting strategies.

Perishable parts of hunted animals, such as hide, had non-food uses that may have influenced hunting strategies. For example, Indigenous peoples met critical shelter and clothing needs by hunting bison: rawhide and leather from bison hides were essential for lodge covers, tipi liners, robes, tailored clothing, footwear, cordage and items like travois baskets (Brink 2008: 47, 67, 224-225). Sexual dimorphism, animal age, and seasonality all influence skin thickness, which will affect hide pliability, which is an aspect of bison morphology that humans considered (Brink 2008:142; LeBlanc 1999; Speth and Staro 2012, 2013).

Here, we capitalize on the exceptional preservation of hundreds of moccasins preserved in the dry cave assemblages of Promontory Caves, Utah, to explore the sex

assemblage of bison used for this purpose. We extracted ancient DNA (aDNA) from 38 hide fragments recovered from the caves and that range in age from 703±23 to 886±27 radiocarbon years before present Ives et al. 2014) and therefore come from nearly the entire early Promontory Phase time range AD 1248-1290. From these, we determined both species and the sex of the animals used to make these moccasins. Determining sex ratios from perishable leather artifacts provides an opportunity to evaluate a more complete suite of Indigenous decision-making factors extending beyond dietary matters to other important needs that would include securing raw materials for hide processing. The sex ratio of Promontory bison leather samples is therefore of considerable interest when determining if, and how, the Promontory community was selective in its hunting practices.

Background

The Promontory Cave Context

The early Promontory Phase on Promontory Point marks a significant discontinuity in the late period archaeological record of the northeastern Great Basin (Ives 2014, 2020). Taking place in the twilight of the Fremont phenomenon—the last traces of which would vanish by the dawn of the fourteenth century— the rich material culture preserved in the dry Promontory Caves of Utah differs from local Fremont expressions in several key ways (Figure 3.1). At the time the Promontory Caves were occupied, local Freemont populations relied exclusively on wild food

resources primarily from wetlands, some large game hunting, intensive small game capture, and wild seed processing. The substantial archaeological deposits of Promontory Cave 1, which was suddenly and briefly occupied during AD 1248-1290 (Bayesian modelled), have scant evidence of small game animals and wild seed processing (Ives et al. 2014). Recent small-scale excavations of Cave 1 have unearthed more than 30,000 whole and fragmentary faunal remains that are the subject of ongoing zooarchaeological analysis (Ives and Janetski, in press). Any Minimum Numbers of Individuals (MNI) projection is certain to estimate large values for bison, antelope, elk, deer and sheep. Many dozens of hides would be required for the more than 340 worn out moccasins recovered to date from Cave 1 (which has significant remaining deposits — Supplement 3.1). The presence of heavy bison remains with low food utility, such as skulls and lower limbs with hooves indicate a nearby kill locale. The saddle terrain above Promontory Caves 1 and 2 constrained game animal passage and was well suited for small-scale communal hunting. Many artifacts indicative of hunting have been recovered from this narrow pass including projectile points, pièces esquillées, drills and other artifacts. Fleshers, beamers, lithic scraping implements, and awls of different sizes are common in the Cave 1 assemblage, attesting to significant hide processing activities. Promontory hunters were successful in what was peripheral bison habitat for most of the Holocene (Grayson 2006; Lupo and Schmitt 1997).

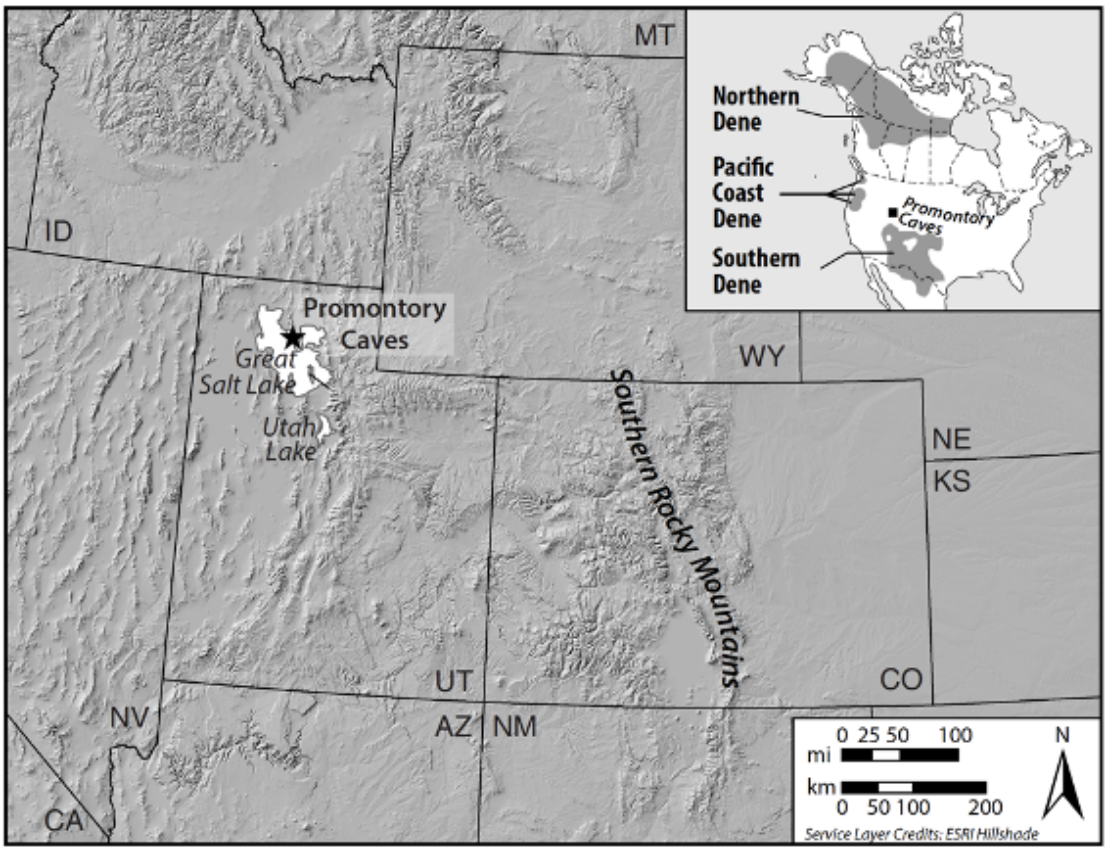

Figure 3.1: The location of the Promontory caves in Utah and the geographic distribution of Athapaskan or Dene languages in North America.

These findings remain the subject of specific regional interest, yet the Promontory record also has a bearing on a more transcendent matter in western North American pre-contact history. Julian Steward (1937) proposed that this unusual assemblage resulted from the presence of Apache or Navajo ancestors, midway between a Subarctic point of origin and ultimate Southwestern and southern Plains homelands. The large assemblage of Promontory footwear was particularly important

to Steward's reasoning: he knew that the 245 soft-soled, fine leather moccasins he recovered were made in a fashion characteristic of Dene (or Athapaskan) and Algonquian speakers in Subarctic Canada. The moccasins were completely unlike contemporary Fremont moccasins (in the form of Fremont, Hogup and hock style moccasins), coarsely executed on poorly tanned leather, or later Numic forms (Aikens 1970; Hatt 1916; Steward 1937).

Our more recent research, involving renewed excavations in Promontory Caves 1 and 2, has reinforced Steward's suspicions. Steward was correct in his attribution of the moccasin pattern to the Subarctic region. In fact, archeologists recovered a 1400-year-old antecedent moccasin form from a southern Yukon ice patch within the northern Dene homeland (Ives, et al. 2014; Hare et al. 2012). The presence of unique stone hide softening implements (*chi-thos* or tabular bifaces still in use today in the Subarctic, yet unknown in Fremont assemblages), instances of intricate plant sinnet weaving (in a style used in the Subarctic for moccasin garters, mitten strings and birch bark vessel handles otherwise unknown among Great Basin fiber perishables), and a probable dog travois basket, extra-local obsidian, and uniquely Fremont rock art (including some closely resembling Promontory Cave 1 images) far to the north in southwestern Alberta all suggest that the Promontory caves were inhabited by a small co-residential group that had Subarctic and northern Plains heritage.

Our research was inspired by one facet of the Promontory project. A significantly anomalous $\delta^{13}C$ value noted during AMS radiocarbon dating for the

ankle wrap of one Promontory Cave 1 moccasin triggered more detailed exploration of a bison isotopic landscape (Metcalfe et al. 2021). An ankle wrap is the one portion of a moccasin most likely to remain serviceable; in the Promontory collections, they were often cut away for re-use once the body of a moccasin had been worn and patched beyond further repair. Isotopic evidence indicated that the bison leather for one Promontory Cave 1 ankle wrap came from an animal that was likely killed several hundred kilometers away, possibly in northern Arizona, eastern Colorado, or somewhere in an arc of regions in between (Metcalfe et al. 2021). As part of this study, we used ancient DNA to determine the sex of the bison whose hide comprised the ankle wrap leather. Ancient DNA analysis allowed us to rule out the possibility that a stray animal wandered into the Great Salt Lake region from afar: a female bison (part of a cow-calf herd) would be much less likely to make a long, rapid voyage than a lone male bison. This test sample provided the impetus for the present sex-determination study to examine sex-biased hunting strategies.

Bison Herd Composition and Hunting Strategies

Reynolds et al. (2003:1028) noted that a slight excess in favour of males in the primary sex ratio is common among mammals, a trend borne out in the bison literature they surveyed. Several studies have identified a higher proportion of males at birth than females (53-62% male: Fuller, 1962; Haugen, 1974; Rutberg, 1986). Wolff (1988) found that cows may put more nursing energy into male calves, but in a

later study (1998) found equal proportions of male and female offspring at birth in a 260-360 animal bison herd in Nebraska. Rutberg (1986) found that cows who had no offspring in a previous year produced more male calves. Green and Rothstein (1991) reported that females may favor caring for males while final calves of older females were nearly always female.On balance, parity in the bison sex ratio at birth is a reasonable working assumption, although there is a tendency for male skewing in a larger number of cases.

Bison social groups do not have equal sex ratios. All bison spend much of their early life in matriarchal herds, with cows and calves forming the largest proportion of the population. As males mature, they leave the cow-calf herd around age four (Olson, 2005), forming small bachelor groups or living as isolated bulls. Bulls rejoin cow-calf herds temporarily during the mid to late summer rut (Brink, 2008). Bulls are known to join cow-calf herds at varying counts throughout the year, outside of the rut season (Olson, 2005). Cow-calf herds vary in proportions of cows, yearlings, and calves (Van Vuren and Bray, 1986; Larter et al., 2000; DelGiudice et al. 2001; Bradley and Wilmshurst 2005; Fuller et al. 2007).

From data gathered on the Henry Mountains bison herds (Van Vuren and Bray, 1986), we estimate cow-calf herds to consist of a maximum 76.5% female bison (calculations described in Supplement 2). From data collected during the post rut season (September to December) of 2002-2004 in Elk Island National Park, we estimated the maximum female proportion at 78% (calculations described in Supplement 3.2, data presented in Supplements 3-4), very similar to the estimated

amount in the Henry Mountains data. These estimations for both populations are made under the assumption that no bulls are in a cow-calf herd post rut which we know is not the case (Olson, 2005) and calves are in equal sex ratio which we know often skews towards male generally, and specifically within the Elk Island herd (Supplement 3.4). Calculations made from the Henry Mountains herd do not take into account the licensed hunting for male bison only. Additionally, the Elk Island calculations include male bison only up to 2 years old, and male bison often stay in the cow-calf herd until age four (Olson, 2005). With the assumptions taken and limitations of observing modern populations, our estimates of 76-78% female bison in a cow-calf herd is likely an overestimation.

The physiological status of bison influenced their desirability as a prey species for human hunters (Speth, 2010). By late winter and early spring, both males and females are in poor condition. During this time, females carry late term calves which they must nurse once born. Males have been in poor condition since the tumultuous activities of the rut. Both male and female physiological conditions decline with the poorer winter foraging conditions. Non-pregnant, non-lactating cows are uncommon but prime hunting targets in the difficult late winter to early spring time frame. Once green-up occurs, bison quickly recover. Cows are in optimal condition by late summer or fall when calves from the previous spring are weaned and gestation after the rut begins (Brink, 2008). In 1830, William Ferris reported the condition of bison in the Bear River valley east of Promontory Point. He commented on the poor status and nutritional value of bison prior to the spring phenological burst and noted how

rapidly female bison became the most desirable hunting target by mid-summer (Phillips, 1940:42; see Speth, 2010:52-56 for this and several other examples, as well as Brink 2008:57-60).

The "overwintering model" suggests that hunters took cow-calf herds in fall so that they could store provisions for winter (Frison, 1978). During late summer-early fall, cow-calf herds were an attractive target for hunting communities because of both the favourable condition of the cows and behavioural characteristics (a tendency of cow-calf herds to bunch when manoeuvred) that could be exploited (Brink 2008; Speth 2013:177). While cow-calf herds were likely a preferred target in many cases, Speth (1983, 2010, 2013; Speth and Rautman 2004) has shown spring hunting instances where hunters focused on male bison when their condition was superior to late term and calving females.

While the majority of bison-related archaeological literature devotes attention to bison as a significant food source, the literature also acknowledges their role as a source for other raw materials, hide in particular. In Plains contexts, Indigenous peoples hunted antelope and deer specifically for their hides at times to use for finer garments, such as dresses and tunics, where thin soft leather was desirable (e.g. Grinnell 1972: vol. 1, 189-224). In contrast, bison have unusually heavy hides, in some places on the animal's body exceeding a centimeter in thickness (Brink 2008:171-175). Thick hides were useful for objects such as shields where the thick neck hide of bulls was sought after (Brink 2008:142; LeBlanc 1999; Speth and Staro 2012, 2013). Heavy hides nevertheless make sewing difficult. While the skill levels

apparent in the Promontory moccasin assemblages vary, the bison leather that Indigenous people used is uniformly of high quality. A number of moccasins feature exquisite sewing by any standard, prehistoric or modern. The quality and craftsmanship indicate that cave artisans had two factors in mind: leather that was thick and durable enough for practical wear but thin and supple enough to allow the characteristic intricate seaming and stitching of the moccasins (Figure 3.2). Approaches to obtain thinner hides included hunting bison in the spring when their hides are naturally thinnest, the traditional time for securing raw materials for lodge coverings (where thinner hides were again desirable) (e.g., Brink 2008:69, 224-225; Southesk 1969:307, writing in 1859-60).

Figure 3.2. A complete Promontory moccasin (UMNH.A.8011.18, FS 42Bo1.801.1) from Cave 1, with its sole folding upwards to meet its vamp and inset, creating a puckered, round toe. In this case, the ankle wrap remains attached to the moccasin,

along with laces for tying it. Photographed courtesy of the Natural History Museum of Utah.

Since these and undoubtedly many other nuances were known to Plains Indigenous peoples, the bison sex ratio of any given site assemblage could fall along a spectrum of values, with various factors causing skew. For example, if Indigenous hunters specifically targeted bachelor herds or lone males, males would predominate site assemblages. In contrast, if hunters preferred cow-calf herds as prey, then females may dominate the assemblage. Furthermore, if Indigenous peoples prefered particular bison ages/sexes to construct moccasins, the observed sex ratio may not be equivalent to the sex ratio of animals hunted.

Materials and Methods:

Archaeological Sample Information:

The hide samples in our study were excavated from Promontory Cave 1, UT between 2011 and 2014 (Figure 3.1). The samples are part of an early Promontory Phase assemblage with an estimated human occupation spanning from AD 1248-1290 (Ives et al. 2014; Ives et al. in press). The dry cave conditions preserved hundreds of worn and discarded moccasins as well as mittens, bison robe fragments, gaming pieces, matting, basketry, cordage, bows, arrows, stone tools, ceramics and a range of hide processing tools (Ives et al. 2014; Yanicki and Ives 2017).

We processed two hide fragments, two bison robe fragments and 34 moccasin samples at the University of California, Santa Cruz Paleogenomics Lab (Table S3.1). Miscellaneous hide fragments recovered from these Promontory sites could have been part of, or intended for, moccasins, thong sandals, drum tops, bags, clothing, mittens, or other items (Steward, 1937). Two samples (FS 305) are from the same moccasin, but were sampled from the moccasin body and ankle wrap and originated from a different, non-local bison (Metcalfe et al., 2021).

DNA extraction, library preparation, and sequencing:

We extracted DNA from hides and prepared the extracted DNA into genomic libraries for sequencing in the UCSC Paleogenomics Lab (PGL), following protocols developed specifically for working with degraded DNA. The PGL space is physically isolated from PCR products and strict protocols are used to avoid contamination, including wearing sterile gloves, full body Tyvek suits, hairnets, face masks, and face shields. We bleached all surfaces in the lab before, throughout, and after sample processing. Prior to extraction, we washed all samples with ultrapure water to remove surface debris. We extracted DNA following Dabney et al. (2013), and included one DNA extraction negative (no sample) in each group of extractions of up to eleven samples. Following extraction, we purified darkly colored extracts with

polyvinylpolypyrrolidone in columns to remove PCR inhibitors following Arbeli and Fuentes (2007).

We generated shotgun Illumina sequencing libraries from these extracts following either the Meyer and Kircher (2010) or Santa Cruz Reaction (SCR) protocol (Kapp et al., 2021) (Table S3.1), the latter of which is a single-stranded library preparation approach that more efficiently converts extracted DNA into sequenceable molecules. For consistency, we prepared SCR libraries from any sample with remaining tissue that was initially prepared as a Meyer-Kircher library. For those samples for which the first preparation exhausted the DNA extract (n=3), we kept the Meyer-Kircher library. For the Meyer-Kircher libraries, we labeled each molecule on both ends via a dual indexing PCR (iPCR) using KAPA Hifi (Roche, Pleasanton, CA, USA), unique indexes, and TruSeq Illumina sequencing primers, which we amplified in the modern DNA lab for 25 cycles. Following SCR library preparation, we performed quantitative PCR for each library to determine the optimal cycle number for dual indexing PCR, as described in Kapp et al. (2021). We cleaned all indexed libraries with a 1.5x concentration of SPRI beads (Beckman, Indianapolis, IN, USA). We quantified library concentration with a Qubit fluorometer (Thermo Fisher, Waltham, MA, USA) and pooled libraries at equimolar ratios. We sequenced all libraries across several Illumina Miseq 2x75bp and NextSeq 2x150bp runs.

Data Analysis:

We trimmed reads of adapters, removed low quality reads, and merged reads using *SeqPrep2* (https://github.com/jeizenga/SeqPrep2). We then used *prinseq* (v.0.20.4, Schmieder and Edwards, 2011) for complexity filtering and removal of duplicate reads. Based on site locality, a previous analysis of similar moccasins (Steward, 1937), and physical evaluation of the leather, all samples were believed to originate from bison. Moccasins at the Promontory Caves are constructed with the hide of multiple animals, most commonly bison, but also including deer, antelope, and (rarely) elk or bear (Steward, 1937). We aligned our data to the BLAST nucleotide database and visualized the results with MEGAN (v. 6.18.0) which identified all samples as bison. To further confirm this species assignment, we mapped all reads using BWA (aln-v. 0.7.12-r1039) to cattle (*Bos taurus* ARS-UCD1.2_Btau5.0.1Y - bosTau9+Y), bighorn sheep (*Ovis canadensis; NCBI CP011912.1*), and roe deer (*Capreolus capreolus* CCMK01) nuclear genomes. We used cow rather than bison because the recently published *Bison* genome (Oppenheimer et al., 2021) was made from an F1 cattle/bison hybrid and lacks an X chromosome. These alignments provided additional confirmation that all samples were bison.

We then determined sex for all samples with at least 150 reads aligned to the cattle X chromosome following Flamingh et al. (2020), which used a script adapted from Mittnik et al. (2016), for chromosome-level alignments to determine the sex of an individual. Sex was predicted based on the number of reads aligned to autosomes

compared to the X chromosome, normalizing the number of reads aligned to each chromosome by the assembled chromosome lengths. The expected alignment ratio (Rx) is different for males and females as males have only one X chromosome.

To develop an understanding of the natural range of observed Rx of bison data aligned to a cattle genome, we analyzed previously-generated shotgun sequencing data from four modern female bison and four male bison (Table S3.2). We used the data generated in Wu et al (2018) for one female American bison (SRR6448737), and data from Yang et al. (2020) for the remaining three (SRR12514558, SRR12514559, and SRR12514560). For the four male bison, we used one individual from Oppenheimer et al. (2021) (SRS7735511), one from Heaton et al. (2016) (SRS1620843), and two from unpublished data presented here for the first time (NAGP 14568/LIB100490 and NAGP 5852/LIB100491; see Supplement 5 for details of data generation). For all eight samples, we trimmed adapters with Seqprep2 and performed complexity filtering with prinseq, as we did for the archaeological samples. To simulate ancient DNA, we trimmed all reads to 58 base pairs, which was the average fragment size from the alignments of our archaeological samples to cattle. We aligned the trimmed reads to the bosTau9+Y genome as above. The minimum number of reads aligned to the cattle genome of any archaeological sample was 3747 reads, so we subsampled 3747 aligned reads from each sample ten times, with replacement, to observe variation in alignment proportions by random sampling of reads. We then used the Flamingh et al (2020) script to calculate the Rx for these five bison of known sex.

Results:

Sequencing Results:

We generated 153,966-8,181,119 read pairs per sample (average 1,879,334 reads; sample-specific counts in Table S3.1). Alignment to cattle, deer, and sheep supported the conclusion observed with BLAST that all samples are bison (read alignment counts per genome in Table S3.1). The 38 sequenced libraries had 135-570,183 reads aligned to the cattle genome (average 65,846 reads; sample-specific counts in Table S3.1).

Reference bison mapped to cattle:

To determine the expected range of Rx values for male and female bison aligned to cattle, we modelled ten bootstrapped observed Rx values for each of our eight reference bison (4 males and 4 females; Figure 3.3). Our observed alignment ratios for females ranged from 0.77 to 1.20, and for males 0.38 to 0.61.

Sex determination of archaeological hides:

Of the initial 38 samples, 1 hide, 2 robe fragments, and 20 moccasin samples yielded data with at least 150 reads aligned to the cattle X chromosome and were used in further sex determination analysis (Table S3.1). The alignment ratios of our samples fall into two distinct Rx clusters, one from 0.76 to 0.97, and the other 0.46-0.61 (Table S1) (Figure 3.3). The separation between the two data clusters is consistent with two distinct categories of sample: male and female. We observe 3

samples in the male cluster and 20 samples in the female cluster (87% female) (Figure 3.3).

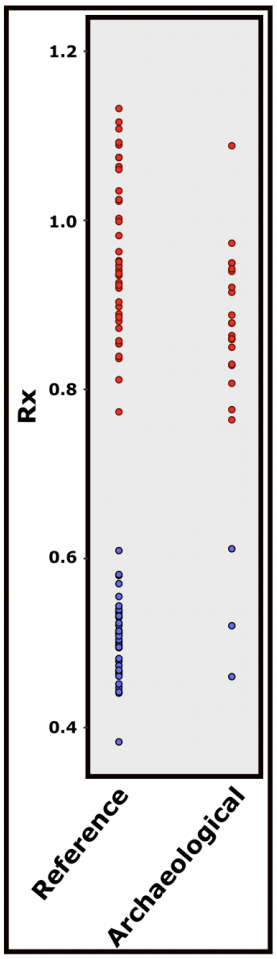

Figure 3.3- Rx values for reference bison and archaeological bison tissue samples. Blue dots are within the male range, and red dots within the female range. Reference bison include four female and four male bison subsampled ten independent times to 3747 reads aligned to the cattle genome.

Discussion and Conclusion

Our results indicate that Promontory people selectively used hides from female bison to produce the leather used in moccasins and robes. Of our samples, 87% were identified as female. As the samples used in this study range from 703±23 to 886±27 radiocarbon years before present and therefore come from nearly the entire early Promontory Phase, they did not come from a single hunting event. The bison sex determinations we report thus reflect decisions made about hunting and hide processing over one or two human generations of Cave 1 occupation.

Just as people have targeted the thicker skin from a bull's hump when constructing shields (LeBlanc, 1999), the soft skin of deer (e.g. Grinnell 1972: vol. 1, 189-224) and juvenile caribou (Binford, 1978) for clothing, and the spring-time thinner hides of bison for lodge coverings (e.g., Brink 2008:69, 224-225; Southesk 1969:307, writing in 1859-60), we have observed that female bison hides were targeted for moccasins. Several factors may explain the observed female dominance of hides used in moccasins in terms of sex-specific behavior, anatomy, and seasonal biology.

As Frison (1978) proposed with the "overwintering model", bison cows were targeted in the fall and early winter to prepare meat, fat, and hides for winter. Cow hides are in the best condition during late fall and early winter, when female bison are

in better condition as a food source (Brink, 2008). In fact, at this time meat from bulls could have been so low in levels of fat that protein poisoning would be a serious risk (Phillips, 1940; Speth, 2020). Seasonality indices for Promontory Cave 1 are still being assessed, but there is evidence for occupations taking place in all seasons. It could be that seasonal hunting activities conducted from the caves would meet the provisions of the overwintering model, explaining a fall and early winter presence. Whether or not that is the case, the female skew in our results indicates that cow-calf herds were the preferred hunting target.

The location of Promontory Caves suggests that ambush hunting was the most likely strategy that the people used to hunt bison, and this, too, would have been simpler to achieve with cow-calf herds. Above and to the east of the Promontory Caves is a "saddle" in the landscape with an extensively used game and cattle trail that would have been well suited for ambushing bison herds. Within this saddle, projectile points along with a stone feature resembling a hunting blind have been found (Supplemental Material 1). The proximity of this site to the Promontory Caves, along with the artefacts and feature associated with bison hunting found, provide evidence for the Promontory peoples use of the saddle landscape for hunting and likely specifically ambush hunting.

Third, the quality of female hides may have made them preferable to males. The skin of bulls would likely be too thick to achieve the fine sewing observed in moccasins made by promontory people. Likewise, the thinner skin of juveniles may have made them less favorable as they would not last as long. Further, cow skin

would have been thinner in the springtime, indicating a possible seasonal practice of gathering hide, providing further evidence for Frison's model.

It is also possible that the selection for female hides by Promontory people occured after the hunt, with female hides being used for moccasins and male hides for other purposes. Resolving this timing of selection could be further addressed by examining the ratio of other bison remains recovered from the caves (ex- bones, other loose hide fragments).

The inability to directly determine seasonality of hunt based on data from moccasin hides, as is possible with some skeletal remains, and direct selection for hides or selection after hunt make it difficult to concretely determine hunting the hunting strategy used by Promontory people. Considering the above points, it is likely the Promontory people used ambush hunting of cow-calf herds in the saddle region nearby Promontory caves during the late fall and early winter, and prioritized the cow hides for moccasins.

Demonstrating here the ability to determine species and sex from archaeological material remains should open new doors to questions regarding hunting strategies, as shown here, as well as use of unidentifiable items among other routes.

The Promontory cave record is unique in the northeastern Great Basin and represents a significant discontinuity from preceding and contemporary late Fremont assemblages. This pilot study confirms another unique feature of this record: the Promontory cave occupants exercised a high degree of selectivity in raw material

used for a sophisticated hide processing tradition, suggesting yet another way that the early Promontory Phase cave occupants were different from their neighbours. They possessed specialized knowledge of bison hunting strategies and desired products highly suggestive of a Plains background, just as Steward (1937) initially proposed.

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Synthesis

Methodological and technological advancements continue to improve the amount of DNA we can isolate, sequence and study from environmental samples and organismal tissue. As we improve our methods and increase our understanding of biases that may be introduced in the field, laboratory, or bioinformatic settings, we increase the accuracy with which we can reconstruct genomes and ecological communities. With increased access to degraded DNA, the field continues to grow in its application and use in studies across a wide array of fields.

With this thesis, I added to the general understanding of how methodological choices made while designing experiments and processing degraded DNA affect the data that we observe (chapter 1). I then applied these techniques to study how plant and animal communities changed over time and human biocultural interactions, targeting degraded DNA protected by the cold and dry conditions of permafrost and caves. First, I used ancient environmental DNA from 33 permafrost samples collected from the Yukon, Canada, representing the last 50,000 years to characterize shifts in floral and mammalian communities (chapter 2). Then, I used ancient DNA isolated from moccasins constructed by the Promontory Cave (UT) occupants to examine hunting strategies used when gathering resources for non-food material goods.

My results have broad application to the ancient DNA and environmental DNA research communities. In chapter 1, I found stochasticity between PCR replicates and the depth of sequencing both significantly influence alpha but not beta diversity. Rare taxa are stochastically detected with PCR and may require saturation

in sequencing depth of each replicate. Despite stochasticity in rare taxa, the occurrence and relative abundance of more common taxa are consistently tracked with each PCR replicate with low occurrence of outliers. This stability in amplification of more common taxa makes eDNA metabarcoding a robust method in consistently estimating beta diversity, even with low sequencing depth.

In chapter 2, I characterized the turnover of plant and mammalian communities over the last 50,000 years in the Klondike territory of Canada. I observed a coupled shift of local ground squirrel extinction and a turnover in plant communities from a mammoth steppe habitat to boreal forest 13,000 years ago. I confirmed expected patterns in community composition and turnover observed with other bioindicators such as arctic ground squirrel nests and pollen analyses. I confirmed the presence of Spruce refugia within the Klondike through the last glacial maximum, a hypothesis of ongoing debate. Consistent amplification of ground squirrel nests from eDNA aligning with fossil observations of nests, an indicator of the mammoth steppe habitat, illustrates the robustness of eDNA in characterizing general habitats.

In chapter 3, I found 87% of moccasins excavated from the Promontory Caves site in Utah were constructed with female bison hide. This finding supports prior hypotheses describing the bison hunting specialists' practices as targeting cow-calf herds and performing the majority of hunting at the beginning of winter.

Degraded DNA preserved in space and time is an informative indicator of paleoecosystems and can be used to answer questions spanning human bioculture and

climate change. Future technological advances and continued studies that address methods used in DNA processing will not only increase the amount of DNA we can isolate and study, but also increase the accuracy of the conclusions we make from the data observed.

Supplemental Materials

Text S1.1

Anacapa is a metabarcoding data processing pipeline that enables simultaneous data processing of multiplexed barcodes and libraries. Within *Anacapa*, we trimmed the TruSeq adapters using *cutadapt* (Martin, 2011), removed bases with Q-scores below 35 with the *FastX-Toolkit* (Gordon and Hannon, 2010), and then trimmed, again using cutadapt. We trimmed the first 5 bases on the 5' end of the forward read and the first 10 bases of the 5' end of the reverse read for the PITS data set. We trimmed 40 bases off the 5' end of the forward read and the first 50 bases of the 5' end of the reverse read for the FITS data set. We used *dada2* (Callahan et al. 2016) to merge the forward and reverse reads, remove chimeric sequences, and identify amplicon sequence variants (ASVs). We performed merged, unmerged paired, and single read ASV assignment to taxa via global and local alignment using *Bowtie2* (Langmead and Salzberg, 2012) to reference CRUX databases released in Curd et al. 2019. The top 100 hits of the *Bowtie2* alignment to reference were bootstrapped with *BLCA* (Gao et al, 2017) to assign each ASV to a taxon and provide uncertainty estimates. We used a 60% bootstrap confidence threshold of taxonomic assignment, as suggested in the *Anacapa* documentation (Curd et al, 2019). *Anacapa* outputs two taxonomy tables (one per amplicon) formatted as matrices of the number of reads from each PCR replicate assigned to a given taxa.

As a recently published database generation tool, CRUX databases are created using a multi-step process that begins with an EcoPCR using primer sequences and a

target amplicon length to pull out e-amplicons from the EMBL nucleotide database (Stoesser et al., 2002). This creates a set of seed marker sequences that are used to query the NCBI nr/nt database twice, first accepting only full length reads, then accepting reads at 70% full length, retrieving up to 10,000 sequences per seed sequence query. This latter step allows us to find and include in the database sequence entries that do not include the primer sites and that are not sequenced across the entire barcode region. The resulting data are dereplicated by retaining only unique sequences, and any taxon labeled as an 'environmental sample' is removed. Closed reference databases such as CRUX databases are commonly used in metabarcoding because they are more rapidly queried compared to open databases and can be quality curated. Prior to generating the eDNA results used in this study, we compared CRUX databases published in Curd et al., (2019) to the UNITE (Nilsson et al., 2018) and found CRUX databases identified more taxa when applied to our eDNA data.

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Text S1.2

As there is no standard way to differentiate false and true positives, we used cross-validation with traditional observation data, similarly to recent studies (McElroy et al., 2020; Lin et al., 2021), to characterize whether taxa occurring at low frequency were likely to be false positives. However, due to the enormous uncharacterized biodiversity within Fungi, we employed this cross-validation with PITS results only. We found 31 of the 161 genera from the PITS dataset were from Chlorophyta and only two of these were cross-validated in traditional observation datasets (Table S1.8). We attributed this low overlap to observation bias and chose not to consider Chlorophyta further. In contrast, 100 of 129 Streptophyta genera were cross-validated (Table S1.8). Those 29 other genera occurred in nine orders, eight of which contained cross-validated genera (Klebsormidiales was the exception). We hypothesized that if these 29 genera represented some false positives they would be found at lower frequency in PCR replicates than cross-validated genera, and observed that the 29 genera had significantly lower frequency of detection than the 100 cross-validated Streptophyta genera based on a one-tailed t-test (p=0.038, t-value=-1.79).

To further assess putative false positives, we examined the frequency of congener species across PCR replicates. We observed that 42% of recovered PITS genera have multiple congener species in our dataset. When we tested whether taxa in a single replicate were congeners with taxa found in other replicates, we found that taxa present in a single replicate (group 1, M=5.35, ss=4853.41) were no more likely

to belong to congener groups than taxa in multiple PCR replicates (group 2, M=4.98, ss=9063.94; t=0.48252, *p*=0.31487), indicating that these congeneric taxa, whether true positives or not, are not overrepresented as singleton observations in PCR replicates. We concluded that our metabarcoding data represent a mixture of low frequency taxa that are largely true positives but contain some false positives, as is common and expected in metabarcoding, and therefore that evaluating similarity among our PCR replicates has the potential power to inform decisions about further filtering based on taxa shared among replicates.

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Table S1.1 Read processing summary data for the PITS metabarcode.

Sample	Total Read Pairs Processed	Total assigned reads	Number of reads assigned to taxa
FO.1_replicate_PITS	129,933	79639	157
FO.1_replicate1_PITS	55,165	32507	140
FO.1_replicate17_PITS	45,636	27730	86
FO.1_replicate18_PITS	40,222	24406	97
FO.1_replicate19_PITS	69,934	43208	54
FO.1_replicate2_PITS	85,342	52106	107
FO.1_replicate20_PITS	58,451	37433	56
FO.1_replicate21_PITS	45,518	28263	120
FO.1_replicate22_PITS	66,253	40293	81
FO.1_replicate23_PITS	45,241	27819	85
FO.1_replicate24_PITS	76,612	48156	127
FO.1_replicate3_PITS	68,532	42715	99
FO.1_replicate4_PITS	58,943	37520	55
FO.1_replicate5_PITS	161,947	97598	335
FO.1_replicate6_PITS	42,970	26226	60

FO.1_replicate7_PITS	9,352	6148	4
FO.1_replicate8_PITS	66,403	17411	53
FO.1_replicate10_PITS	119,493	28342	69
FO.1_replicate11_PITS	59,401	27156	46
FO.1_replicate12_PITS	108,331	22969	19
FO.1_replicate13_PITS	106,118	30496	8
FO.1_replicate14_PITS	87,222	28601	54
FO.1_replicate15_PITS	84,763	27012	290
FO.1_replicate16_PITS	119,599	22288	823
FO.2_replicate9_PITS	33,518	20601	1131
FO.2_replicate1_PITS	29,676	18841	357
FO.2_replicate17_PITS	45,589	30089	531
FO.2_replicate18_PITS	62,185	40232	888
FO.2_replicate19_PITS	28,311	18422	312
FO.2_replicate2_PITS	56,430	37013	1602
FO.2_replicate20_PITS	62,127	40319	240
FO.2_replicate21_PITS	58,615	37820	1444
FO.2_replicate22_PITS	62,547	40460	1231
FO.2_replicate23_PITS	105,562	65059	3874
FO.2_replicate24_PITS	50,505	32676	290

FO.2_replicate3_PITS	47,114	30076	188
FO.2_replicate4_PITS	50,713	32468	624
FO.2_replicate5_PITS	35,028	21570	383
FO.2_replicate6_PITS	24,371	15775	466
FO.2_replicate7_PITS	55,022	29121	21072
FO.2_replicate8_PITS	113,608	34252	577
FO.2_replicate10_PITS	185,900	38188	1381
FO.2_replicate11_PITS	145,178	42911	413
FO.2_replicate12_PITS	111,549	47549	1227
FO.2_replicate13_PITS	156,919	53528	594
FO.2_replicate14_PITS	156,521	62153	1710
FO.2_replicate15_PITS	106,219	25910	957
FO.2_replicate16_PITS	122,831	32207	428
YL.1_replicate9_PITS	81,170	52816	494
YL.1_replicate1_PITS	51,682	27670	438
YL.1_replicate17_PITS	57,082	30654	15998
YL.1_replicate18_PITS	68,969	36533	640
YL.1_replicate19_PITS	69,502	37801	602
YL.1_replicate2_PITS	27,741	14782	144
YL.1_replicate20_PITS	75,007	37564	553

YL.1_replicate21_PITS	92,497	49284	506
YL.1_replicate22_PITS	26,066	13133	171
YL.1_replicate23_PITS	59,740	29411	369
YL.1_replicate24_PITS	45,853	23821	171
YL.1_replicate3_PITS	65,717	35344	463
YL.1_replicate4_PITS	84,967	44043	304
YL.1_replicate5_PITS	73,324	39127	376
YL.1_replicate6_PITS	56,253	28524	225
YL.1_replicate7_PITS	72,525	39556	791
YL.1_replicate8_PITS	175,715	34722	309
YL.1_replicate10_PITS	149,384	28437	301
YL.1_replicate11_PITS	112,632	26141	327
YL.1_replicate12_PITS	174,820	33306	235
YL.1_replicate13_PITS	143,124	8224	27
YL.1_replicate14_PITS	170,523	35437	228
YL.1_replicate15_PITS	185,544	24459	136
YL.1_replicate16_PITS	140,591	34255	1444
YL.2_replicate9_PITS	50,531	32403	22
YL.2_replicate1_PITS	86,360	55672	0
YL.2_replicate17_PITS	113,024	74558	68

YL.2_replicate18_PITS	80,409	52178	7
YL.2_replicate19_PITS	69,203	43842	27
YL.2_replicate2_PITS	80,289	51813	44
YL.2_replicate20_PITS	66,544	43545	9
YL.2_replicate21_PITS	133,082	85792	10
YL.2_replicate22_PITS	100,671	65231	73
YL.2_replicate23_PITS	40,255	26665	0
YL.2_replicate24_PITS	94,385	60806	0
YL.2_replicate3_PITS	51,793	33045	54
YL.2_replicate4_PITS	29,176	18841	2
YL.2_replicate5_PITS	67,851	43348	43
YL.2_replicate6_PITS	79,893	53238	159
YL.2_replicate7_PITS	65,185	43550	12
YL.2_replicate8_PITS	120,586	22952	26
YL.2_replicate10_PITS	164,320	53825	1
YL.2_replicate11_PITS	108,400	14761	7
YL.2_replicate12_PITS	116,969	25795	0
YL.2_replicate13_PITS	51,259	8661	0
YL.2_replicate14_PITS	93,552	12869	0
YL.2_replicate15_PITS	133,866	12561	0

YL.2_replicate16_PITS	111,509	14067	0
StP.1_replicate9_PITS	66,181	41788	51
StP.1_replicate1_PITS	39,026	24860	136
StP.1_replicate10_PITS	69,745	45479	21
StP.1_replicate11_PITS	41,767	27920	5
StP.1_replicate12_PITS	57,342	36744	26
StP.1_replicate13_PITS	48,206	30690	2
StP.1_replicate14_PITS	34,108	22095	70
StP.1_replicate15_PITS	40,643	25073	56
StP.1_replicate16_PITS	59,420	36359	844
StP.1_replicate17_PITS	75,524	47375	367
StP.1_replicate18_PITS	103,431	61977	196
StP.1_replicate19_PITS	61,946	40619	51
StP.1_replicate2_PITS	48,448	30782	8
StP.1_replicate20_PITS	45,652	28100	1
StP.1_replicate21_PITS	42,864	26795	0
StP.1_replicate22_PITS	48,354	29396	1
StP.1_replicate23_PITS	48,620	29372	7
StP.1_replicate24_PITS	70,160	45667	1
StP.1_replicate3_PITS	66,377	42667	1

StP.1_replicate4_PITS	49,845	31808	3
StP.1_replicate5_PITS	57,653	35611	250
StP.1_replicate6_PITS	56,118	34807	656
StP.1_replicate7_PITS	38,584	23229	46
StP.1_replicate8_PITS	134,619	86786	847
StP.2_replicate9_PITS	76,644	48892	106
StP.2_replicate1_PITS	94,851	57463	199
StP.2_replicate10_PITS	282,579	166279	85154
StP.2_replicate11_PITS	16,943	11281	0
StP.2_replicate12_PITS			
StP.2_replicate13_PITS	60,457	36264	118
StP.2_replicate14_PITS	61,062	37905	602
StP.2_replicate15_PITS	59,227	35774	7904
StP.2_replicate16_PITS	52,804	31763	103
StP.2_replicate17_PITS	98,578	62476	147
StP.2_replicate18_PITS	96,603	57886	290
StP.2_replicate19_PITS	71,478	45828	84
StP.2_replicate2_PITS	22,592	14610	0
StP.2_replicate20_PITS	34,396	21566	16
StP.2_replicate21_PITS	66,379	40347	73

StP.2_replicate22_PITS	77,417	45884	150
StP.2_replicate23_PITS	55,648	33919	79
StP.2_replicate24_PITS			
StP.2_replicate3_PITS	34,151	21774	279
StP.2_replicate4_PITS	56,091	35319	77
StP.2_replicate5_PITS	80,815	53082	16
StP.2_replicate6_PITS	60,986	39514	54
StP.2_replicate7_PITS	75,530	48082	532
StP.2_replicate8_PITS	164,050	94635	20741
SBS7_replicate9_PITS	56,184	29608	29608
SBS7_replicate1_PITS	169,767	86273	86273
SBS7_replicate3_PITS	3,236	1658	1658

Table S1.2 - Read processing summary data for the FITS metabarcode.

Sample	Total Read Pairs Processed	Total assigned reads	Number of reads assigned to taxa
FO.1_replicate1_FITS	265,630	218818	177794
FO.1_replicate17_FITS	85,517	69392	56691
FO.1_replicate18_FITS	92,469	74850	60697
FO.1_replicate19_FITS	49,312	39796	31858
FO.1_replicate2_FITS	212,249	174707	142977
FO.1_replicate20_FITS	69,998	55819	44694
FO.1_replicate21_FITS	55,608	44802	35856
FO.1_replicate22_FITS	110,922	89832	72722
FO.1_replicate23_FITS	62,181	49870	40437
FO.1_replicate24_FITS	47,254	38293	31657
FO.1_replicate3_FITS	81,250	64242	51489
FO.1_replicate4_FITS	104,081	83818	67082
FO.1_replicate5_FITS	71,169	58184	46503
FO.1_replicate6_FITS	76,096	61409	50279
FO.1_replicate7_FITS	55,427	43432	35287

FO.1_replicate8_FITS	57,842	46640	37617
FO.1_replicate10_FITS	66,403	30315	24828
FO.1_replicate11_FITS	119,493	58134	46788
FO.1_replicate12_FITS	59,401	14156	11549
FO.1_replicate13_FITS	108,331	58165	46323
FO.1_replicate14_FITS	106,118	47005	38065
FO.1_replicate15_FITS	87,222	32283	26128
FO.1_replicate16_FITS	84,763	29015	23393
FO.1_replicate9_FITS	119,599	66115	53488
FO.2_replicate1_FITS	91,327	74483	49684
FO.2_replicate17_FITS	96,789	79941	53719
FO.2_replicate18_FITS	77,403	64467	43151
FO.2_replicate19_FITS	93,302	77453	52068
FO.2_replicate2_FITS	22,807	18586	12578
FO.2_replicate20_FITS	76,679	61762	41782
FO.2_replicate21_FITS	67,767	54961	36646
FO.2_replicate22_FITS	59,542	48567	32508
FO.2_replicate23_FITS	87,827	72288	48033
FO.2_replicate24_FITS	129,715	107300	72574
FO.2_replicate3_FITS	18,820	15259	10210

FO.2_replicate4_FITS	15,409	12360	8417
FO.2_replicate5_FITS	36,100	29214	19434
FO.2_replicate6_FITS	81,374	67338	44824
FO.2_replicate7_FITS	27,925	22792	14884
FO.2_replicate8_FITS	37,037	30569	20290
FO.2_replicate10_FITS	113,608	49571	33449
FO.2_replicate11_FITS	185,900	103483	70475
FO.2_replicate12_FITS	145,178	62806	42289
FO.2_replicate13_FITS	111,549	30102	20143
FO.2_replicate14_FITS	156,919	58956	39717
FO.2_replicate15_FITS	156,521	47332	31641
FO.2_replicate16_FITS	106,219	53448	35809
FO.2_replicate9_FITS	122,831	57189	38633
YL.1_replicate1_FITS	60,044	47733	35990
YL.1_replicate17_FITS	102,825	81871	61162
YL.1_replicate18_FITS	58,406	46034	34977
YL.1_replicate19_FITS	153,408	123499	93445
YL.1_replicate2_FITS	41,691	32668	24163
YL.1_replicate20_FITS	51,401	40120	29910
YL.1_replicate21_FITS	62,157	49579	37759

YL.1_replicate22_FITS	72,897	57578	43469
YL.1_replicate23_FITS	35,485	28300	21194
YL.1_replicate24_FITS	48,203	38503	28929
YL.1_replicate3_FITS	75,427	60414	45352
YL.1_replicate4_FITS	36,161	28491	21347
YL.1_replicate5_FITS	67,937	54335	41357
YL.1_replicate6_FITS	44,335	34523	25987
YL.1_replicate7_FITS	43,349	33087	25043
YL.1_replicate8_FITS	19,883	15294	11478
YL.1_replicate10_FITS	175,715	85013	65250
YL.1_replicate11_FITS	149,384	76040	57782
YL.1_replicate12_FITS	112,632	51740	39601
YL.1_replicate13_FITS	174,820	88023	67176
YL.1_replicate14_FITS	143,124	100369	77555
YL.1_replicate15_FITS	170,523	81589	63927
YL.1_replicate16_FITS	185,544	110261	86322
YL.1_replicate9_FITS	140,591	60861	46502
YL.2_replicate1_FITS	121,891	99078	69352
YL.2_replicate17_FITS	76,161	61688	45460
YL.2_replicate18_FITS	30,163	23705	17197

YL.2_replicate19_FITS	72,143	58314	42097
YL.2_replicate2_FITS	79,307	64111	46127
YL.2_replicate20_FITS	25,803	20020	14694
YL.2_replicate21_FITS	67,584	54502	39895
YL.2_replicate22_FITS	52,074	41044	29909
YL.2_replicate23_FITS	51,123	41326	30462
YL.2_replicate24_FITS	50,095	40094	29172
YL.2_replicate3_FITS	145,620	120291	87503
YL.2_replicate4_FITS	102,906	83787	60049
YL.2_replicate5_FITS	74,549	60498	43779
YL.2_replicate6_FITS	97,619	79847	57667
YL.2_replicate7_FITS	84,501	67491	48866
YL.2_replicate8_FITS	90,890	73716	52799
YL.2_replicate10_FITS	120,586	69222	48224
YL.2_replicate11_FITS	164,320	67383	48114
YL.2_replicate12_FITS	108,400	69291	49283
YL.2_replicate13_FITS	116,969	61297	43639
YL.2_replicate14_FITS	51,259	29768	21217
YL.2_replicate15_FITS	93,552	58919	42909
YL.2_replicate16_FITS	133,866	93999	68691

YL.2_replicate9_FITS	111,509	72204	51284
StP.1_replicate1_FITS	90,454	75399	63826
StP.1_replicate10_FITS	259,609	216338	185905
StP.1_replicate11_FITS	45,009	37198	32153
StP.1_replicate12_FITS	256,234	173193	154785
StP.1_replicate13_FITS	121,368	58056	49923
StP.1_replicate14_FITS	72,513	60420	52166
StP.1_replicate15_FITS	59,285	49759	43093
StP.1_replicate16_FITS	75,781	63189	54284
StP.1_replicate17_FITS	80,644	67753	57219
StP.1_replicate18_FITS	59,133	49812	42309
StP.1_replicate19_FITS	28,513	23729	20202
StP.1_replicate2_FITS	87,145	73003	62323
StP.1_replicate20_FITS	45,822	38295	32596
StP.1_replicate21_FITS	26,925	22477	19577
StP.1_replicate22_FITS	38,623	32577	28289
StP.1_replicate23_FITS	25,332	21355	18761
StP.1_replicate24_FITS	58,850	49516	41934
StP.1_replicate3_FITS	382,888	323210	273720
StP.1_replicate4_FITS	96,310	80638	69590

StP.1_replicate5_FITS	186,627	156968	132357
StP.1_replicate6_FITS	114,799	95097	81024
StP.1_replicate7_FITS	89,173	75177	64065
StP.1_replicate8_FITS	70,918	60197	51664
StP.1_replicate9_FITS	80,667	66752	56986
StP.2_replicate1_FITS	103,844	87914	68034
StP.2_replicate10_FITS	41,278	34461	25480
StP.2_replicate11_FITS	67,729	56571	41199
StP.2_replicate12_FITS	47,408	39088	29264
StP.2_replicate13_FITS	63,793	52553	38910
StP.2_replicate14_FITS	29,282	23951	17241
StP.2_replicate15_FITS	70,357	58470	43599
StP.2_replicate16_FITS	26,324	22236	17904
StP.2_replicate17_FITS	95,097	80241	59411
StP.2_replicate18_FITS	109,576	92280	68934
StP.2_replicate19_FITS	67,312	56948	41765
StP.2_replicate2_FITS	80,935	67767	51947
StP.2_replicate20_FITS	70,866	59973	44085
StP.2_replicate21_FITS	83,559	70667	51504
StP.2_replicate22_FITS	73,066	61757	45031

StP.2_replicate23_FITS	39,873	32890	23800
StP.2_replicate24_FITS	41,674	34743	26226
StP.2_replicate3_FITS	55,376	46435	34766
StP.2_replicate4_FITS	126,416	104419	79217
StP.2_replicate5_FITS	73,309	61368	44483
StP.2_replicate6_FITS	127,138	106745	80456
StP.2_replicate7_FITS	40,190	33762	25131
StP.2_replicate8_FITS	50,985	42923	33336
StP.2_replicate9_FITS	60,466	49708	38384
extraction.neg_replicate1_FITS	83,988	74009	73853
extraction.neg_replicate2_FITS	34,326	28727	8941
extraction.neg_replicate3_FITS	38,488	32521	27593
extraction.neg_replicate4_FITS	50,108	43518	33460
pcr.neg_replicate1_FITS	62,216	54519	30560
pcr.neg_replicate2_FITS	2,684	88	88

Table S1.3

Available as a separate file.

Table S1.4

Available as a separate file.

Table S1.5

Table S5. Alpha diversity for PITS and FITS datasets with 1,000 and 10,000 reads, and a minimum read cutoff of 5, calculated with Observed, Shannon, and Simpson metrics. "Fold inc." is the fold increase in α diversity observed by increasing the sequencing depth from 1,000 to 10,000 reads. P-values from t-test comparing the α diversity calculated from datasets of 1,000 and 10,000 reads.

	PITS									FITS								
α diversity measure	Observed				Shannon				Simpson									
	Avg α diversity with 1k reads	Avg α diversity with 10k reads	Fold inc.	P value	Avg α diversity with 1k reads	Avg α diversity with 10k reads	Fold inc.	P value	Avg α diversity with 1k reads	Avg α diversity with 10k reads	Fold inc.	P value	Avg α diversity with 1k reads	Avg α diversity with 10k reads	Fold inc.	P value	Avg α diversity with 1k reads	Avg α diversity with 10k reads	Fold inc.	P value
FO.1	7.000	12.478	1.783	0.000	1.246	1.299	1.043	0.1529	0.604	0.612	1.013	0.6072	14.458	41.846	2.894	0.000	2.038	2.254	1.106	0.000	0.837	0.849	1.015	0.000
FO.2	7.625	13.739	1.802	0.000	0.903	0.953	1.056	0.3734	0.408	0.416	1.019	0.7393	11.208	24.944	2.226	< 2.2e-16	1.016	1.164	1.146	0.000	0.395	0.429	1.085	0.0003432
YL.1	9.786	23.739	2.426	0.000	1.387	1.511	1.089	0.0584	0.645	0.659	1.022	0.5425	18.833	46.176	2.452	0.000	1.819	2.048	1.126	0.000	0.679	0.708	1.043	0.0002844
YL.2	8.708	11.652	1.338	0.000	1.310	1.327	1.012	0.227	0.642	0.644	1.003	0.6817	24.625	63.429	2.576	< 2.2e-16	2.396	2.712	1.132	0.000	0.817	0.841	1.029	0.000
StP.1	9.333	14.667	1.571	0.000	1.140	1.059	0.0481	0.451	0.463	1.026	0.3645	16.667	27.600	1.656	0.000	2.361	2.454	1.039	0.000	0.878	0.884	1.007	0.0003045
StP.2	8.176	13.417	1.641	0.000	0.718	0.768	1.068	0.3503	0.291	0.293	1.007	0.9144	7.042	18.688	2.654	0.000	0.499	0.621	1.245	0.01407	0.198	0.227	1.144	0.2598
Average	8.438	14.949	1.760		1.107	1.166	1.055		0.507	0.514	1.015		15.472	37.114	2.410		1.688	1.875	1.132		0.634	0.656	1.054	

Table S1.6

Extrapolated richness for each PCR replicate using the iNext program that estimates richness at 2x the empirical sequencing depth. Outliers were calculated using the two-sided Iglewicz and Hoaglin's robust test for multiple outliers with modified Z score ≥ 3.5. Outliers are bolded for ease of viewing.

Sample Name	Site	iNext Extrapolated Richness	Outlier?	Sample Name	Site	iNext Extrapolated Richness	Outlier?
FO.1_replicate1_FITS	FO.1	**148.794**	O	FO.1_replicate1_PITS	FO.1	29.332	
FO.1_replicate17_FITS	FO.1	94.656		FO.1_replicate10_PITS	FO.1	23.148	
FO.1_replicate18_FITS	FO.1	98.516		FO.1_replicate11_PITS	FO.1	34.078	
FO.1_replicate19_FITS	FO.1	85.905		FO.1_replicate12_PITS	FO.1	**58.225**	O
FO.1_replicate2_FITS	FO.1	**151.435**	O	FO.1_replicate13_PITS	FO.1	28.729	
FO.1_replicate20_FITS	FO.1	97.886		FO.1_replicate14_PITS	FO.1	32.963	

FO.1_replicate21_FITS	FO.1	73.324		FO.1_replicate15_PITS	FO.1	27.945	
FO.1_replicate22_FITS	FO.1	95.535		FO.1_replicate16_PITS	FO.1	30.332	
FO.1_replicate23_FITS	FO.1	101.972		FO.1_replicate17_PITS	FO.1	34.793	
FO.1_replicate24_FITS	FO.1	73.255		FO.1_replicate18_PITS	FO.1	**79.194**	O
FO.1_replicate3_FITS	FO.1	97.956		FO.1_replicate19_PITS	FO.1	30.089	
FO.1_replicate4_FITS	FO.1	111.208		FO.1_replicate2_PITS	FO.1	21.19	
FO.1_replicate5_FITS	FO.1	92.383		FO.1_replicate20_PITS	FO.1	30.295	
FO.1_replicate6_FITS	FO.1	99.583		FO.1_replicate21_PITS	FO.1	30.953	
FO.1_replicate7_FITS	FO.1	86.039		FO.1_replicate22_PITS	FO.1	35.945	
FO.1_replicate8_FITS	FO.1	87.382		FO.1_replicate23_PITS	FO.1	**141.709**	O

FO.1_replicate10_FITS	FO.1	70.531		FO.1_replicate24_PITS	FO.1	27.935	
FO.1_replicate11_FITS	FO.1	91.542		FO.1_replicate3_PITS	FO.1	**3**	O
FO.1_replicate12_FITS	FO.1	46.442		FO.1_replicate4_PITS	FO.1	37.883	
FO.1_replicate13_FITS	FO.1	101.761		FO.1_replicate5_PITS	FO.1	33.295	
FO.1_replicate14_FITS	FO.1	80.486		FO.1_replicate6_PITS	FO.1	**62.583**	O
FO.1_replicate15_FITS	FO.1	70.483		FO.1_replicate7_PITS	FO.1	32.07	
FO.1_replicate16_FITS	FO.1	69.851		FO.1_replicate8_PITS	FO.1	26.121	
FO.1_replicate9_FITS	FO.1	83.694		FO.1_replicate9_PITS	FO.1	48.321	
FO.2_replicate1_FITS	FO.2	96.558		FO.2_replicate1_PITS	FO.2	29.945	
FO.2_replicate17_FITS	FO.2	97.748		FO.2_replicate10_PITS	FO.2	18.945	

FO.2_replicate18_FITS	FO.2	82.572		FO.2_replicate11_PITS	FO.2	16.148	
FO.2_replicate19_FITS	FO.2	97.104		FO.2_replicate12_PITS	FO.2	33.962	
FO.2_replicate2_FITS	FO.2	44.221		FO.2_replicate13_PITS	FO.2	30.725	
FO.2_replicate20_FITS	FO.2	80.363		FO.2_replicate14_PITS	FO.2	45.242	
FO.2_replicate21_FITS	FO.2	76.766		FO.2_replicate15_PITS	FO.2	68.307	
FO.2_replicate22_FITS	FO.2	69.663		FO.2_replicate16_PITS	FO.2	47.566	
FO.2_replicate23_FITS	FO.2	95.821		FO.2_replicate17_PITS	FO.2	17.19	
FO.2_replicate24_FITS	FO.2	105.149		FO.2_replicate18_PITS	FO.2	48.332	
FO.2_replicate3_FITS	FO.2	39.266		FO.2_replicate19_PITS	FO.2	46.883	
FO.2_replicate4_FITS	FO.2	24.528		FO.2_replicate2_PITS	FO.2	22.297	

FO.2_replicate5_FITS	FO.2	59.851		FO.2_replicate20_PITS	FO.2	63.167	
FO.2_replicate6_FITS	FO.2	77.906		FO.2_replicate21_PITS	FO.2	50.963	
FO.2_replicate7_FITS	FO.2	52.401		FO.2_replicate22_PITS	FO.2	22.442	
FO.2_replicate8_FITS	FO.2	61.156		FO.2_replicate23_PITS	FO.2	60.038	
FO.2_replicate10_FITS	FO.2	69.039		FO.2_replicate24_PITS	FO.2	49.758	
FO.2_replicate11_FITS	FO.2	106.706		FO.2_replicate3_PITS	FO.2	20.442	
FO.2_replicate12_FITS	FO.2	93.142		FO.2_replicate4_PITS	FO.2	32.332	
FO.2_replicate13_FITS	FO.2	55.177		FO.2_replicate5_PITS	FO.2	2	
FO.2_replicate14_FITS	FO.2	90.034		FO.2_replicate6_PITS	FO.2	2	
FO.2_replicate15_FITS	FO.2	63.591		FO.2_replicate7_PITS	FO.2	25.528	

FO.2_replicate16_FITS	FO.2	77.886		FO.2_replicate8_PITS	FO.2	18.148	
FO.2_replicate9_FITS	FO.2	98.323		FO.2_replicate9_PITS	FO.2	15.148	
YL.1_replicate1_FITS	YL.1	94.975		YL.1_replicate1_PITS	YL.1	48.321	
YL.1_replicate17_FITS	YL.1	127.072		YL.1_replicate10_PITS	YL.1	100.383	
YL.1_replicate18_FITS	YL.1	101.103		YL.1_replicate11_PITS	YL.1	65.342	
YL.1_replicate19_FITS	YL.1	156.076		YL.1_replicate12_PITS	YL.1	31.332	
YL.1_replicate2_FITS	YL.1	76.323		YL.1_replicate13_PITS	YL.1	50.143	
YL.1_replicate20_FITS	YL.1	87.772		YL.1_replicate14_PITS	YL.1	64.069	
YL.1_replicate21_FITS	YL.1	94.043		YL.1_replicate15_PITS	YL.1	56.413	
YL.1_replicate22_FITS	YL.1	124.834		YL.1_replicate16_PITS	YL.1	68.56	

YL.1_replicate23_FITS	YL.1	62.883		YL.1_replicate17_PITS	YL.1	61.142	
YL.1_replicate24_FITS	YL.1	80.81		YL.1_replicate18_PITS	YL.1	122.773	
YL.1_replicate3_FITS	YL.1	110.416		YL.1_replicate19_PITS	YL.1	47.433	
YL.1_replicate4_FITS	YL.1	89.234		YL.1_replicate2_PITS	YL.1	2	
YL.1_replicate5_FITS	YL.1	104.255		YL.1_replicate20_PITS	YL.1	93.925	
YL.1_replicate6_FITS	YL.1	81.121		YL.1_replicate21_PITS	YL.1	64.069	
YL.1_replicate7_FITS	YL.1	93.098		YL.1_replicate22_PITS	YL.1	81.925	
YL.1_replicate8_FITS	YL.1	48.568		YL.1_replicate23_PITS	YL.1	74.851	
YL.1_replicate10_FITS	YL.1	128.26		YL.1_replicate24_PITS	YL.1	**227.555**	O
YL.1_replicate11_FITS	YL.1	131.469		YL.1_replicate3_PITS	YL.1	107.383	

YL.1_replicate12_FITS	YL.1	97.92		YL.1_replicate4_PITS	YL.1	110.517	
YL.1_replicate13_FITS	YL.1	145.519		YL.1_replicate5_PITS	YL.1	73.477	
YL.1_replicate14_FITS	YL.1	145.952		YL.1_replicate6_PITS	YL.1	63.413	
YL.1_replicate15_FITS	YL.1	105.696		YL.1_replicate7_PITS	YL.1	24.072	
YL.1_replicate16_FITS	YL.1	145.244		YL.1_replicate8_PITS	YL.1	7	
YL.1_replicate9_FITS	YL.1	124.468		YL.1_replicate9_PITS	YL.1	33.952	
YL.2_replicate1_FITS	YL.2	182.806		YL.2_replicate1_PITS	YL.2	45.443	
YL.2_replicate17_FITS	YL.2	140.649		YL.2_replicate10_PITS	YL.2	**2**	O
YL.2_replicate18_FITS	YL.2	97.038		YL.2_replicate11_PITS	YL.2	**2**	O
YL.2_replicate19_FITS	YL.2	159.21		YL.2_replicate12_PITS	YL.2	37.314	

YL.2_replicate2_FITS	YL.2	147.33		YL.2_replicate13_PITS	YL.2	48.19	
YL.2_replicate20_FITS	YL.2	**85.559**	O	YL.2_replicate14_PITS	YL.2	26.729	
YL.2_replicate21_FITS	YL.2	131.654		YL.2_replicate15_PITS	YL.2	33.793	
YL.2_replicate22_FITS	YL.2	118.363		YL.2_replicate16_PITS	YL.2	38.883	
YL.2_replicate23_FITS	YL.2	132.179		YL.2_replicate17_PITS	YL.2	44.058	
YL.2_replicate24_FITS	YL.2	126.071		YL.2_replicate18_PITS	YL.2	41.967	
YL.2_replicate3_FITS	YL.2	186.648		YL.2_replicate19_PITS	YL.2	35.701	
YL.2_replicate4_FITS	YL.2	155.329		YL.2_replicate2_PITS	YL.2	**98.707**	O
YL.2_replicate5_FITS	YL.2	163.158		YL.2_replicate20_PITS	YL.2	**1**	O
YL.2_replicate6_FITS	YL.2	160.296		YL.2_replicate21_PITS	YL.2	30.078	

YL.2_replicate7_FITS	YL.2	159.222		YL.2_replicate22_PITS	YL.2	35.295	
YL.2_replicate8_FITS	YL.2	160.172		YL.2_replicate23_PITS	YL.2	37.242	
YL.2_replicate10_FITS	YL.2	158.785		YL.2_replicate24_PITS	YL.2	44.222	
YL.2_replicate11_FITS	YL.2	164.658		YL.2_replicate3_PITS	YL.2	28.92	
YL.2_replicate12_FITS	YL.2	153.029		YL.2_replicate4_PITS	YL.2	27.102	
YL.2_replicate13_FITS	YL.2	142.896		YL.2_replicate5_PITS	YL.2	28.379	
YL.2_replicate14_FITS	YL.2	104.425		YL.2_replicate6_PITS	YL.2	3	
YL.2_replicate15_FITS	YL.2	141.14		YL.2_replicate7_PITS	YL.2	29.102	
YL.2_replicate16_FITS	YL.2	199.833		YL.2_replicate8_PITS	YL.2	49.816	
YL.2_replicate9_FITS	YL.2	148.834		YL.2_replicate9_PITS	YL.2	26.912	

StP.15_replicate1_FITS	StP.15	58.222		StP.15_replicate10_PITS	StP.15	11.245	
StP.15_replicate10_FITS	StP.15	**91.996**	O	StP.15_replicate11_PITS	StP.15	2	
StP.15_replicate11_FITS	StP.15	44.758		StP.15_replicate12_PITS	StP.15	1	
StP.15_replicate12_FITS	StP.15	78.363		StP.15_replicate13_PITS	StP.15	30.102	
StP.15_replicate13_FITS	StP.15	58.046		StP.15_replicate14_PITS	StP.15	19.865	
StP.15_replicate14_FITS	StP.15	47.816		StP.15_replicate15_PITS	StP.15	34.628	
StP.15_replicate15_FITS	StP.15	49.89		StP.15_replicate16_PITS	StP.15	24.935	
StP.15_replicate16_FITS	StP.15	64.323		StP.15_replicate17_PITS	StP.15	21.896	
StP.15_replicate17_FITS	StP.15	59.664		StP.15_replicate18_PITS	StP.15	31.295	
StP.15_replicate18_FITS	StP.15	49.97		StP.15_replicate19_PITS	StP.15	27.379	

StP.15_replicate19_FITS	StP.15	42.241		StP.15_replicate2_PITS	StP.15	3	
StP.15_replicate2_FITS	StP.15	50.295		StP.15_replicate20_PITS	StP.15	35.295	
StP.15_replicate20_FITS	StP.15	41.056		StP.15_replicate21_PITS	StP.15	**121.497**	O
StP.15_replicate21_FITS	StP.15	31.088		StP.15_replicate22_PITS	StP.15	**70.591**	O
StP.15_replicate22_FITS	StP.15	36.347		StP.15_replicate23_PITS	StP.15	33.379	
StP.15_replicate23_FITS	StP.15	30.7		StP.15_replicate24_PITS	StP.15	34.963	
StP.15_replicate24_FITS	StP.15	48.204		StP.15_replicate3_PITS	StP.15	16.432	
StP.15_replicate3_FITS	StP.15	**103.255**	O	StP.15_replicate4_PITS	StP.15	21.19	
StP.15_replicate4_FITS	StP.15	50.566		StP.15_replicate5_PITS	StP.15	51.434	
StP.15_replicate5_FITS	StP.15	68.142		StP.15_replicate6_PITS	StP.15	48.816	

StP.15_replicate6_FITS	StP.15	63.488		StP.15_replicate7_PITS	StP.15	20.19	
StP.15_replicate7_FITS	StP.15	55.058		StP.15_replicate8_PITS	StP.15	46.569	
StP.15_replicate8_FITS	StP.15	50.883		StP.15_replicate9_PITS	StP.15	2	
StP.15_replicate9_FITS	StP.15	56.477		StP.20_replicate1_PITS	StP.20	9.864	
StP.20_replicate1_FITS	StP.20	75.187		StP.20_replicate10_PITS	StP.20	13.865	
StP.20_replicate10_FITS	StP.20	37.962		StP.20_replicate11_PITS	StP.20	26.912	
StP.20_replicate11_FITS	StP.20	45.057		StP.20_replicate12_PITS	StP.20	37.442	
StP.20_replicate12_FITS	StP.20	52.342		StP.20_replicate13_PITS	StP.20	40.494	
StP.20_replicate13_FITS	StP.20	40.171		StP.20_replicate14_PITS	StP.20	34.442	
StP.20_replicate14_FITS	StP.20	35.883		StP.20_replicate15_PITS	StP.20	56.178	

StP.20_replicate15_FITS	StP.20	55.342		StP.20_replicate16_PITS	StP.20	24.491	
StP.20_replicate16_FITS	StP.20	36.478		StP.20_replicate17_PITS	StP.20	34.072	
StP.20_replicate17_FITS	StP.20	44.883		StP.20_replicate18_PITS	StP.20	39.162	
StP.20_replicate18_FITS	StP.20	73.275		StP.20_replicate19_PITS	StP.20	46.172	
StP.20_replicate19_FITS	StP.20	53.215		StP.20_replicate2_PITS	StP.20	5	
StP.20_replicate2_FITS	StP.20	61.194		StP.20_replicate20_PITS	StP.20	45.478	
StP.20_replicate20_FITS	StP.20	63.842		StP.20_replicate21_PITS	StP.20	38.314	
StP.20_replicate21_FITS	StP.20	51.142		StP.20_replicate22_PITS	StP.20	31.945	
StP.20_replicate22_FITS	StP.20	55.591		StP.20_replicate23_PITS	StP.20	30.379	
StP.20_replicate23_FITS	StP.20	36.433		StP.20_replicate24_PITS	StP.20	31.102	

StP.20_replicate24_FITS	StP.20	43.004		StP.20_replicate3_PITS	StP.20	9.864	
StP.20_replicate3_FITS	StP.20	37.777		StP.20_replicate4_PITS	StP.20	35.07	
StP.20_replicate4_FITS	StP.20	62.451		StP.20_replicate5_PITS	StP.20	27.379	
StP.20_replicate5_FITS	StP.20	60.81		StP.20_replicate6_PITS	StP.20	26.121	
StP.20_replicate6_FITS	StP.20	61.758		StP.20_replicate7_PITS	StP.20	18.264	
StP.20_replicate7_FITS	StP.20	34.161		StP.20_replicate8_PITS	StP.20	16.919	
StP.20_replicate8_FITS	StP.20	51.662		StP.20_replicate9_PITS	StP.20	15	
StP.20_replicate9_FITS	StP.20	56.342					

Table S1.7

Available as a separate file.

Table S1.8

Available as a separate file.

Table S2.1

Permafrost soil plug sample information including sample name, site, library ID, sample age, and number of PCR replicates successfully amplified for both primer sets. An 'x' in the normalized Ct columns represent samples that did not amplify during qPCR. For those samples, we used a 1:8 dilution and 40 cycles in PCR.

Sample ID	Site	Sample Age (Cal yr BP)	trnL normalized Ct	16Smamm normalized Ct
MM13-20	Upper Quartz	5,165	0.691351202	0.7709072478
MM13-18	Upper Quartz	5,165	0.765404808	0.78205778
MM13-16	Upper Quartz	5,165	0.7383255043	x
MM12-91	Upper Quartz	5,925	0.6507322465	0.7181956412
MM12-39	Upper Goldbottom	5,945	0.7891682785	x
MM12-49	Upper Hunker	6,090	x	0.8925494171
MM13-15	Upper Quartz	12,255	1	0.9515965535
MM13-13	Upper Quartz	13,048	0.7821221332	0.8360364927
MM13-28	Upper Quartz	13,110	0.6983973473	0.7359351242
MM13-21	Upper Quartz	13,461	0.8208068527	0.8819057273
MM13-25	Upper Quartz	14,080	0.5580270793	0.656614293
MM13-17	Upper Quartz	14,335	0.6670350926	0.6452103396
MM12-QC-04	Upper Quartz	14,927	0.6244819011	0.7589964521
MM13-11	Upper Quartz	15,740	0.5012434374	0.7192093259
MM13-22	Upper Quartz	16,270	0.5614810721	x
MM13-29	Upper Quartz	16,310	0.6561204753	0.8864673087
MM13-24	Upper Quartz	16,385	0.6522520033	0.786112519
MM12-112	Upper Goldbottom	18,820	0.6794694667	0.7569690826
MM12-116A	Upper Goldbottom	19,264	0.6424426637	0.7830714648
MM12-113	Upper Goldbottom	20,375	0.5661785023	0.6340598074
MM12-117A	Upper Goldbottom	20,375	0.7391544626	0.7475924987
MM12-28	Lower Hunker	22720	0.6642718983	0.7597567157
MM13-46	Upper Goldbottom	24,335	0.6883116883	0.8329954384
MM13-44	Upper Goldbottom	24,525	0.6090080133	0.764318297
MM13-45	Upper Goldbottom	24,715	0.6457584968	0.9216928535
MM13-42	Upper Goldbottom	25,200	0.7021276596	0.7840851495
MM13-41A	Upper Goldbottom	25,285	0.6825089804	0.7557019767
MM13-40	Upper Goldbottom	31,633	0.8814589666	0.8634059807
MM12-134	Upper Goldbottom	35,895	0.6725614811	0.8258996452
MM12-125A	Upper Goldbottom	42,100	x	0.8793715155
MM12-42	Upper Goldbottom	42536	0.848576955	0.9153573239
MM12-127A	Upper Goldbottom	43,200	x	x
MM12-128A	Upper Goldbottom	43,804	0.7963525836	0.9143436391

Table S2.2: Dates of arctic ground squirrel nests.

Arctic Ground Squirrel Nest Ages from the Klondike							
no.	Sample ID	Lab ID	Locality	Cal. yr BP	^{14}C yr BP	±	Source
1	UCIAMS-131092	DF13-05	Lucky Lady 2	13,675	11,875	35	Froese Lab
2	-	DF13-06	Lucky Lady 2	*ca.* 14,000	-	-	Froese lab
3	GSC-2641	-	Hunker	14,135	12,200	100	Harington, 1977
4	UCIAMS-142195	MM13-11	Upper Quartz	15,740	13,110	35	This paper
5	UCIAMS-114721	DF12-61b	Lucky Lady 2	15,990	13,300	30	Froese Lab
6	QC-664	-	Lower Sulfur	16,070	13,350	265	Guthrie, 1990
7	UCIAMS-131096	MM13-22	Upper Quartz	16,270	13,510	45	This paper
8	UCIAMS-51324	DF08-97	Lucky Lady 2	16,500	13,680	35	Froese Lab
9	Beta-111606	DG-O-01	Lower Hunker	16,850	13,910	70	Kotler and Burn, 2000
10	UCIAMS-67157	DF-09-29	Hunker	17,155	14,100	40	Froese lab
11	-	MM13-09	Upper Quartz	*ca.* 20,000	-	-	Froese Lab
12	-	MM13-10	Upper Quartz	*ca.* 20,000	-	-	Froese Lab
13	UCIAMS-114712	MM12-113	Upper Goldbottom	20,375	16,895	45	Froese Lab
14	UCIAMS-131093	MM13-44b	Upper Goldbottom	24,525	20,310	720	Froese Lab
15	-	MM13-42	Upper Goldbottom	*ca.* 25,000	-	-	Froese Lab
16	UCIAMS-73889	GZ-2009-33	Little Blanche	25,140	20,840	100	Froese Lab
17	OS-75964			25,225	20,900	110	Froese Lab
18	UCIAMS-131095	MM13-41b	Goldbottom	25,285	20,960	150	Froese Lab
19	Beta-136365		Hunker	26,320	22,090	140	Storer, 2002
20	UCIAMS-73888		Lindo	27,485	23,220	140	Froese Lab
21	UCIAMS-114718	MM12-67	Black Hills	27,575	23,360	100	Froese Lab
22	Beta-161238		Quartz	28,040	23,990	130	Froese et al., 2002
23	UCIAMS-101864	DF10-25	Quartz	28,210	23,990	560	Froese Lab
24	Beta-161239		Quartz	28,315	24,280	130	Froese et al., 2002
25	Beta-202416	GZ.04.07 (YG 343.15)	Quartz	28,935	24,880	170	Zazula et al., 2007
26	OxA-16062		Quartz	29,320	25,270	120	Demuro et al., 2008
27	OxA-16063		Quartz	29,365	25,310	110	Demuro et al., 2008
28	Beta-202421	GZ.02.07.01.42 (YG 343.12)	Quartz	29,970	25,750	200	Zazula et al., 2007
29	-	GZ.02.07.01.36 (YG 343.8)	Quartz	*ca.*30,000	In Dt	-	Froese Lab
30	-	GZ.05.29 (YG 343.28)	Quartz	*ca.*30,000	2.5m below Dt		Zazula 2006b
31	-	GZ.05.32 (YG 343.31)	Quartz	*ca.*30,000	below Dt	-	Zazula 2003
32	Beta-210522	GZ.04.44 (YG 343.42)	Quartz	30,025	25,800	240	Zazula et al., 2006b
33	Beta-210521	GZ.05.34 (YG 343.33)	Quartz	30,100	25,870	190	Zazula et al., 2006b
34	Beta-202419	GZ.04.69 (YG 343.3)	Quartz	30,210	25,970	200	Zazula et al., 2007
35	OxA-16044		Quartz	30,470	26,180	160	Demuro et al., 2008
36	OxA-18330	CQC 4	Quartz	30,530	26,220	140	Brock et al., 2010
37	Beta-202417	GZ.04.13 (YG 343.21)	Quartz	33,165	29,030	310	Zazula et al., 2007
38	Beta-202418	GZ.04.46 (YG.350.2)	Goldbottom	33,600	29,450	320	Zazula et al., 2006b
39	114714	MM12-134	Upper Goldbottom	35,895	32,000	320	Froese Lab

Table S2.3: Plants detected with trnL meta barcode. We categorized plants into three groups (forb, graminoid, and woody plant). Numbers in sheet 1 correlate to the number of PCR replicates (out of 5) that the taxa was detected in each sample with, and in sheet 2 signify the cumulative number of reads (across all PCR replicates) that the taxa was detected in.

Figure S2.1: Ct values from the 16Smamm and trnL 1:3 dilution qPCR showing DNA preservation for all samples (a lower number represents a higher proportion of amplifiable DNA). The yellow bar denotes the presence of arctic ground squirrel nests which are a strong indicator of a steppe.

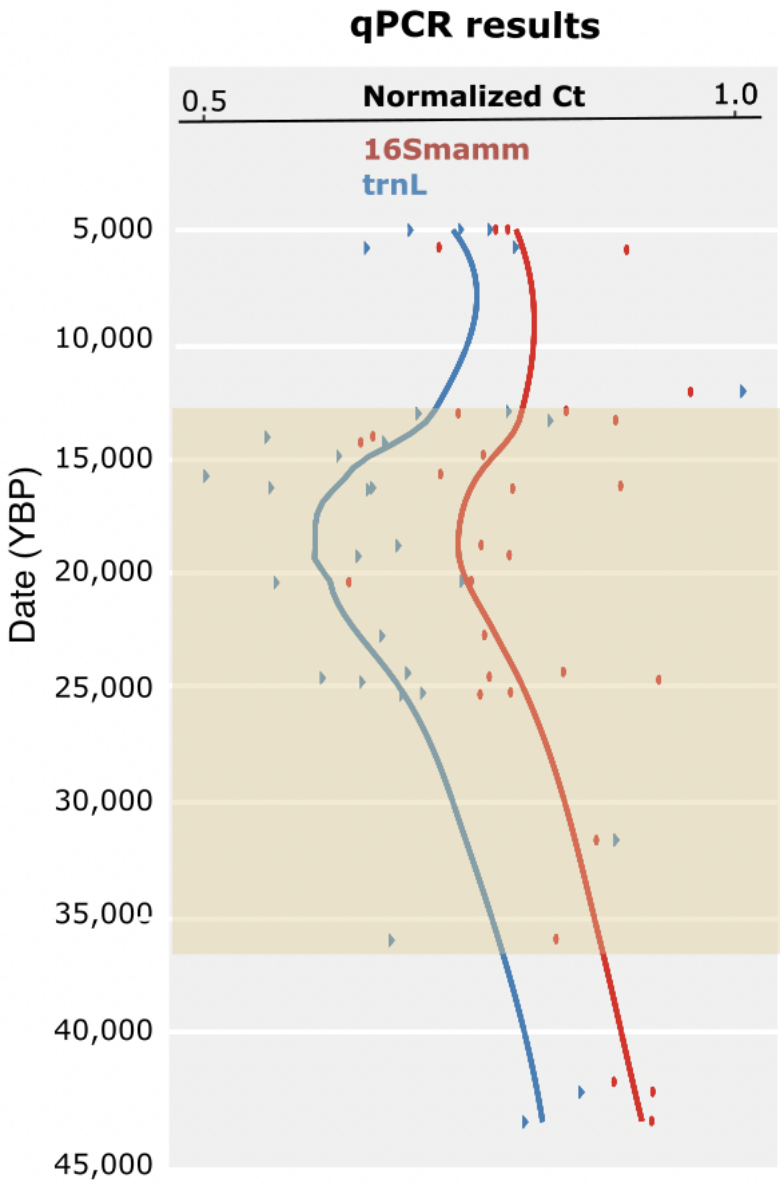

Figure S2.2: Mammal plot as shown in figure 2.1, here also including the distribution of bison clades observed (red box). The vertical columns indicate how many PCR replicates bison clades could be identified from, while the pie charts represent the total proportion of reads, cumulatively across PCR replicates, supporting bison clades 1 and siberian lineage versus clade 2. The yellow bar denotes the presence of arctic ground squirrel nests which are a strong indicator of a steppe.

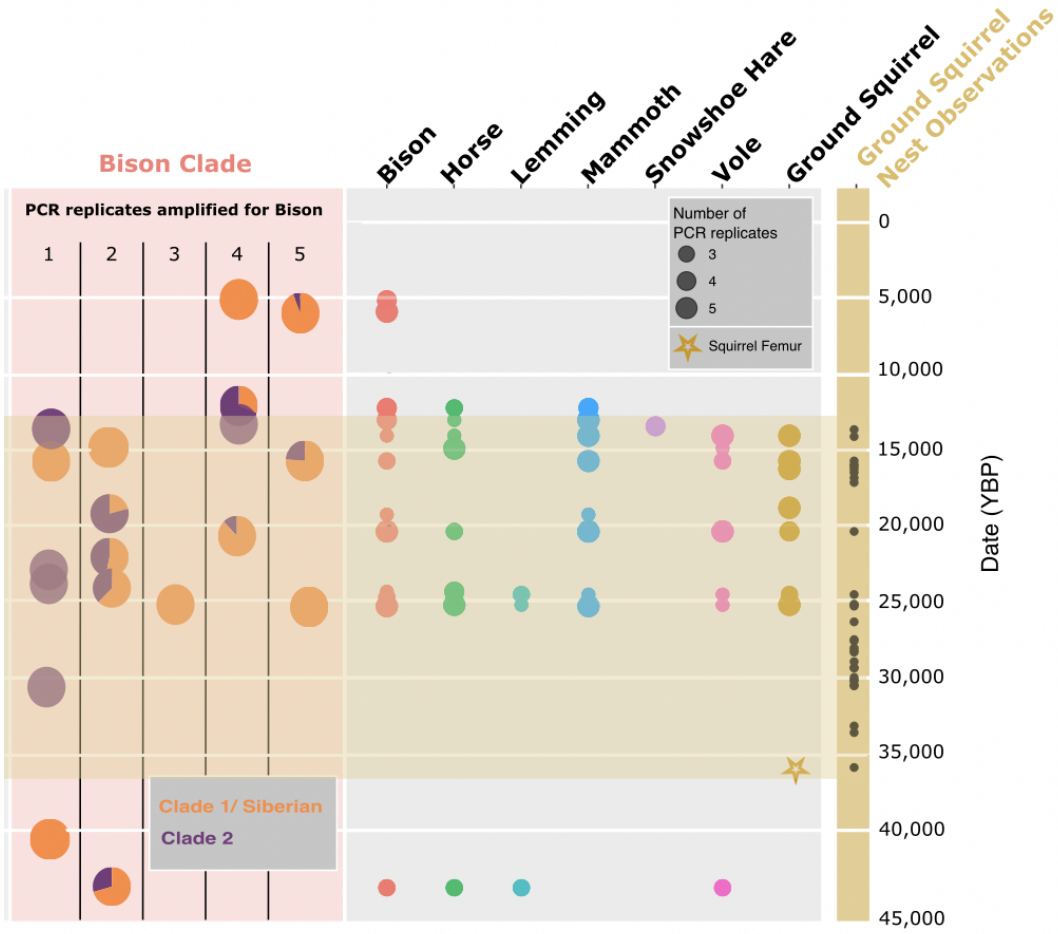

Figure S2.3: MEGAN phylogeny of reads from rodent femur aligned to BLAST database. Numbers show the number of reads aligned to each animal.

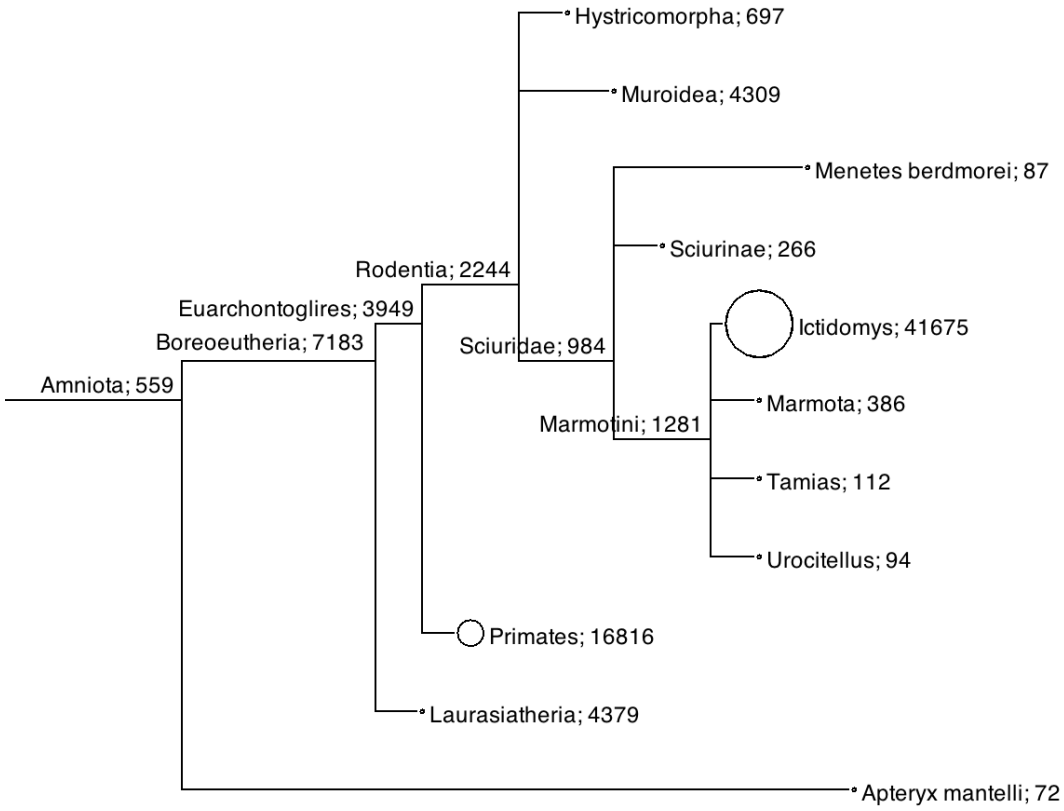

Table S3.1. Table of archaeological samples metadata and sequencing statistics.

Sample Metadata					Library Prep protocol	Total read pairs processed	Alignment Statistics			Rx ratio of Cattle genome	Predicted Sex based on Rx
Library #	FS #	Radiocarbon Lab #	RCYBP	Object type			Unique reads aligned to Cattle	Unique reads aligned to Deer	Unique reads aligned to Bighorn Sheep		
1383	305	OxA-25234	725 ± 24	Moccasin Ankle Wrap Fragment	SC	2585396	71911	3608	4163	0.86	Female
1365	235			Moccasin	SC	2392792	42779	2675	2771	0.81	Female
1416	1677	OxA-30544	818 ± 26	Moccasin	SC	3494342	563834	31144	36794	0.83	Female
SC17.SBS.021	1244	OxA-28320	742 ± 23	Moccasin	SC	1360746	10406	5478	4560	0.83	Female
1379	1290	OxA-30782	804 ± 28	Moccasin	SC	2683825	24891	1539	1712	0.85	Female
1384	945	OxA-28316	708 ± 24	Moccasin	SC	2627479	55923	4886	5670	0.86	Female
1412	1340			Moccasin	SC	3440009	48708	2901	3338	0.86	Female
1374	1408	OxA-30701	783 ± 23	Moccasin	SC	2758066	19381	1624	1522	0.88	Female
SC17.SBS.028	1145			Moccasin	MK	8181119	570183	262485	270789	0.88	Female
1370	291 / 42BO1			Moccasin	SC	2707406	15938	1047	1107	0.89	Female
1401	799	OxA-26097	741 ± 23	Moccasin	SC	3699976	70199	4645	5609	0.91	Female
SC17.SBS.002	1120	OxA-28314	751 ± 24	Hide	MK	1219721	225945	96784	107489	0.92	Female
1400	131			Moccasin	SC	3318920	16128	1419	1415	0.94	Female
SC17.SBS.026	1505	OxA-30542	886 ± 27	Moccasin	MK	153966	3747	1883	2041	0.94	Female
1369	1433			Moccasin	SC	2079796	18707	1565	1453	0.95	Female
1371	117			Moccasin	SC	2449758	247092	18292	22785	0.95	Female
1398	126			Moccasin	SC	2207673	22420	2242	2340	0.97	Female
1397	173	OxA-25183	706 ± 25	Moccasin	SC	2276168	14442	1555	1507	1.09	Female
1385	303			Moccasin	SC	2827506	18228	3944	4452	0.76	Female
1409	1676	OxA-30705	841 ± 23	Bison Robe Fragment	SC	6275013	252894	24697	27072	0.78	Female
1376	1675	OxA-30703	799 ± 23	Bison Robe Fragment	SC	3106197	28307	1595	1860	0.52	Male
1382	1596	OxA-30702	777 ± 23	Moccasin	SC	364820	7795	1025	1724	0.46	Male
1414	1223	OxA-28319	703 ± 23	Moccasin	SC	3453590	136378	8243	10270	0.61	Male
1366	801	OxA-26098	777 ± 22	Moccasin	SC	396709	2791	1722	1443	NA	NA
1367	225			Moccasin	SC	362272	1334	926	624	NA	NA
1368	305			Moccasin	SC	366720	684	1148	505	NA	NA
1372	263			Moccasin	SC	415957	501	443	190	NA	NA
1373	1318			Hide	SC	393194	175	372	82	NA	NA
1375	1568			Moccasin	SC	291637	589	326	249	NA	NA
1377	1117			Moccasin	SC	320699	809	438	367	NA	NA
1378	291			Moccasin	SC	386328	1628	817	788	NA	NA
1380	116	OxA-25180	730 ± 26	Moccasin	SC	319580	1871	1166	966	NA	NA
1381	168			Moccasin	SC	416765	1071	1383	1310	NA	NA
1399	130			Moccasin	SC	429686	711	560	350	NA	NA
1402	615			Moccasin	SC	483442	2165	1033	925	NA	NA
1403	969	OxA-28317	752 ± 23	Moccasin	SC	479421	608	550	319	NA	NA
1410	568			Moccasin	SC	429584	135	275	71	NA	NA
1413	1431			Moccasin	SC	258429	840	568	379	NA	NA

Table S3.2. Table of metadata and Rx values for eight reference bison.

Reference Number	Sex	Reads subsampled	Average Rx ratio (across ten subsamples)	Assigned sex by Rx ratio	Source
100490	Male	3747	0.52	Male	Published with this study
100491	Male	3747	0.51	Male	Published with this study
SRS7735511	Male	3747	0.52	Male	Oppenheimer et al., 2021
SRS1620843	Male	3747	0.46	Male	Heaton et al., 2016
SRR12514558	Female	3747	0.99	Female	Yang et al., 2020
SRR12514559	Female	3747	0.89	Female	Yang et al., 2020
SRR12514560	Female	3747	1.06	Female	Yang et al., 2020
SRR6448737	Female	3747	1.08	Female	Wu et al., 2018

Supplement 3.1. Further background on the Promontory Caves

Steward (1937) recorded 12 Promontory caves, undertaking significant excavations in Caves 1 and 2 in 1930-31. He excavated a large area of the most habitable portion of Cave 1, yielding large collections held at the Natural History Museum of Utah; these included stone tools, ceramics, abundant faunal remains, and hundreds of perishable items such a moccasins, mittens, matting, cordage, and gaming pieces. Ives and Janetski undertook additional sondages in Caves 1 and 2 between 2011 and 2014 (Ives et al.2014; Ives 2020). Cave 1 is the largest of the caves and has produced by far the largest assemblage of early Promontory Phase artifacts (Steward's Promontory Culture). These materials come from a narrow thirteenth century interval: 95 AMS dates have been Bayesian modelled indicating an occupation range from AD 1248-1290 (Ives et al. 2014; Ives and Janetski in press). Space per person needs and artifact discard formulae suggest that roughly 25-40 persons likely inhabited the cave, at times interacting with late Fremont populations who had reverted from maize horticulture to securing wild foods themselves (Hallson 2017; Lakevold 2017; Yanicki and Ives 2015; Yanicki 2019). The cave population was highly mobile, with a variety of indicators (such as ceramic and obsidian source studies) suggesting that they circulated seasonally in northern Utah and southern Idaho (Ives 2020; Yanicki 2019). Seasonality indicators range from deer skulls with cast antlers (winter) to abundant bulrush seeds associated with mat making (later summer); from time to time, the Promontory cave inhabitants must have also made extended stays in Caves 1 and 2.

While the existing early Promontory Phase collections from both episodes of work are imposing to view, it is important to realize that significant midden deposits nevertheless remain in Cave 1, which had also been looted prior to Steward's work. The total amount of material culture that once existed or is still present must be

significantly larger, to give a better approximation of the scale of site occupation coupled with excellent preservation conditions.

The volume of the original Cave 1 deposits can be calculated by Geographic Information System means of a triangulated irregular network (TIN), as Hallson (2017) has done. Volumetric densities from our more recent excavations can be used to project ranges for the total number of artifacts that still are or were present. Modal values for these calculations suggest roughly 1.8 million fragmentary and whole faunal remains were originally present (extrapolated from the >30,000 fragmentary and whole faunal remains recovered in our 2011-2014 testing), along with about 2400 moccasins. Although these numbers seem staggering, they are predictable when artifact accumulation and discard formulae are applied for the one to two human generation occupation span, as Hallson (2017) showed.

While bison materials are occasionally found in contemporary late Fremont sites, they are limited in quantity, in line with general perceptions that the Great Basin has not been a prime bison habitat. In literature and collection surveys for the Great Basin, bison were somewhat more numerous just prior to and during the thirteenth century, they became scarce again after AD 1300. (Grayson 2006; Lupo and Schmitt 1997). Although our zooarchaeological analyses remain ongoing, it is clear from our volumetric analyses that the Minimum Number of Individual (MNI) values for the entire Cave 1 deposits will be large for both bison and other large game species including antelope and deer. A logical question arising from these collections and our projections would be how did the Promontory cave occupants secure so many bison and other large game animals? The presence of bison skulls and lower limbs with hooves, along with the sheer volume of faunal debris imply that a kill locus must be nearby. Movement along the shoreline of Great Salt Lake is difficult, and movement down the centre of the Promontory range would be virtually impossible. Along the west side of Promontory Point, however, there is an elevated trail system slightly

inland, at higher elevation. Trails both north and south converge in a relatively narrow saddle or pass just above Promontory Caves 1 and 2. The sharply convex topography of the saddle apex, hidden lookouts allowing visibility for great distances both north and south, the presence of boulders providing cover, and a possible hunting blind could all readily have been exploited by skilled hunters, as the Promontory cave occupants most certainly were. Surface collections from the saddle area have yielded projectile points, pieces esquillées, drill fragments and other artifacts one would expect for hunting or related activities hunters would undertake while awaiting game in hunting stand settings. Altogether, the Promontory record is consistent with a relatively small, intrusive population that engaged in a brief thirteenth century period of highly successful large game hunting, particularly of bison, in conjunction with a sophisticated hide processing and sewing tradition especially important for the replacement of numerous worn out moccasins.

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Supplement 3.2. Bison demographics background

Here, we estimated demographics of two surveyed bison populations to generate estimates of the male and female proportions of cow-calf herds. We estimate demographics from the Henry Mountains herd from data published by Van Vuren and Bray (1986). The second population we estimate demographics from is the Elk Island National Park herd which was surveyed by co- authors Wes Olson and Rob Found (data included in additional supplements).

Henry Mountains herd:

We estimated cow-calf herd proportions from the data presented in Van Vuren and Bray (1986). Bison herds in the Henry Mountains were calculated to include 23% bulls, 40% cows, 17% yearling, and 21% calves (Van Vuren and Bray, 1986). Disregarding bulls, which are known to be present in cow-calf herds at times, cow-calf herd proportions would have been 51.5% cows, 21.5% yearlings, and 27% calves. Van Vuren and Bray (1986) observed 47% of yearlings were male, meaning 10% of the cow calf herd would be male yearlings. Assuming an equal sex ratio of calfs, we expect a cow-calf herd to include 13.5% male calfs. With these calculations, we estimate a minimum 23.5% males in a cow-calf herd. This estimation is likely an underestimate as bulls are known to be present in cow-calf herds outside of rut season (Olson, 2005) and calf ratios often favor males. Additionally, hunting of only male bison is licensed for this reason, meaning male proportions in the cow-calf herds of an unmanaged population would be higher.

Elk Island National Park herd:

We estimated cow-calf her demographics from Elk Island National Park bison herds in Alberta's Aspen Parkland ecotone from 2002-2004 during the post rut season (September to December) (data in Supplement 3). Bison populations were calculated with 21% calves (bison sex could not be consistantly determined), 12% yearlings of near equal sex ratio, 25% bulls, and 42% cows (Supplement 3). Assuming no bulls (mature or immature) in the cow-calf herd and parity in calf sex ratio (Supplement 4), the Elk Island cow-calf herds would have consisted of 28% calves (14% male adn

14% female), 16% yearlings (here including bison age 1-2, 8% male and 8% female), and 56% cows. Therefore, we estimated the total male proportion at a minimum of 22%. In addition to the known presence of bulls in a cow-calf herd, presence of 3-4 year old male bison in a cow-calf herd (which were not accounted for here), and a slight bias towards male offspring (Supplement 4), we expect the male proportion of bison in a cow calf herd to be higher than the estimated 22%.

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Supplement 3.3: Elk Island National Park bison post-rut (September-December) herd demographics gathered by Wes Olson 2002-2004

DATE	CALVES					COWS					BULLS										
	B0	C0			C1	C2	C3	C4			B1	B2	B3	B4	B5		Total	Proportion	Proportion	Proportion	Proportion	Proportion
D/M/YR	Male	Female	Unknown	Total	yearling	Immature	Adult	Aged	Total		yearling	Immature	Adult	Breeding	Aged	Total	in	calf	F yearling	M yearling	Bull	Cow
						2 year old	3 to 15	15 plus				2 year old	3 to 7 yrs	8 to 15 yrs	15 +	Males	Group					
2-Sep-02	2	2		4			5		5		1					1	10	0.40	0.00	0.10	0.00	0.50
2-Sep-02	4	2		6			9	1	10		2			1		3	19	0.32	0.00	0.11	0.05	0.53
2-Sep-02		4		4	2		6		8		3				1	4	16	0.25	0.13	0.19	0.06	0.38
9-Sep-02	2	5	1	8	1	2	19	1	23			3	1	3		7	38	0.21	0.08	0.08	0.11	0.53
9-Sep-02				0				1	1							0	1	0.00	0.00	0.00	0.00	1.00
11-Oct-02	8	11	12	31	5	2	34	1	42		2	3	1		1	7	80	0.39	0.09	0.06	0.03	0.44
13-Oct-02	3	12		15	2	1	18	5	26		3	4	1	1		9	50	0.30	0.06	0.14	0.04	0.46
13-Oct-02				0					0					2		2	2	0.00	0.00	0.00	1.00	0.00
13-Oct-02				0					0					3	2	5	5	0.00	0.00	0.00	1.00	0.00
13-Oct-02				0					0						2	2	2	0.00	0.00	0.00	1.00	0.00
13-Oct-02				0					0						2	2	2	0.00	0.00	0.00	1.00	0.00
16-Oct-02	1		1	2			2	1	3				1		1	2	7	0.29	0.00	0.00	0.29	0.43
16-Oct-02	7	6	2	15	2		19	4	25		2	2	1		1	6	46	0.33	0.04	0.09	0.04	0.50
16-Oct-02	14	15	1	30	6	5	42	6	59		4	4	2	3	3	16	105	0.29	0.10	0.08	0.08	0.46
4-Nov-02			13	13		1	21	4	26		1	3	2		2	8	47	0.28	0.02	0.09	0.09	0.53
4-Nov-02			5	5	1		6	1	8		1	2			1	4	17	0.29	0.06	0.18	0.06	0.41
10-Nov-02			13	13		1	22	4	27					1	1	2	42	0.31	0.02	0.00	0.05	0.62
10-Nov-02			4	4			7		7		1	2		1		4	15	0.27	0.00	0.20	0.07	0.47
10-Nov-02			4	4	2		7		9					1		1	14	0.29	0.14	0.00	0.07	0.50
11-Nov-02			4	4			4		4			2		2		4	12	0.33	0.00	0.17	0.17	0.33
11-Nov-02				0					0				1	2	1	4	4	0.00	0.00	0.00	1.00	0.00
11-Nov-02				0				1	1				1	1		2	3	0.00	0.00	0.00	0.67	0.33
11-Nov-02				0					0						2	2	2	0.00	0.00	0.00	1.00	0.00
11-Nov-02			31	31	3	2	43	3	51			5	1			6	88	0.35	0.06	0.06	0.01	0.52
25-Nov-02			5	5		2	6	1	9			1				1	15	0.33	0.13	0.07	0.00	0.47
25-Nov-02			3	3			6		6							0	9	0.33	0.00	0.00	0.00	0.67
25-Nov-02			12	12	2		21		23							0	35	0.34	0.06	0.00	0.00	0.60
25-Nov-02				0					0					2	2	4	4	0.00	0.00	0.00	1.00	0.00
25-Nov-02				0			1	4	5							0	5	0.00	0.00	0.00	0.00	1.00
26-Nov-02			13	13	4	1	15	4	24		4	5	1	3		13	50	0.26	0.10	0.18	0.08	0.38
27-Nov-02			5	5	1	1	8		10							0	15	0.33	0.13	0.00	0.00	0.53
13-Sep-03	1	1		2	1	4	2	1	8							0	10	0.20	0.50	0.00	0.00	0.30
26-Sep-04			22	22		4	34	7	45	65%	4	4	5	1		14	81	0.27	0.05	0.10	0.07	0.51
26-Sep-04			28	28	3	9	35	11	58	80%	4	14	8	3		29	115	0.24	0.10	0.16	0.10	0.40
30-Nov-04				0			8	4	12	0%		2	1			3	15	0.00	0.00	0.13	0.07	0.80
30-Nov-04			4	4		3	5	3	11	80%		2	1			3	18	0.22	0.17	0.11	0.06	0.44
30-Nov-04			6	6		1	7	1	9	86%		1				1	16	0.38	0.06	0.06	0.00	0.50
17-Dec-04			79	79	13	25	103	22	163	77%	4	29	28	17	3	81	323	0.24	0.12	0.10	0.15	0.39
AVERAGE																		0.21	0.06	0.06	0.25	0.42

Supplement 3.4: Elk Island National Park Bison calf counts gathered from 1963-2019 by Wes Olson and Rob Found

Recruitment Year	Female	Male	Total Calves	Female:Male Ratio
1963	43	79	122	0.35
1964	20	11	31	0.65
1965	10	9	19	0.53
1966	42	43	85	0.49
1967	53	70	123	0.43
1969	13	14	27	0.48
1980	8	7	15	0.53
1981	43	31	74	0.58
1982	51	62	113	0.45
1984	33	41	74	0.45
1986	57	47	104	0.55
1988	55	50	105	0.52
1990	69	68	137	0.50
1992	65	80	145	0.45
1993	119	120	239	0.50
1994	107	114	221	0.48
1995	80	101	181	0.44
1996	40	88	128	0.31
1997	38	29	67	0.57

1998	**61**	**69**	**131**	**0.47**
1999	**66**	**69**	**135**	**0.49**
2000	**62**	**49**	**111**	**0.56**
2001	**43**	**51**	**94**	**0.46**
2003	**48**	**61**	**109**	**0.44**
2005	**56**	**65**	**121**	**0.46**
2007	**36**	**1**	**37**	**0.97**
2009	**45**	**24**	**69**	**0.65**
2011	**34**	**36**	**70**	**0.49**
2013	**77**	**92**	**169**	**0.46**
2015	**40**	**50**	**90**	**0.44**
2016	**29**	**24**	**53**	**0.55**
2018	**32**	**38**	**70**	**0.46**
2019	**40**	**36**	**76**	**0.53**
Total	***1616***	***1731***	***3348***	***0.48***

Supplement 3.5. Methods for data generation for two reference male bison

We generated shotgun data for two bison bulls (NAGP 14568/LIB100490,NAGP 5852/LIB100491) for comparison to archaeological samples. We obtained semen samples from the ARS National Animal Germplasm Repository for the two male bison. Semen was collected under Colorado State University IACUC protocol 17-7117A (Dr. J. Barfield).

Genomic DNA was extracted from the semen samples using a standard phenol:chloroform method as described in Heaton et al. (2001). DNA was then sheared using sonication on a Covaris S220 instrument (Covaris Inc., Woburn, MA) to generate fragment sizes less than 800 base pairs in length. We constructed indexed libraries from the sheared DNA using the Tru-Seq PCR-Free Kit (Illumina Inc., San Diego CA) and sequenced these libraries on an Illumina NextSeq500 instrument using a 2x150 cycle paired end kit following the approach described in Oppenheimer et al. (2021).

Heaton, M.P., Grosse,W.M., Kappes, S.M., Keele, J.W., Chitko-McKown, C.G., Cundiff, L.V., Braun, A., Little, D.P., Laegreid, W.W. (2001). Estimation of DNA sequence diversity in bovine cytokine genes. Mamm Genome 12(1):32-7.