DEVELOPMENT OF FECAL DNA SAMPLING METHODS TO ASSESS GENETIC POPULATION STRUCTURE OF GREATER YELLOWSTONE BISON

By

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Development of fecal DNA sampling methods to assess genetic population structure of Greater Yellowstone bison

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The bison (*Bison bison*) of Yellowstone National Park (YNP) and Grand Teton National Park (GTNP) represent two of only three remaining populations of plains bison that have no evidence of hybridization with cattle. Therefore, these bison are an important source for ecological and genetic restoration of wild bison. Little is known regarding genetic population structure and gene flow among the Greater Yellowstone Area (GYA) bison herds. I evaluated the feasibility of fecal DNA sampling for genetic analyses of wild bison populations. I used matched blood and fecal samples from eight radio-collared bison from Hayden Valley breeding group (YNP), and multiplex polymerase chain reaction (PCR) of four microsatellite loci to assess amplification success and genotyping error rates. The amplification success rate was 92% and the genotyping error rate was 12% on average across all individuals, and loci. Exclusion of two poor quality samples from data analyses increased amplification success to 97%, and reduced the genotyping error rate to 4%. I PCR amplified a 470 bp mitochondrial DNA (mtDNA) fragment for sequencing, and successfully identified haplotypes for120 individuals. The error rate for mtDNA sequencing was 0.0005 nucleotide mis-incorporations across all samples. Sequencing and RFLP analysis of mtDNA control region from 179 fecal samples collected over two consecutive seasons was conducted to evaluate population structure among YNP breeding groups, and between GTNP and YNP bison populations. I found significant genetic distinction between YNP and GTNP bison populations ($F_{ST} = 0.191$, $p < 0.001$). The differences in haplotype frequencies between Hayden Valley and Lamar Valley breeding groups were highly significant ($F_{ST} = 0.367$, $p < 0.001$), and nearly two times greater than between GTNP and YNP thus providing evidence for at least two genetically distinct breeding groups within YNP. Differences between breeding groups remained significant even though haplotype frequencies were different between years within Hayden Valley ($F_{ST} = 0.054$, $p < 0.05$). The techniques and protocols developed have allowed high amplification success, low genotyping and sequencing error rates. This study demonstrated that non-invasive fecal DNA sampling is feasible for bison, and detected fine-scale population genetic structure in among GYA bison, suggesting female philopatry.

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Dedication

The work presented within the body of this thesis is dedicated to all of those who have subsisted in respectful co-existence with bison in the past, and to those who dream of a time when bison thundered across the plains, and work toward their restoration. Mostly, I would like to dedicate this thesis to the tribal elders of the North American First Nations, who guide our path to the future through teaching us about our past. One such elder who has played a major role in my life, is my adopted Mom and spiritual mentor, Dr. Henrietta Mann, who inspires me to be the best scientist and human being I can possibly be. She has taught me to be generous, respectful, strong, how to feel the earth under my feet, and stretch my hands to the sky. I would also like to dedicate this thesis to my beautiful daughters, Jasmine, Juanita, and Jade for their love and patient support. Finally I offer honor, respect and gratitude to the bison of Greater Yellowstone for their generous contributions to this study.

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Preface

Dale Lott grew up on the National Bison Range, and spent most of his adult life studying bison behavior and ecology. This work culminated in a book titled, “American Bison: A Natural History”. Chapter five of this publication, amusingly titled, “Digestion: Grass to Gas and Chips”, discusses the remarkable digestive system of bison. At the end of this chapter he touts the usefulness of fecal studies in wildlife, and for bison in particular; “In their passage chips also pick up bison cells that contain the individual’s complete genome. It is possible that they could reveal not only the individual’s identity but perhaps the identity of its parents as well. So science will just keep chipping away at the secrets in the belly of the beast….but few other ways are as humane and efficient as chip analysis. No need to subdue the buffalo with a tranquilizing darts–and no worries that hormone levels in the blood sample reflect short-term peaks or bottoms caused by the trauma of the sampling. Little wonder then, that when the chips are down, the biologist’s spirits are up. The investigator that at first seems a figure of fun, a dedicated pooper-scooper, is really the very model of a modern-day mammalogist.”

Dale was right! The non-invasive fecal DNA sampling protocols I have developed for population genetic studies of free-ranging bison, has just begun to reveal “the secrets in the belly of the beast”. I have become the dedicated “pooper-scooper”, and hope to continue the use of non-invasive fecal sampling to learn as much as I can about the wild bison of Greater Yellowstone. And, who knows how much we will continue to learn about these amazing animals through just sampling of their feces? Hopefully, we will gain the information and insights we need in order to conserve them for future generations.

Chapter 1.

Introduction

Conservation of wild bison (*Bison bison*) is crucial to conservation of North American Tribal cultures and the biodiversity of the plains ecosystem. Bison are an ecological and cultural keystone species (McHugh 1972; Erdoes and Ortiz 1984; Knapp *et al*. 1999). Concern for the persistence of wild bison has increased since conservation status reviews revealed that they are ecologically extinct from over 90% of their former habitats. Of the estimated >450,000 bison in the United States (U.S.), 95% of them reside on private ranches where they have been subjected to hybridization with cattle and domestication (Boyd 2003). In addition, at least seven of ten federal bison herds show evidence of hybridization (Halbert and Derr 2007).

Prior to Euro-American settlement, the bison populations that roamed North America were reduced from an estimated 30 million (Seton 1937; Barsness 1985; Hornaday 2002) to less than 1000 by the late 1800's (Coder 1975; Hornaday 2002; Smits 1994). Shortly after this near extermination, the few hundred remaining bison were either captured and sent to zoos or adopted by private ranchers (Coder 1975; Hornaday 2003). The conservation efforts of the past have insured that the American bison is no longer at risk of demographic extinction. However, the loss of genetic diversity due to multiple bottlenecks, founder effects, hybridization, and domestication pose the risk of genomic extinction, and reduced evolutionary potential (Freese *et al*. 2007).

Following Boyd's (2003) recommendations, the Wildlife Conservation Society has initiated a comprehensive review of the status of bison in collaboration with the IUCN Bison Specialist Group. In 2005, the WCS began the revitalization of the

principles of the historic American Bison Society in an effort to bring about the ecological recovery of bison populations across their historic range (Freese *et al*. 2007).

Individual and collaborative efforts directed at ecological restoration of bison to their former ranges have been initiated by government agencies, private citizens, non-governmental organizations, and Native American tribes (Freese *et al*. 2007). They face two major issues in pursuing these efforts; locating large tracts of suitable habitat for bison, and identifying source populations with sufficient genetic diversity and no evidence of hybridization that can contribute to the conservation of the bison genome (Freese *et al*. 2007).

The Greater Yellowstone Area (GYA) bison herds represent an evolutionary legacy for conservation of bison because they are the only surviving naturally occurring wild bison population in the United States (Freese *et al*. 2007). Two large herds currently reside within Yellowstone and Grand Teton National Parks (YNP and GTNP). Previous genetic studies revealed that the GYA bison have a relatively high degree of genetic variation and no evidence of hybridization (Ward *et al*. 1999; Halbert and Derr 2007). The GYA bison may also represent an ecological microcosm of historic bison populations, thus requiring careful conservation efforts to ensure their persistence. Population genetic studies would provide crucial information for agencies charged with the management and conservation of these bison populations:

- National Park Service (NPS)
- U.S. Fish and Wildlife Service (USFWS)
- U.S. Forest Service (USFS)

- United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS)
- Montana Fish, Wildlife, and Parks (MFWP)
- Wyoming Game and Fish (WGF)
- Montana Department of Livestock (MDOL)

Previous genetic studies of the GYA bison relied upon opportunistic sampling animals captured on winter ranges outside of park boundaries. Over forty microsatellite loci were used to assess genetic diversity and evaluate whether more than one subpopulation exists within YNP bison (Halbert 2003). Population substructure analyses conducted in STRUCTURE (Pritchard *et al*. 2000) suggested the presence of three subpopulations (or breeding groups) for 166 bison exiting the park though the northern boundary, and one primary subpopulation for 63 bison exiting through West Yellowstone (Halbert 2003). Although, Halbert (2003) tested for genetic differentiation between YNP and GTNP bison herds (F_{ST} = 0.102), the rate and direction of gene flow between these parks was not thoroughly assessed. Because bison were not sampled at the geographic locations of breeding groups within the parks, population structure could not be confirmed, and the partitioning of genetic diversity among breeding groups remained unknown.

An expanded assessment of population structure and gene flow between GYA bison populations through non-invasive fecal sampling, microsatellite studies, and the addition of mtDNA sequencing could confirm whether population structure among breeding groups truly exists. YNP bison breeding groups are known to congregate in two primary geographic locations (Lamar Valley/ Mirror Plateau and Hayden Valley), and

within a possible third location (Mirror Plateau/Pelican Valley) during the summer rut season (Fig. 1-1) (Meagher *et al*. 2002; Geremia *et al*. 2005; Jones *et al*. 2006). Genetic variation and population substructure, for these breeding groups is unknown. This is, in large part, due to the difficulty obtaining blood or tissue samples from wild, free ranging bison during the summer. Also, the high risk of undue physiological stress from live capture, and potential mortality associated with immobilizing agents further hinder invasive approaches to sample collection. Sampling bison with traditional methods while they are congregated in large groups during the breeding season can be complicated, dangerous and stressful for the bison. Therefore, an alternative sampling approach was considered for acquiring genetic data from bison within the parks. Non-invasive fecal DNA sampling offers an efficient means of acquiring genetic data from GYA bison populations across their geographic range, with minimum risk to wild bison, and personnel collecting samples. This approach will also allow us to associate the genetic data with specific locations of breeding groups.

Most importantly, the non-invasive sampling approach is highly compatible with NPS wildlife management philosophy which mandates the following: "The Wildlife Management Program will achieve the NPS's primary mission by…minimizing human impacts on native animals, populations, communities, and ecosystems, and the processes that sustain them" (USDOI-NPS 2006). This study will also assist the NPS with meeting the Genetic Resource Management Principles "to protect the full range of genetic types (genotypes) of native plants and animal populations in the parks by perpetuating natural evolutionary processes and minimizing human interference with evolving genetic diversity…the Service will maintain the appropriate levels of natural genetic diversity",

through providing genetic information crucial for management of the GYA bison (USDOI-NPS 2006).

Non-invasive sampling for genetic studies

Non-invasive sampling has the advantage of extracting DNA from alternative sources of cellular material without physically handling wildlife. DNA has been extracted from hairs, feces, urine, feathers, snake skins, eggshells, and even skulls found in owl pellets (Bricker *et al.* 1996; Morin and Woodruff 1996; Taberlet and Fumagali 1996). Analysis of genetic material, particularly microsatellites, obtained through non-invasive sources collected in the field can be highly informative. Fecal DNA samples can provide individual identification, relatedness estimates, pedigree construction, sex identification, estimates of census and effective population size, mark-recapture data, and determine genetic variation within and between populations (Luikart and England 1999; Taberlet *et al*. 1999; Frantz *et al.* 2003; Wilson *et al.* 2003). Non-invasive fecal sampling has been used successfully in studies of ungulates, such as alpine ibex (*Capra ibex*), Corsican mouflon (*Ovis musimo*), and bighorn sheep (*Ovis canadensis*) (Maudet *et al.* 2004; Luikart *et al*. 2007). Fecal sampling in wild, free ranging ungulates, such as bison, can allow observation of groups or individuals at safe distances for social dominance ranking, sex, and age group prior to collection of feces, thus, providing an opportunity to collect additional data with respect to their ecology.

DNA amplification from fecal samples (as well as other non-invasive sample types) can present several challenges to successful genotyping that may potentially offer false results (Taberlet *et al.* 1999). Genotyping error rates can be high in certain types of

samples. However, repeated genotyping of heterozygous individuals can be used to accurately determine genotyping error rates (Taberlet *et al.* 1999; Maudet *et al.* 2004).

Seasonal differences in forage quality can potentially affect genotyping error rates from ungulate feces (Maudet *et al.* 2004). Maudet *et al*. (2004) found significant differences for DNA recovery in fecal samples collected in winter versus summer samples. DNA recovery rates were much lower, and genotyping error rates were much higher in seasons when forage quality is quite high, such as spring or summer. Recovery of DNA from bison fecal samples collected during the summer rut season could be challenging and error rates high. Bison typically form pie-shaped fecal depositions that may be more difficult to extract DNA from compared to pellet-form fecal depositions. However, non-invasive fecal DNA sampling has been successfully employed for genetic studies in African and Asian elephants, which form dung piles that are similar to, but larger than bison feces (Vidya *et al*. 2005; Archie *et al*. 2006).

Feasibility studies should be conducted on all novel non-invasive sample types, before approaching a large scale genetic analysis to insure that DNA analysis is reliable (Taberlet *et al.* 1999; Maudet *et al.* 2004). Non-invasive fecal DNA samples can produce high error rates due to allelic dropout and false alleles, therefore it is necessary to estimate the number of replicate genotypings needed for confidently assigning correct genotypes (Taberlet *et al.* 1999; Maudet *et al.* 2004). We therefore assessed amplification success and error rates for fecal DNA extracted from bison feces.

mtDNA and population structure

Our primary objective was to assess population structure among the GYA bison breeding groups. We chose to use the mtDNA control region for this study because it is

useful for evaluating genetic population structure and gene flow in mammal species that exhibit strong matrilineal social structure, similar to that found in bison. (Nyakaana *et al*. 2002; Archie *et al*. 2006; Parsons *et al*. 2006). Female philopatry to natal ranges can play an important role in determining the genetic structure of populations (Chesser 1991; Storz 1999). Philopatry has been documented in other ungulates such as roe deer (*Capreolus capreolus*), bushbuck (*Tragelaphus scriptus*), Svalbard reindeer (*Rangifer tarandus platyrhynchus*), desert bighorn sheep (*Ovis canadensis nelsoni*), and Soay sheep (*Ovis aries*) (Côté *et al*. 2002; Coltman *et al*. 2003; Epps *et al*. 2005; Nies *et al*. 2005; Wronski and Apio 2006). Bison have been observed to assemble in matrilineal groups which may include several generations of related individuals which travel together (McHugh 1972; Lott 2002; Halbert 2003). However, no studies have confirmed female philopatry free ranging, wild bison through the use of genetic markers such as mtDNA.

The mtDNA control region has been widely used for assessing population genetic structure because of its high variability. This region of the mtDNA has been primarily used for surveying bison herds for evidence of hybridization with cattle, and evaluating phylogenetic relationships between North American bison populations. There are 10 unique *B. bison* haplotypes and 12 variable sites that were previously detected within a 470 bp section within the mtDNA control region. Only five haplotypes were detected among U.S. bison herds. However, there were no studies using this region of the mtDNA to study population structure at a fine-scale in large ungulates.

Objectives

This thesis focuses on two primary objectives: 1) develop and evaluate methods for using fecal DNA samples for population genetic studies of wild bison; 2) assess

population structure within YNP breeding groups, and between YNP and GTNP bison populations using a 470 bp mtDNA control region sequence.

In chapter 2, I addressed the following objectives:

1) Determine whether non-invasive fecal samples can provide a reliable source of DNA for genetic studies in bison.
    a. Assess the success of PCR amplification and genotyping error rates for four microsatellite loci co-amplified in a single multiplex from bison fecal samples.
    b. Assess PCR amplification rates for and sequencing error rates for an mtDNA control region sequence from bison fecal DNA samples.

In chapter 3, my objectives were the following:

1) Evaluate population structure among the GYA bison using mtDNA amplified from bison feces.
    a. Test for genetic differences among the three breeding groups within YNP bison.
    b. Test for genetic differences between YNP and GTNP bison populations.

Summary and Synthesis

We had high amplification success (97%) and low error rates (4%), excluding two extremely poor quality samples, for genotyping using four microsatellite loci in a single multiplex PCR amplification. These results suggest that at least 25% more fecal samples should be collected than are necessary to insure adequate sample sizes. A single multiplex PCR using three to four microsatellite loci should be used to initially screen samples for quality before they are included in a full population genetics study. We

demonstrated that multiplex PCR can be efficiently employed for amplifying several loci at once from fecal DNA samples, screening for sample quality, and genotyping individual bison, while achieving low genotyping error rates. Microsatellite genotyping error rates can be further reduced by screening many loci and using only those with low error rates, (error rates varied among loci from 1% to 8%).

The per nucleotide error rate for mtDNA amplified from feces has not been previously published for any species. For our mtDNA study, we detected an error rate of 0.0005 nucleotide mis-incorporations across all samples. This is approximately five times higher than published error rates for high quality DNA sources with the Platinum *taq* we used in our PCR amplification (0.0001) (Tindall and Kunkel 1988). We successfully identified haplotypes for 120 of 127 individuals through mtDNA sequencing. Sequencing of the mtDNA control region, and RFLP analysis, revealed two haplotypes among 179 bison sampled from both parks over two consecutive rut seasons. The 470 bp sequence we amplified matched with the first 408 bp of haplotypes 6 and 8 previously defined by Ward *et al*. (1999).

The frequencies of haplotypes 6 and 8 among the GYA breeding groups revealed surprising strong evidence for genetic population structure. Significant differentiation was detected between YNP and GTNP bison populations ($F_{ST} = 0.191$, $p < 0.001$). However, the most intriguing result was the substantial genetic differences we found among breeding groups within YNP. The differentiation between the Lamar Valley and Hayden Valley was nearly twice that found between the parks ($F_{ST} = 0.367$, $p < 0.001$). And, the differences in haplotype frequencies between Mirror Plateau and Hayden Valley breeding groups were higher than between the parks as well ($F_{ST} = 0.231$, $p < 0.001$).

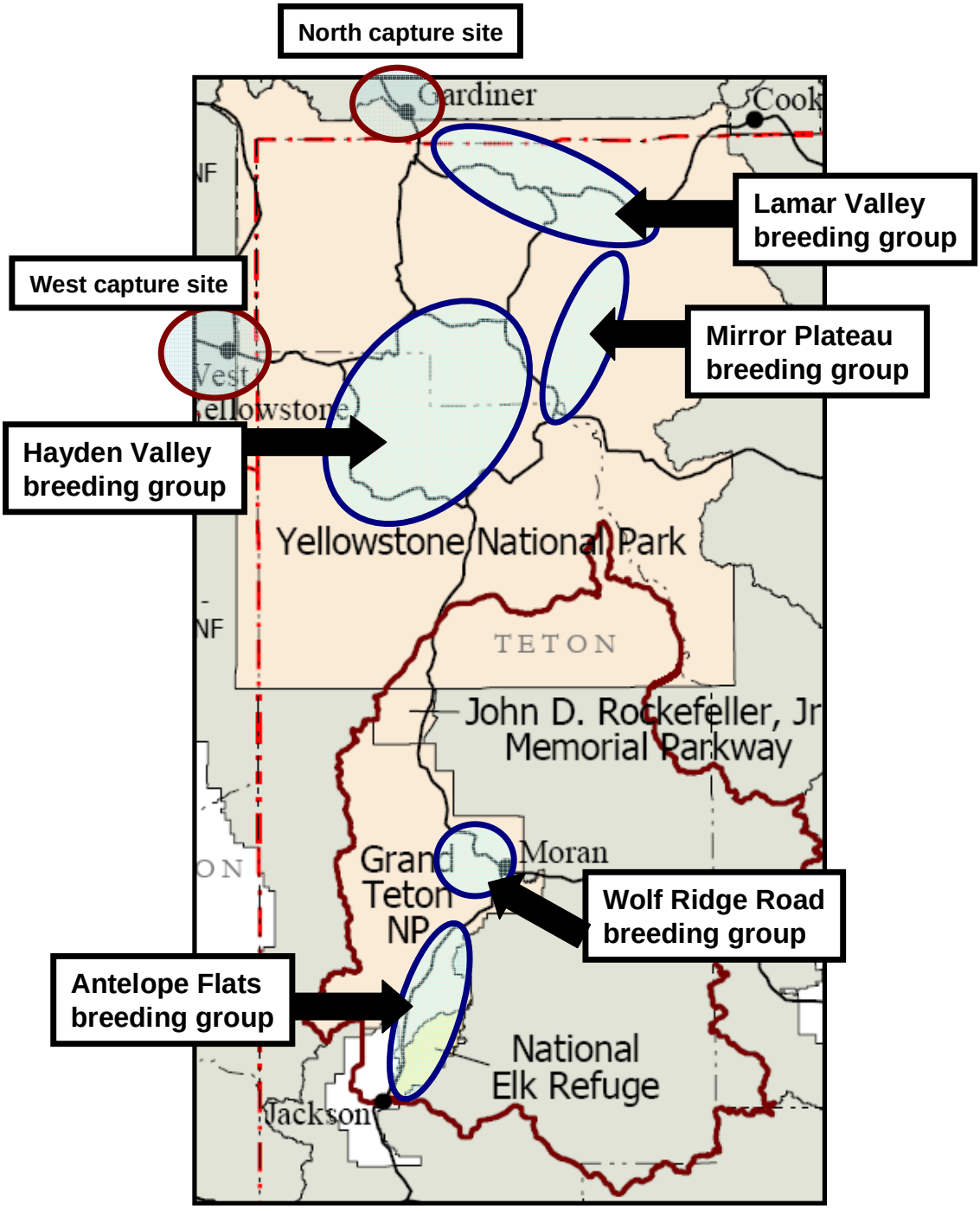
The highly differentiated population structure observed among the YNP breeding groups suggests female philopatry to natal ranges. However, this begs the question; why would bison exercise this behavior at such a fine scale? Is female philopatry a response to limited resources within the park, or have these modern bison carried on historical movement patterns of their ancestors? The long distance migrations of immense herds of bison across the plains were well documented prior to their near extirpation (McHugh 1972; Barsness 1985). The GYA bison exhibit fairly predictable, limited seasonal migratory movements that may represent a microcosm of bison migration patterns of the past. Future genetic studies using DNA extracted from historic bison skulls found within the GYA, and collaboration with archeologists may provide answers to some of these questions (Cannon 2001; Cannon 2007).

This history of bottlenecks and founder events, rather than natural bison ecology, may provide the most plausible explanation for the lower genetic differentiation we observed between the YNP and GTNP bison populations. Bison from YNP were used to found the GTNP bison population in 1948 (USDOI-NPS *et al*. 1996). In 1963, brucellosis was discovered in the GTNP bison population, which had been maintained between 15 to 30 animals, the herd was subsequently reduced to nine calves, and supplemented a year later with 12 adult bison from Theodore Roosevelt National Park (TRNP) (USDOI-NPS *et al*. 1996). The TRNP population was founded by bison from Ft. Niobrara National Wildlife Refuge (FNNWR), which has only haplotype 6 (Ward *et al*. 1999). Both TRNP and FNNWR have evidence of hybridization with cattle (Halbert and Derr 2007). Therefore, the rate and direction of gene flow between GTNP and YNP should be further

investigated using microsatellite loci, along with additional surveillance for hybridization.

The field collection, laboratory, and molecular genetic techniques and protocols developed through this study provide an approach that will allow an expanded assessment of genetic diversity and population structure for the GYA bison. The high amplification success and low error rates we achieved demonstrate that non-invasive fecal DNA sampling is a viable approach for conducting population genetic studies of free ranging bison. This study has provided evidence for population structure among the GYA breeding groups, advanced the field of non-invasive studies in wildlife, and opened the door to future studies that will provide crucial information for the genetic conservation and ecological restoration of wild bison.

Figure 1-1. Map of YNP showing locations of bison breeding groups as described by Meagher 1973, Taper and Meagher 2000, Meagher et al. 2002 (GYA map from USDOI-NPS 2007)



Chapter 2.

Non-invasive fecal DNA sampling and low genotyping error rates in the Greater Yellowstone Area bison.

Abstract: We assessed the feasibility of non-invasive fecal sampling in the Greater Yellowstone Area (GYA) bison using four microsatellite loci co-amplified in a multiplex PCR and a 470 bp mitochondrial DNA (mtDNA) control region sequence. Individual samples had significantly different error rates ($p < 0.01$). This was caused primarily by two samples that had relatively low amplification success and high error rates. Amplification success for all PCR amplifications was 92%, and increased to 97% after removing the two poor quality samples. Microsatellite genotyping error rates averaged 14%, and were reduced to 4% after removing these two samples. We detected no significant difference in error rates between DNA extractions. However, there were significantly different error rates among the four microsatellite loci ($p < 0.02$). We successfully identified mtDNA haplotypes for 94% of individuals sequenced. For mtDNA sequencing, the nucleotide mis-incorporation rate during PCR was 0.0005 per bp across all 127 individual samples (including 470 bp per sample). This study demonstrated the feasibility of using non-invasive fecal DNA sampling in wild bison. The techniques and protocols we developed will be useful for future population genetic studies that will provide answers to questions regarding the ecology and evolution of wild bison, and information crucial to their conservation.

INTRODUCTION

Wild bison are at risk of genomic deterioration as a result of population bottlenecks, hybridization, and domestication (Freese *et al*. 2007). The Greater Yellowstone Area (GYA) bison represent two of only three remaining populations in the U.S. without hybridization with cattle (Freese *et al*. 2007; Halbert and Derr 2007). Knowledge regarding the distribution of genetic diversity among bison would help managers to conserve the diversity remaining in bison. GYA bison breeding groups, which congregate during the summer breeding season, should be targeted for sampling to evaluate genetic population structure because this is when most gene flow between these groups occurs. Unfortunately, traditional invasive methods of obtaining blood or tissue samples by capturing free ranging bison are extremely difficult, costly, and dangerous for both the bison and research personnel. Traditional sampling entails a high risk of physiological stress and potential mortality associated with immobilizing agents.

We developed an alternative approach for acquiring DNA samples from wild bison in the field. Non-invasive fecal sampling has been used, with a high degree of success, in studies of ungulates, such as alpine ibex (*Capra ibex*) and Corsican mouflon (*Ovis musimon*), bighorn sheep (*Ovis canadensis*) (Maudet *et al.* 2004; Luikart *et al*. 2007). Benefits of fecal sampling in wild, free ranging animals, such as bison, are three fold. First, individuals may be observed at safe distances for social dominance ranking, sex, and age group prior to collection of feces, thus, providing an opportunity to collect additional data, without disturbing individuals of interest. Second, fecal samples provide information for infectious diseases (i.e. bacteria, viruses, and parasites, etc.), diet, or hormone status (e.g. pregnancy or stress) (Borjesson *et al*. 1996; Möstl and Palme 2002;

Waits and Paetkau 2005). Third, non-invasive fecal sampling can be used to address questions regarding population genetics of free ranging bison populations.

DNA amplification from fecal samples (as well as other non-invasive sample types) often yields high genotyping error rates. However, seasonal differences in forage quality affect genotyping error rates in ungulates. Therefore, genotyping error rates may be higher in spring and summer due to forage quality (Maudet *et al.* 2004). An assessment of genetic variation and substructure among GYA bison breeding groups requires sampling during summer months, when genotyping error rates might be highest due to forage quality (Maudet *et al.* 2004). Bison typically form pie-shaped fecal depositions that are likely more difficult to extract DNA from compared to fecal pellets (Manel *et al.* 2004). However, non-invasive fecal DNA sampling has been successfully employed for genetic studies in African and Asian elephants, which form dung piles larger than bison feces (Vidya *et al*. 2005; Archie *et al*. 2006).

The primary objective of this study was to develop and evaluate non-invasive fecal sampling techniques to facilitate genetic studies of free ranging bison. We quantified PCR amplification success, microsatellite genotyping error rates, and mtDNA sequencing error rates from bison fecal DNA samples.

METHODS

Sampling and extraction

Matched blood and fecal samples were collected from eight radio-collared female adult bison by the YNP Bison Ecology Program staff in September 2006. Each fecal

sample was extracted twice using the extraction process described below (Fig. 1). Each extraction was genotyped five times with four microsatellite loci.

Blood samples were applied to Whatman© FTA cards according to manufacturer's instructions. For DNA recovery for PCR, we used two separate punches from each FTA card that were purified according to manufacturers' instructions. Two independent extractions (purifications) per individual were genotyped with the four microsatellite loci.

Fecal samples for mtDNA studies were collected from 127 bison within YNP and GTNP, during the rut (July 2006). Most samples (~ 5 grams) were collected within 10-15 minutes of defecation and placed into vials containing ~20 mls of 95% ethanol (ETOH), and immediately placed into portable coolers until frozen at -20º C for up to 1 year prior to extraction in a laboratory designated for non-invasive samples.

All fecal extractions were carried out in a designated non-invasive laboratory. Each of the eight fecal samples was extracted twice for microsatellite analyses, and 127 fecal samples only extracted once mtDNA studies. Sterile filter tips, transfer pipettes, collection tubes, and microtubes were used. The QIAamp© Stool Mini Kit (QIAGEN) was used to extract genomic DNA from all fecal samples according to manufacturer's protocol with modifications as described below. Fecal samples were mixed well to insure relatively homogeneous distribution of cellular material prior to sub-sampling for extraction.

We transferred approximately 1.5 to 2 ml of fecal suspension into Eppendorf microtubes which were centrifuged at 16,100 rcf (relative centrifugal force) for 5 minutes. As much ethanol as possible was drawn off, leaving behind approximately 200 µl of feces required for the QIAamp© extraction protocol. The QIAamp protocol was then

carried out with one last modification. The final elution of DNA was repeated by passing the eluate through the column once more. This last step was modified in an attempt to recover as much DNA as possible. The tubes were centrifuged for one minute at 16,100 rcf. Negative extraction controls, containing sterile distilled water, were used along with each set of fecal extractions to monitor for possible cross contamination.

Microsatellite PCR and genotyping error rates

We screened a set of 36 microsatellite loci for use with fecal DNA samples, which were previously used in other genetic studies of bison (Halbert (2003). Only loci that were previously found to have a minimum heterozygosity of 0.50 in GYA bison were used to provide high power for future population genetic studies. We identified four dinucleotide loci with allele ranges less than 200 bp, BM 711(157-163 bp; NED), BM2113 (123-143 bp; NED), BMS1001 (101-109 bp; 6- FAM), and BMS2258 (123-144 bp; VIC) for use in a single multiplex PCR to estimate genotyping error rate (Appendix 1).

Multiplex PCR was carried out in 10 µl volumes containing 2 µl sterile HPLC H_2O, 5 µl QIAGEN Multiplex PCR Mix, 1 µl QIAGEN Q-Solution, 1 µl 10x primers (Table 1), and 1 µl template DNA. PCR was performed in an MJ Research PTC-200 Peltier Thermal Cycler using the following touchdown profile: 95º C for 5 min, followed by one cycle of 94º C for 30 s, 58º C for 1 min 30 s, and 72º C for 20 s. For the subsequent 19 cycles, all conditions remained the same except that the annealing temperature decreased by 0.5 degrees per cycle. This was followed by 26 cycles of 94º C for 30 s, 48º C for 1 min 30 s, and 72º C for 20 s. The profile concluded with a single extension of 72º C for 10 min.

Fragment analysis was carried out on an ABI 3130*xl* using the GS-600 LIZ size standard and GeneMapper v3.7 software was used to size fragments and call alleles for each locus. Peaks less than 50 relative fluorescent units were not scored. Two people independently scored all electropherograms for each locus. True genotypes were assumed to be those obtained from DNA amplified from high quality blood samples. Amplification success (AS) was calculated by the proportion of PCR amplifications that resulted in a scoreable genotype. Allelic dropout (AD) rate was inferred when a homozygous genotype was scored for a heterozygous individual known from analysis of the corresponding blood sample. Allelic dropout rate was computed as the proportion of all genotypes among loci and individuals with a dropout. False alleles (FA) were inferred when a different allele was observed in a replicate PCR compared to true genotype known from blood.

A generalized linear mixed model (GLMM) was used to test for differences in error rates among individual samples, loci, and between extractions per Steele (1996), and implemented using Gauss 7.0 Aptech Systems (http://www.aptech.com/). GLMM allows us to broaden the scope and inference of the individual samples used for evaluating genotyping error rate to the "population" of samples randomly collected in the field (Steele and Hogg 2003).

mtDNA PCR and error rate

We surveyed NCBI-GenBank and relevant literature to identify a short (< 500 bp) mtDNA sequence that could potentially be amplified from bison feces. Alignment of bison mtDNA D-loop sequences; AF083357 through AF083364 (Ward *et al*. 1999), CIC1 and CIC2 (Vogel *et al*. 2006) in MEGA 3.1 (Kumar *et al*. 2004), reveals 10 unique

B. bison haplotypes and twelve variable sites within a 408bp section of this region. Primers BISCR-16348F and BISCR-16990R were used to amplify the first 470 bp of the mtDNA control region (Vogel *et al*. 2006). These same primers were used by Shapiro *et al*. (2004) to amplify this same region of the D-loop from fossil *Bison bison* bones ranging in age from modern to >60 ka BP.

Primers BISCR-16348F 5'-CTACAGTCTCACCGTCAACCC-3' and BISCR-16990R 5'-GATGAGATGGCCCTGAAGAA-3' were used to amplify a 470 bp segment of the bison mtDNA control region (Shapiro *et al*. 2004; Vogel *et al*. 2006). PCR was carried out in 25 µl volumes containing; 8.95 µl sterile HPLC H_2O, 2.5 µl Invitrogen©10X PCR buffer, 1 µl dNTP's, 0.5 µl of each primer, 2.5 µl BSA (2ng/µl), 1.25 µl MgCl (50 mM), 0.3 µl Invitrogen© Platinum *Taq* Polymerase (5 units/µl), and 7.5 µl of template DNA. PCR was performed in an MJ Research PTC-200 Peltier Thermal Cycler using the following touchdown protocol: 94º C for 5 min, followed by one cycle of 94º C for 30 s, 60º C for 30 s, and 72º C for 30 s. For the subsequent 10 cycles, all conditions remained the same except that the annealing temperature decreased by 0.5 degrees per cycle. This was followed by 25 cycles of 94º C for 30 s, 55º C for 30 s, and 72º C for 30 s. The profile concluded with a single extension of 72º C for 5 min.

PCR products were purified using QIAquick© purification columns according to manufacturers' instructions with one exception; the final elution was carried out with 20 µl of Buffer EB instead of the recommended 30-50 µl to compensate for potential low quantity template DNA . The amount of purified post-PCR product was quantified by fluorometry prior to sequencing to insure that 5-10 ng/µl of amplified DNA was present in the sample. Sequencing was performed on the ABI 3100*xl* sequencer. Sequences were

visualized, assessed for quality, and edited with Chromas 2.31 (http://www.technelysium.com.au/chromas.html). MEGA 3.1 (Kumar *et al*. 2004) was used to align edited mtDNA sequences with known bison haplotypes. Comparison of sequence nucleotide variation at known variable sites determined the haplotype of each sample. PCR was repeated for sequences that matched a known haplotype at all known variable sites, but had nucleotide mis-incorporations at other sites, to rule out the existence of novel haplotypes. Repeated PCR amplification and sequencing was performed to resolve these ambiguities and determine the correct haplotype for those samples.

Ambiguous sequences that could not be scored primarily due to numerous overlapping nucleotide peaks were re-extracted, PCR amplified, and sequenced again to determine the correct haplotype. Samples that repeatedly yielded ambiguous sequences post re-extraction were not assigned haplotypes. The per nucleotide error rate for mtDNA amplified from feces has not been previously published. We estimated the *nucleotide mis-incorporation error rate* is the number of erroneous nucleotide mis-incorporations (that occurred during PCR) divided by the total number of nucleotides sequenced.

RESULTS

Microsatellite loci

Individual sample quality had a significant overall effect on microsatellite genotyping error rates ($p < 0.01$) (Table 1). Two samples contributed to the majority of genotyping errors. The combined errors from these two samples accounted for 34 of 72 (47%) allelic dropout errors and produced the one false allele. Removal of these two

individuals from the data set decreased the total overall genotyping error rate from 15% to 5% across all loci and samples, and increased amplification success from 92% to 97% (Table 1). There were no significant differences in error rates between extractions. This provided evidence that our extraction techniques are relatively consistent across all samples.

There were significant differences in error rates among loci ($p < 0.01$) (Table 1). Error rates varied among loci from 12% to 20%, and were reduced to 1% to 8% when YELL-024 and YELL-030 are excluded from the analysis. Even with the exclusion of the two poor quality samples from the GLMM, we still detected significant differences in error rates among loci ($p < 0.02$). We observed no tendency for larger alleles to have higher error rates. BM 711, which has the largest allele range (157-163), produced fewer errors than loci in the median range (BM2113; 123-143 and BMS2258; 123-144). Within loci, we found no association between larger allele size and error rates.

mtDNA and error rate

We obtained sequences from 120 samples (94%) that matched with the first 408 bp of two sequences previously identified as haplotypes 6 and 8 (GenBank accessions AF083362 and AF083364) by Ward *et al*. (1999). There are four base-pair differences between these two haplotypes, and three variable sites; two of which are single nucleotide polymorphisms (SNP's), and one insertion-deletion.

The remaining seven samples produced ambiguous sequences that were not scoreable due to multiple overlapping nucleotide peaks. The initial PCR for two of these samples yielded poor quality ambiguous sequences. Therefore PCR and sequencing was repeated. Two of these samples produced abbreviated or fragmented sequences that

aligned with less than two out of three diagnostic sites, after a second PCR amplification. The remaining five samples consistently produced sequences with multiple overlapping nucleotide peaks, which precluded alignment with any haplotype. Multiple attempts involving repeated extraction, PCR amplification, and sequencing failed to resolve this issue for those samples.

Ambiguous sequences that matched a haplotype at all three diagnostic sites, but had random nucleotide mis-incorporations at other sites, occurred in 4% of all samples. Haplotypes were not identified for these samples until repeated PCR and sequencing yielded a sequence that unambiguously aligned with a known haplotype without mis-incorporations at other sites within the sequence. The nucleotide errors were resolved for all of these samples on the second PCR. The per-nucleotide error rate was low for all of these samples, with the overall per-nucleotide error rate across all samples being 0.05%

DISCUSSION

We successfully extracted and amplified DNA from all eight matched fecal samples. However, because two samples had exceptionally high error rates, we recommend that future studies screen samples and exclude those that yield low amplification and high error rates. Thus, it is important to collect many extra fecal samples in the field to insure that there are sufficient high quality samples available for genetic study of interest. For bison, it may be necessary to collect at least 25% more fecal samples than what is needed to insure an adequate number are available for genetic analyses.

We successfully optimized a multiplex (4-locus) PCR. This is important because multiplexing is especially challenging for poor quality DNA samples, and not widely used in non-invasive studies, but can greatly reduced time and cost of analyses while consuming less DNA (which is limited in low quality non-invasive samples). A single multiplex PCR allows for rapid screening to identify fecal samples with sufficiently high quality or quantity of template DNA. We recommend that future non-invasive studies use multiplexes, which was facilitated here by the use of QIAGEN Multiplex PCR kit.

Only two other studies that employed noninvasive fecal DNA sampling for species that have non-pellet form feces reported higher amplification and lower error rates than we found in our study (Appendices 2-2 and 2-3). Fernando *et al*. (2003) reported amplification success of 97.5 to 100%, for six microsatellite loci, which were slightly higher than our overall AS rate (excluding poor quality samples). Our AD rate (4%) was nearly twice that reported by Fernando *et al*. (2%). Parsons (2001) reported a slightly higher AS rate (98.1%) than our study and a lower error rate (2%) for bottlenose dolphins (*Tursiops truncatus*). However, a noninvasive study of harbor seals (*Phoca vitulina*) reported very a low AS rate (60%) relative to our results (Reed *et al*.1997).

The AS rate (97%) we achieved was also much higher than that found in noninvasive studies of African and forest elephants which ranged from 60% to 72% (Appendix 2-1) (Eggert *et al*. 2003; Fernando *et al*. 2003; Buchan *et al*. 2005). The rate of AD for African elephants ranged from 15% to 25% across 12 loci, and an average of 15% AD rate was reported for 7 loci amplified from forest elephant feces (Appendix 2-2). Our AS rates were much higher, and AD error rates were lower those reported for terrestrial carnivore species such as wolves (*Canis lupus*), coyotes (*Canis latrans,*), black

bears (*Ursus americanus*), and brown bears (*Ursus arctos*) as well (Appendices 2-1 and 2-2) (Wasser *et al*. 1997; Kohn *et al*. 1999; Lucchini *et al*. 2002; Murphy *et al*. 2002). Noninvasive fecal DNA studies in primates reported the lowest AS and highest AD rates relative to our study results (Appendices 2-1 and 2-2) (Bradley *et al*.2000; Lathuillière *et al*. 2001; Morin *et al*. 2001).

We found no large effect of extractions to error rates. Therefore, only one extraction per fecal sample is necessary prior to screening for quality. It would be much more cost effective and efficient to perform one extraction, and screening for sample quality with a single multiplex PCR, before using a sample for complete genetic studies.

For mtDNA, we successfully amplified high quality sequences from 93% of fecal DNA extracts on the first attempt. Sequences that produced nucleotide mis-incorporations that did not match any previously described haplotypes were PCR amplified and sequenced at least once more. This resolved all nucleotide mis-incorporation errors. Seven apparently low quality samples yielded ambiguous sequences with multiple overlapping nucleotide peaks. Repeated PCR amplification and sequencing did not resolve this issue. However, our success rate for amplifying scoreable haplotypes was much higher than for scoreable microsatellite genotypes. The higher success is most likely due to the fact that mtDNA occurs in multiple copies within each cell. Therefore, mtDNA studies from fecal samples may require collection of only about 10% more samples in the field than necessary, as opposed to 25% more for microsatellite studies.

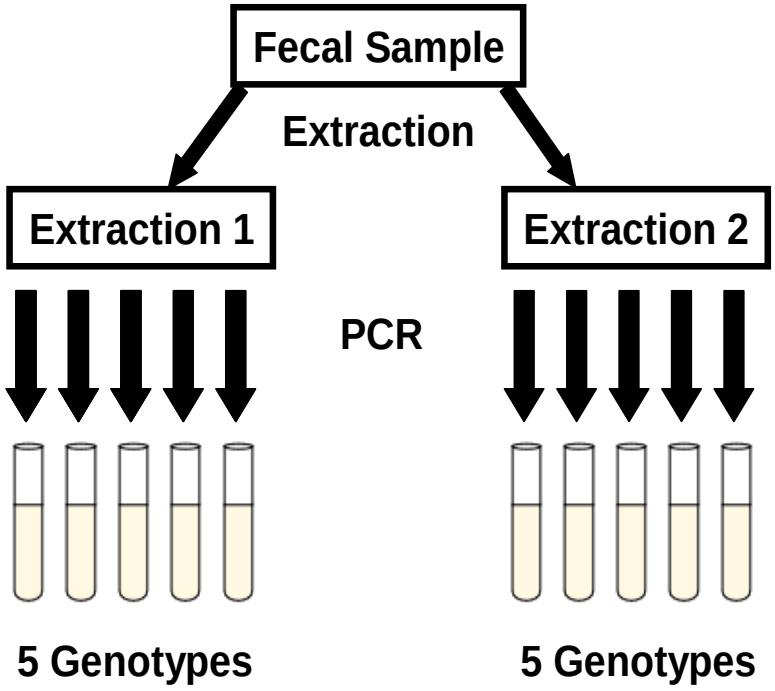
The per nucleotide error rate (0.0005) for fecal DNA generated by our study demonstrates that caution must exercised in identifying new haplotypes through sequencing. Our fecal DNA extracts produced higher than average nucleotide mis-

incorporation error rate (0.0005) for the platinum *taq* polymerase (0.0001) used in our PCR amplifications (Tindall and Kunkel 1988). Samples that do not match previously identified haplotypes should be sequenced in both directions at least twice before the haplotype can be confirmed. An initial subset of fecal DNA extractions should be sequenced in both directions to screen for potential haplotype diversity before proceeding with full mtDNA studies. In addition, initial sequencing in both directions will determine whether individual haplotype assignment can be confidently determined from sequencing in only one direction for cost effectiveness.

This study clearly demonstrates that non-invasive fecal DNA sampling is a feasible approach for conducting population genetic studies of wild bison. We achieved reasonably high amplification success and minimal error rates through strict adherence to systematically developed field and laboratory protocols. This will allow us to use non-invasive fecal DNA sampling in an efficient and cost effective manner. The results of this study further advance the field of non-invasive genetic sampling in wildlife while providing another example of the feasibility and usefulness of this approach.

Figures:

Figure 2-1. Flow diagram showing the two extractions per fecal sample, and five, multiplex PCR amplification (using four microsatellite loci) per extraction, and number of genotypings per individual radio-collared bison used to assess amplification and genotyping error rates.



Tables:

Table 2-1. Amplification success (AS), allelic dropout (AD) rates for all loci co-amplified in the multiplex PCR. Total and average error rates were first estimated across all genotypings (loci and individuals), and then excluding data from two poor quality individual samples, YELL-024 and YELL-030.

	Extraction 1		Extraction 2		Average per locus			
Locus	**AS**	**AD**	**AS**	**AD**	**AS**	**AD**	**Total error rates**	**Total errors w/o 024 & 030**
BM711	0.93	0.11	0.95	0.21	0.94	0.16	0.16	0.03
BM2113	0.93	0.24	0.90	0.06	0.91	0.15	0.15	0.04
BMS1001	0.95	0.18	0.98	0.05	0.96	0.12	0.12	0.01
BMS2258	0.90	0.22	0.85	0.18	0.88	0.20	0.20	0.08
Averages:	**0.93**	**0.19**	**0.92**	**0.12**	**0.92**	**0.16**	**0.14**	**0.04**
Averages excluding YELL-024 & YELL-030:	**0.97**	**0.21**	**0.97**	**0.04**	**0.97**	**0.12**		

Table 2-2. Amplification success (AS), allelic dropout (AD) rates for all individual samples. Averages were first estimated across all individuals, and then excluding data from the two poor quality samples; YELL-024 and YELL-030.

	Extraction 1		Extraction 2		Average per sample		Total
Sample ID	**AS**	**AD**	**AS**	**AD**	**AS**	**AD**	**error rates**
YELL-003	1.00	0.08	1.00	0.00	1.00	0.04	0.04
YELL-011	0.90	0.03	1.00	0.03	0.95	0.03	0.03
YELL-017	1.00	0.03	1.00	0.00	1.00	0.01	0.01
YELL-024	0.95	0.26	0.95	0.21	0.95	0.24	0.24
YELL-030	0.90	0.22	0.88	0.23	0.89	0.23	0.23
YELL-031	0.98	0.13	0.90	0.03	0.94	0.08	0.08
YELL-038	0.98	0.00	0.95	0.00	0.96	0.00	0.00
YELL-039	1.00	0.00	1.00	0.00	1.00	0.00	0.00
Averages:	**0.96**	**0.09**	**0.96**	**0.06**	**0.96**	**0.08**	**0.08**
Averages excluding YELL-024 & YELL-030:	**0.98**	**0.20**	**0.98**	**0.04**	**0.98**	**0.12**	**0.05**

Appendices

Appendix 2-1. Characteristics of microsatellite loci amplified in the multiplex PCR; primers, and chromosome location (Halbert 2003). Allele ranges, and number of alleles (N_A) found in the eight radio-collared bison used to assess amplification success and error rates for this study.

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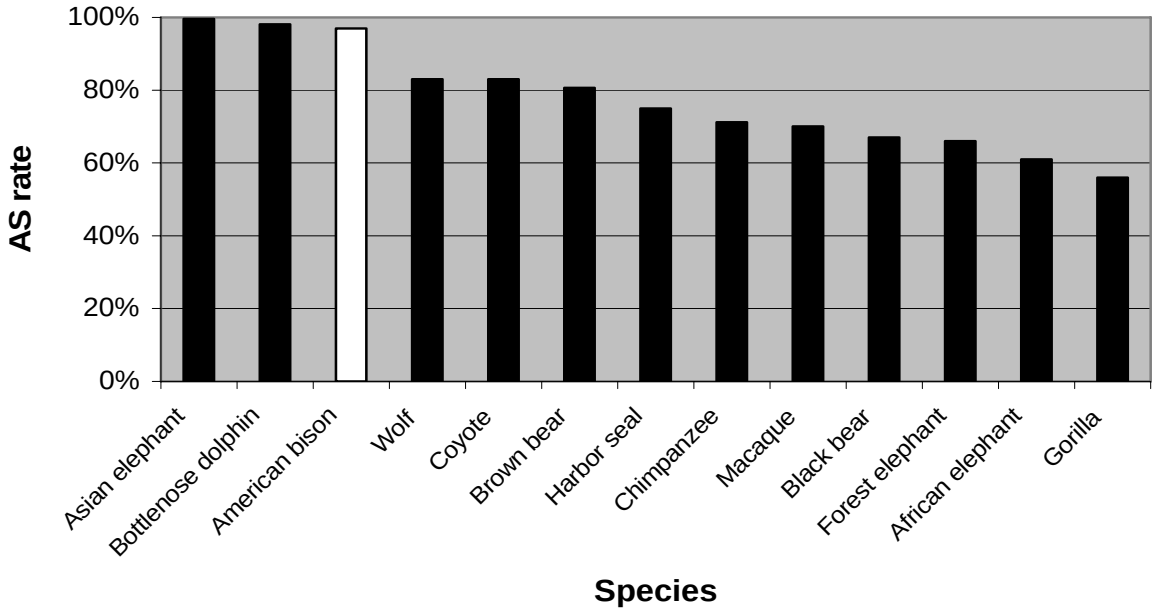
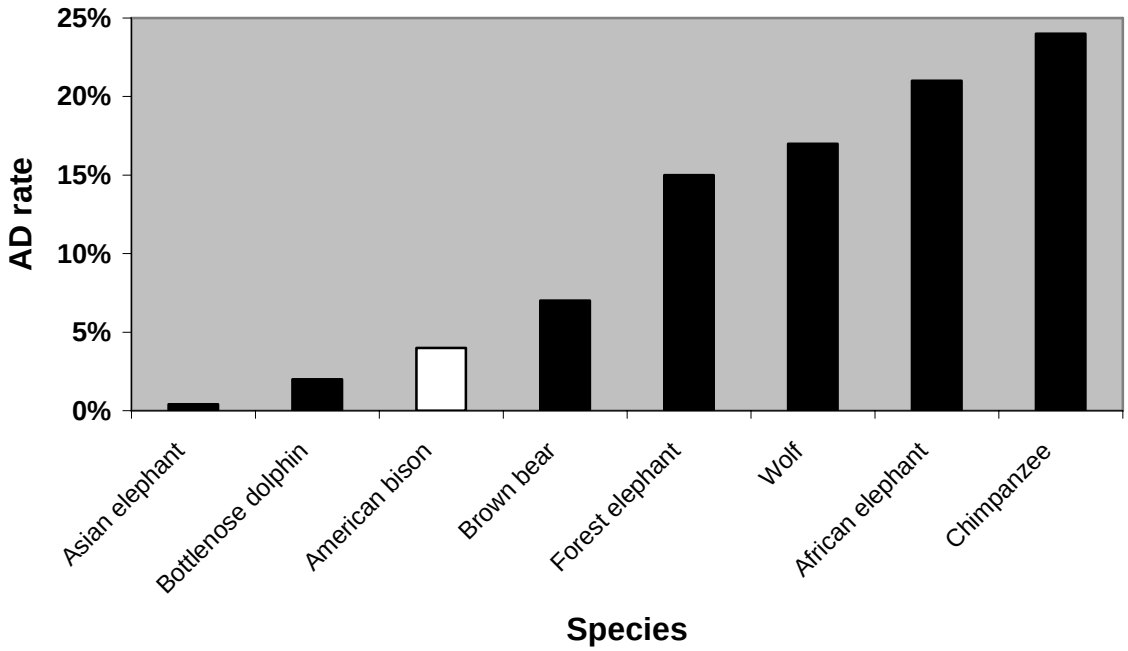
Locus	Primers	Allele Size Range	N_A	Chromosome
BM711	F 5'- CAGCATCAGCAACTAACATAGG -3' R 5'- TGGACCATGAGGGAAGTCTC -3'	157-163	3	8
BM2113	F 5'- GCTGCCTTCTACCAAATACCC -3' R 5'- CTTCCTGAGAGAAGCAACACC -3'	123-143	4	2
BMS1001	F 5'- GAGCCAATTCCTACAATTCTCTT -3' R 5'- AGACATGGCTGAAATGACTGA -3'	101-109	4	27
BMS2258	F 5'- CCAGCAGAAGAGAAAGATACTGA -3' R 5'- AGTGGTAGAACTTCCATCTCACA -3'	123-144	5	7

Appendix 2-2. Amplification rates (AS) reported in our study and for other species with non-pellet form feces (Reed *et al*. 1997; Wasser *et al*. 1997; Kohn *et al*. 1999; Bradley *et al*. 2000; Lathuillière *et al*. 2001; Morin *et al*. 2001; Parsons 2001; Lucchini *et al*. 2002; Murphy *et al*. 2002; Eggert *et al*. 2003; Fernando *et al*. 2003; Buchan *et al*. 2005).



Appendix 2-3. Allelic dropout (AD) rates reported in our study and for other species with non-pellet form feces (Morin *et al*. 2001; Parsons 2001: Lucchini *et al*. 2002; Murphy *et al*. 2002; Eggert *et al*. 2003; Fernando *et al*. 2003; Buchan *et al*. 2005).



Chapter 3.

Genetic Population Structure in the Greater Yellowstone Bison Revealed by Mitochondrial DNA Analyses

Abstract: The Greater Yellowstone Area (GYA) populations are crucial for conservation of wild bison (*Bison bison*). The GYA provides the only place in the United States where the ecology and population genetic structure of free ranging plains bison populations may be observed. There are three locations where breeding groups congregate within YNP during the rut season; Hayden Valley, Lamar Valley, and Mirror Plateau. Bison in Grand Teton National Park (GTNP) primarily congregate in Antelope Flats during the rut. Non-invasive sampling and mitochondrial DNA studies were conducted to assess population subdivision. We hypothesized that fidelity to breeding areas would result in genetic differences among GYA breeding groups. Non-invasive fecal samples were collected from bison within the geographic locations of breeding groups during two consecutive rut seasons. We used sequencing and restriction fragment length polymorphism (RFLP) analysis of a 470 bp segment of the bison mtDNA control region to assess population structure. We found substantial differentiation between YNP and GTNP populations (F_{ST} = 0.1912, $p < 0.001$). We also found substantial differences in haplotype frequencies between the Lamar Valley and Hayden Valley breeding groups (F_{ST} = 0.3667, $p < 0.001$). Within Hayden Valley haplotype frequencies were different between two consecutive years (F_{ST} = 0. 0536, $p < 0.05$). However, the genetic differences between the Lamar Valley and Hayden Valley were highly significant for both 2005 and 2006. The relatively

strong fine scale genetic differentiation among breeding groups within YNP suggests female philopatry to natal ranges.

INTRODUCTION

The most immediate genetic threats to bison conservation are population bottlenecks, hybridization with cattle, domestication, and anthropogenic selection (Freese *et al*. 2007). The Yellowstone bison is one of only two or three remaining non-hybridized source U.S. populations for bison reintroduction and restoration (Freese *et al*. 2007). Previous genetic studies, using microsatellite loci, revealed that the GYA bison have a relatively high degree of genetic variation and no evidence of hybridization (Ward *et al*. 1999; Halbert 2003; Halbert and Derr 2007). This previous study, which relied upon opportunistic sampling of bison on winter ranges outside park boundaries, suggested the presence of three subpopulations (or breeding groups) within YNP (Halbert 2003).

An expanded assessment of population structure and gene flow between GYA bison populations, using mtDNA and direct sampling of breeding groups, would further knowledge of the distribution of genetic variation in the parks. There are three locations where breeding groups congregate within YNP during the rut season; Hayden Valley, Lamar Valley, and Mirror Plateau (Meagher 1973; Taper and Meagher 2000; Meagher *et al*. 2002; Geremia *et al*. 2005; Jones *et al*. 2006). Bison in Grand Teton National Park (GTNP) primarily congregate in Antelope Flats during the rut (USDOI-NPS and USFWS 2007). However, a smaller group of bison are known to range around Wolf Ridge Road near Moran, Wyoming (S. Cain pers. comm., Supervisory Biologist, GTNP, 2006).

Female philopatry to natal ranges can play an important role in determining the genetic structure of populations (Chesser 1991; Storz 1999). Philopatry has been documented in other ungulates such as roe deer (*Capreolus capreolus*), bushbuck (*Tragelaphus scriptus*), Svalbard reindeer (*Rangifer tarandus platyrhynchus*), and Soay sheep (*Ovis aries*) (Côté *et al*. 2002; Coltman *et al*. 2003; Nies *et al*. 2005; Wronski and Apio 2006). Bison have been observed to assemble in matrilineal groups which may include several generations of related individuals which travel together (McHugh 1972; Lott 2002; Halbert 2003). Social structure, polygyny, and dominance hierarchies have been documented in bison as well (Lott 2002; Gates *et al*. 2005). However, no studies have confirmed female philopatry in free ranging, wild bison through the use of genetic markers such as mtDNA.

Mitochondrial DNA (mtDNA) is especially useful for genetic studies involving non-invasive fecal samples where a limited number of cells may be present in fecal samples. We used non-invasive fecal DNA sampling to minimize disturbance to bison activities, and improve our ability to collect a sample of DNA from a significantly large proportion of the respective breeding groups. Previous studies revealed 10 ***B. bison*** haplotypes and 12 variable sites within a 470 bp section of the bison mtDNA control region (Polziehn *et al*. 1995; Polziehn *et al*. 1996; Ward *et al*. 1999; Shapiro *et al*.2004; Vogel *et al*. 2006). The variability of the 470 bp sequence could prove useful for mtDNA studies in the GYA bison populations to assess population structure.

The primary objective of this study was to assess population structure among the GYA bison using mtDNA amplified from bison feces. We hypothesize that because

fidelity to breeding areas may be high, there should be genetic differences among bison breeding groups of the Yellowstone population, and between YNP and GTNP.

METHODS

Sample collection and storage

We collected fecal samples from bison within the geographic range of each breeding group during the 2005 and 2006 breeding seasons. We determined the relative age class of individuals sampled through field observations of horn length and width, body size, and condition (especially for older animals). Social dominance ranking was recorded for individuals who displayed obvious behavioral clues such as displacement of other bison from foraging patches or wallowing pits, false charges, challenges for mates, and leading groups of other bison. Most samples (~ 5 grams) were collected within 10-15 minutes of defecation and placed into vials containing approximately 20 ml of 95% ethanol, and placed into coolers for up to 8 hours before they were frozen. Fecal samples were stored frozen at -20º C for up to 1 year prior to extraction.

Extraction

All fecal extractions were carried out in a designated non-invasive laboratory. Sterile filter tips, transfer pipettes, and microtubes were used. The QIAamp© Stool Mini Kit (QIAGEN) was used to extract genomic DNA from all fecal samples according to manufacturer's protocol with modifications. Negative extraction and PCR controls were used to monitor for possible contamination.

PCR amplification

Primers BISCR-16348F 5'-CTACAGTCTCACCGTCAACCC-3' and BISCR-16990R 5'-GATGAGATGGCCCTGAAGAA-3' (Shapiro *et al*. 2004; Vogel *et al*. 2006) were used to amplify a 470 bp segment of the bison mtDNA control region. PCR was carried out in 25 µl volumes containing; 8.95 µl sterile HPLC H_2O, 2.5 µl Invitrogen©10X PCR buffer, 1 µl dNTP's, 0.5 µl of each primer, 2.5 µl BSA (2ng/µl), 1.25 µl MgCl (50 mM), 0.3 µl Invitrogen© Platinum *Taq* Polymerase (5 units/µl), and 7.5 µl of template DNA. Amplification was performed in an MJ Research PTC-200 Peltier Thermal Cycler using the following touchdown protocol: 94º C for 5 min, followed by one cycle of 94º C for 30 s, 60º C for 30 s, and 72º C for 30 s. For the subsequent 10 cycles, all conditions remained the same except that the annealing temperature decreased by 0.5 degrees per cycle. This was followed by 25 cycles of 94º C for 30 s, 55º C for 30 s, and 72º C for 30 s. The profile concluded with a single extension of 72º C for 5 min.

Sequencing

PCR products were purified using QIAquick© purification columns according to manufacturers' instructions with one exception; the final elution was carried out with 20 µl of Buffer EB instead of the recommended 30-50 µl to compensate for potential low quantity template DNA . The amount of purified post-PCR product was quantified by fluorometry to insure that 5-10 ng/µl of amplified DNA was present for sequencing on the ABI 3100*xl* sequencer. Sequences were visualized, assessed for quality, and edited using Chromas 2.31 (http://www.technelysium.com.au/chromas.html). MEGA 3.1 (Kumar *et al*. 2004) was used to align edited mtDNA sequences with known bison haplotypes.

RFLP

PCR amplification was carried out as described above and digested with *Ssp1*, which cuts haplotype 8, resulting in two fragments (372 bp and 98 bp in length). Restriction digests were conducted in 20 µl volumes consisting of 11.3 µl sterile HPLC water, 2 µl RE 10X buffer, 0.2 µl acetylated BSA (10µg/ µl), and 5 µl PCR product and incubated at 37ºC for four hours. Digested products were run out on 2% agarose gels for two hours. Gels were stained with ethidium bromide solution, and visualized using a Hitachi FMBIOII scanner. We used 16 samples from YNP 2006 previously identified as either haplotype 6 or 8 through sequencing as controls to test the accuracy of our RFLP analysis. A set of 12 YNP 2005 samples identified as haplotype 6 were re-screened to evaluate whether failure to digest could result in erroneous haplotype identification. No haplotype identification errors were detected, thus validating the accuracy of the RFLP analysis.

Data analysis

The combined results of sequencing and RFLP analysis were used to determine the frequency and distribution of these haplotypes among the GYA bison populations, and determine F_{ST} values. Significance of F_{ST} values were tested by contingency chi-square analyses for comparisons among YNP breeding groups, and between parks.

RESULTS

Sequencing revealed two mtDNA control region haplotypes, amplified from 120 GYA bison fecal samples from the 2006 breeding season. These haplotypes matched the first 408 bp of haplotypes 6 and 8 previously defined by Ward *et al*. (1999). RFLP

analysis was used to resolve between haplotypes for 59 bison fecal samples collected in 2005.

Significant differentiation was found between YNP and GTNP populations (F_{ST} = 0.191, $p < 0.001$). Haplotype 6 was the most common in both parks, while haplotype 8 was found only in 34 YNP bison samples tested, occurring most frequently in Lamar Valley (0.54) and Mirror Plateau (0.38), and least common in Hayden Valley 2005 and 2006 (0.10 and 0.00, respectively) (Table 3-1).

Among YNP breeding groups, we found substantial differentiation between the Lamar Valley and Hayden Valley (F_{ST} = 0.367, $p < 0.001$). The bison sampled in Mirror Plateau were more similar to the Lamar Valley breeding group (F_{ST} = 0.026) than they were to bison sampled in Hayden Valley 2005 and 2006 (F_{ST} = 0.103, and F_{ST} = 0.231, respectively, Table 3-2).

Within Hayden Valley, haplotype frequencies were different between two consecutive years (F_{ST}= 0.054, $p < 0.05$). Haplotype 6 was found in 87% of bison samples tested from Hayden Valley in 2005, and in 100% of samples tested from Hayden during 2006 (Table 3-1). However, the genetic differences between Hayden Valley (2005) and Lamar Valley were significant for both years (2005: F_{ST}= 0.218, $p < 0.001$, 2006: F_{ST} = 0.367, $p < 0.001$, Table 3-2).

DISCUSSION

The frequency of mtDNA control region haplotypes among the GYA bison provided strong evidence for genetic differentiation among breeding groups (Fig. 3-1). One of the most striking results of this study was the substantial differentiation we found

between the Lamar Valley and Hayden breeding groups. This was perhaps surprising for several reasons. First, in 1936, 71 bison were translocated from Lamar Valley to Hayden Valley and the Firehole, where bison had been absent for over 30 years (McHugh 1972; USDOI, NPS 2000; Gates et al. 2005). Assuming that this translocation captured a fair representation of haplotype frequencies present within Lamar during this time, we might expect little differentiation between these two groups. Second, bison are highly mobile and have been known to travel long distances in a short period of time (Carbyn 1997), and Hayden and Lamar Valleys are separated by less than 50 kilometers.

However, the difference in haplotype frequencies between Hayden and Lamar breeding groups may not be all that surprising when we consider the role of female philopatry in determining genetic differentiation. Female philopatry has been observed in other highly mobile ungulate species such as roe deer (*Capreolus capreolus*), bushbuck (*Tragelaphus scriptus*), Svalbard reindeer (*Rangifer tarandus platyrhynchus*), desert bighorn sheep (*Ovis canadensis nelsoni*), and Soay sheep (*Ovis aries*) (Côté *et al*. 2002; Coltman *et al*. 2003; Epps *et al*. 2005; Nies *et al*. 2005; Wronski and Apio 2006). Female philopatry to natal ranges can play an important role in determining the genetic structure of populations (Chesser 1991; Storz 1999).

Marked animal re-location data recorded for adult female bison from the central range reveals occasional movements west and north out of YNP park boundaries during winter months (Gates *et al*. 2005). However, these marked bison always return to the central range during the summer rut season (Gates *et al*. 2005). For example, YELL-011 (a radio-collared adult female identified as haplotype 6) has been documented to travel to the northern range during winter month, and primarily range within Hayden Valley

during summer rut season (Gates *et al*. 2005). Interestingly, Christianson *et al*. (2005) found significant differences in incisor wear, between female bison from the northern and central ranges of YNP suggesting limited exchange of individuals between the ranges. Differences in incisor wear were attributed to increased levels of fluoride content in vegetation and water within central Yellowstone's geothermal areas (Shupe *et al*. 1984; Christianson *et al*. 2005)

. Finally, the diverse historic origins of the Lamar Valley bison, relative to the Hayden Valley herd, may explain, in part, the differences in haplotype frequencies we observed here. By 1902, YNP's wild bison herd had been reduced from 200-300, through illegal poaching, to about 25 animals that remained in Pelican Valley. This led to a decision to introduce 21 bison from other sources into Lamar Valley that same year (McHugh 1972; USDOI, NPS 2000; Gates *et al*. 2005). However, the Hayden Valley bison descended from a single translocation of 71 individuals from Lamar Valley in 1936 that may not have captured the range of haplotype frequencies that were present in these bison during that time.

We also observed differences in haplotype frequencies within Hayden Valley between two consecutive breeding seasons. However, we found strong differences between Hayden and Lamar breeding groups for both years (Table 3-2). A few radio-collared adult females have been observed to move between Hayden Valley and Pelican Creek during late summer and early fall (R. Wallen, Bison Ecologist, YNP, pers. comm. 2007). Re-location data from YELL-024 (an adult female identified as haplotype 8), reveal movements between Hayden Valley and Pelican Creek during late summer and early fall (R. Wallen, pers. comm. 2007). The lower F_{ST} values between Mirror Plateau

and Lamar Valley, in comparison to the higher values between these groups and Hayden Valley (2005 and 2006) suggest that genetic exchange between Mirror Plateau and Lamar Valley bison may occur more frequently than with central range animals (Table 3-2).

These data suggest a possible stepping-stone pattern of gene flow within YNP, whereby genetic exchange is more likely to occur between adjacent subpopulations (or breeding groups) than geographically distant ones. Therefore, because adjacent subpopulations are more similar, genetic drift will have more of an effect and result in greater differentiation among breeding groups in this model of gene flow. This observed pattern of genetic differentiation is in contrast to the conclusions of Meagher et al. (2002) who suggested that bison that previously ranged between Mirror Plateau and Pelican Valley may have assimilated into the Hayden Valley bison after the mid to late 80's. Their conclusions were based on observations of large congregations of bison within Hayden Valley during the rut, from air surveys conducted during 1983 to 2001. However, the possible pattern of gene flow among YNP breeding groups, as suggested by the observed haplotype frequencies, does appear to match closely with historic bison travel routes described by Meagher (Taper *et al*. 2000; Gates *et al*. 2005). Therefore, the current YNP bison population may be carrying on historical movement patterns of their ancestors, which in turn has contributed to the pattern of genetic differentiation we observed with mtDNA haplotypes.

Significant genetic differentiation between GTNP and YNP was found previously by Halbert (2003) using 49 microsatellite loci (F_{ST} = 0.102). The F_{ST} (0.191) we found for mtDNA was only twice that estimated from Halbert's microsatellite data. This difference is lower than would be expected, however, since mtDNA generally

represents only about 25% the effective population size of nuclear DNA (Birky *et al*. 1983; Allendorf and Luikart 2007). Furthermore, the F_{ST} values within YNP among breeding groups, at a fine scale, were much higher (with the exception of Mirror Plateau and Lamar Valley, $F_{ST} = 0.026$) than between GTNP and YNP, which are approximately five times further apart (Table 3-2).

The F_{ST} value (0.191, $p < 0.001$) between GTNP and YNP may be attributed to both the history of these populations and limited gene flow. There are isolated accounts of a few individual bison from YNP traveling to GTNP. During the winter of 1996/97, one adult female accompanied by two juvenile females from YNP were discovered on a groomed road heading toward GTNP (S. Cain pers. comm. 2006). These females were radio-collared to track their movements. They became permanent residents of the GTNP herd, and subsequently bred within this population. A single bull from YNP migrated between the parks outside of the breeding season (S. Cain pers. comm. 2006; R. Wallen pers. comm. 2006). No migration of bison from GTNP to YNP has been documented. The F_{ST} (0.191) value observed between YNP and GTNP, and the absence of haplotype 8 within GTNP may be explained by the history of bottlenecks and supplementation experienced by these bison.

The GTNP bison herd originated in 1948 from 20 animals brought in from Lamar Valley, YNP, which may have resulted in similar haplotype frequencies between GTNP and YNP. Furthermore, in 1963 when brucellosis was discovered, all 13 adults were destroyed, leaving only nine calves. The following year, GTNP received 12 adult bison from the TRNP bison herd, which originated from Ft. Niobrara National Wildlife Refuge (FNNWR) which contains only haplotype 6 (Ward *et al*. 1999).

The differences in haplotype frequencies among YNP breeding groups may be attributed to both the natural biology of free ranging bison populations and the historical origins of these bison. However, the history of the GTNP herd may be playing a greater role than the natural ecology of wild bison in determining the genetic differences we observed between the YNP and GTNP populations. Additional sampling should be collected within the same locations for at least one more consecutive breeding season to further evaluate temporal stability of mtDNA haplotype and microsatellite allele frequencies among GYA breeding groups.

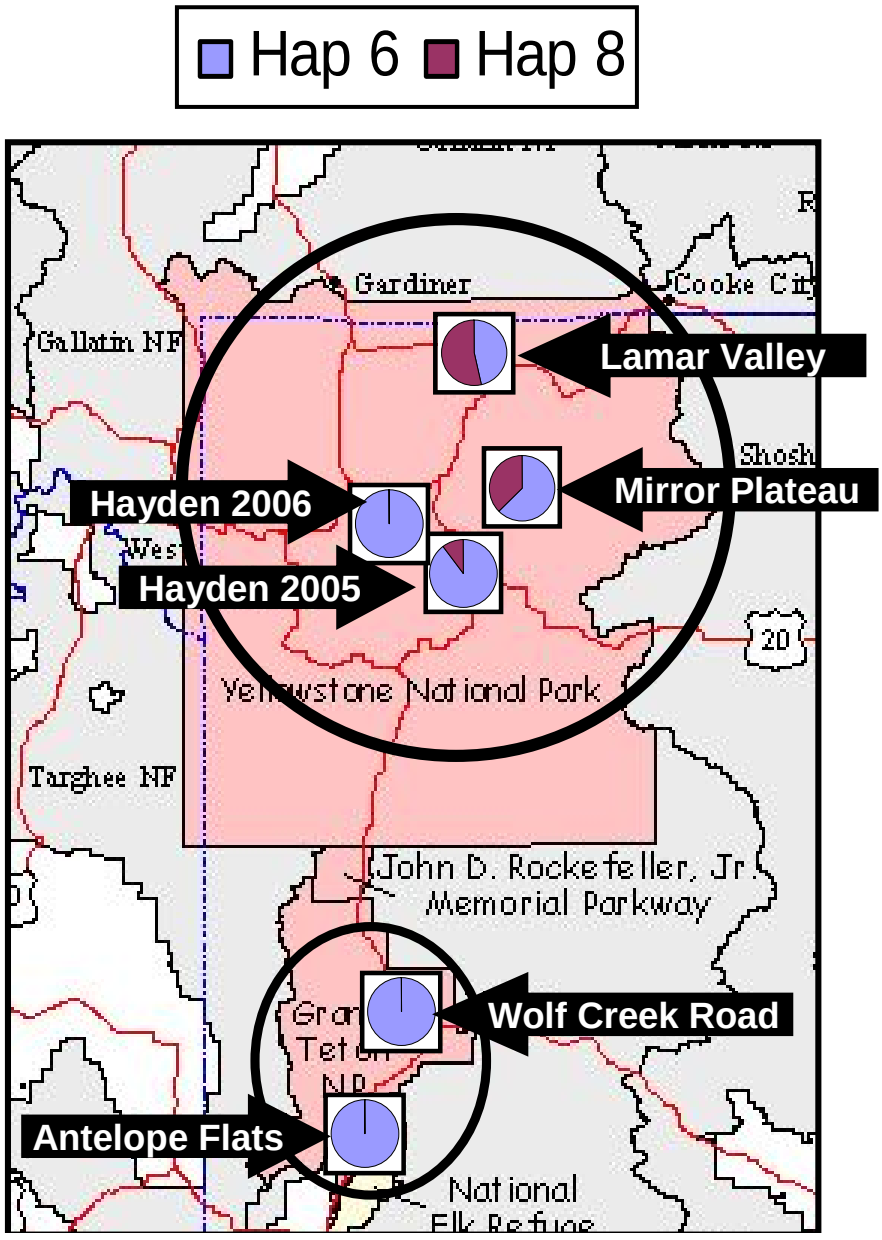
Estimating the rate and direction of gene flow between YNP and GTNP may be especially important with respect to issues such as disease transmission, and the transfer cattle genes to a non-hybridized bison population. No evidence of hybridization with cattle has been found in either YNP or GTNP bison in other studies (Halbert and Derr 2007). Also, we did not detect any cattle haplotype among the samples tested for this study. However, the GTNP bison originate, in part from herds where hybridization is known to exist. There is no way to know whether the bison translocated from TRNP in 1964 were hybrids. Populations that show evidence of introgression can be considered hybrid swarms, the result of generations of backcrossings with parental types and matings among hybrids (Allendorf *et al*. 2001). The small sample size from GTNP ($N = 39$) may have limited the power to detect hybridization within this population (Halbert and Derr 2007). The concern for possible introgression of cattle DNA into YNP warrants further investigation with the addition of microsatellite loci, for estimating the rate and direction of gene flow between GTNP and YNP.

The preferred alternative proposed for managing the GTNP bison population to reduce disease risks and prevent habitat degradation from the rapidly growing herd, is to cull bison through hunting over several years to maintain a population of about 500 individuals (USDOI-USFWS and NPS). The effects of this type of herd reduction to heterozygosity have not been evaluated. Gross and Wang (2005) demonstrated, through modeling of various management scenarios, that a population of 1000 animals would have a 90% probability of maintaining 90% allelic diversity for 200 years. However, these models were based on genetic data from YNP provided by Halbert (2003) without accounting for any existing population subdivision within this herd, and did not include data from GTNP.

Genetic data collected from bison during the breeding season, and on winter ranges within YNP and outside park boundaries should be used to construct models under various management scenarios to evaluate effects of culling to existing genetic diversity. Modeling of various herd reduction scenarios for the GTNP bison population should be conducted as well. The output generated by these models would provide crucial information for agencies to evaluate the effects of current and proposed management regimes to the genetic diversity of the GYA bison populations.

Figures:

Figure 3-1. Approximate locations of GYA bison breeding groups, represented by the haplotype frequencies of bison sampled within each area.



Tables:

Table 3-1. Distribution and frequency of haplotype 6 among the GYA bison populations sampled over two consecutive breeding seasons, 2005 and 2006, and the total number of samples analyzed from each population per season.

		N			Haplotype 6	
Park	**Breeding Group**	**2005**	**2006**	**Total**	**2005**	**2006**
YNP	Hayden	59	35	94	0.898	1.000
	Lamar	–	41	41	–	0.463
	Mirror Plateau	–	16	16	–	0.625
	Total:	**59**	**92**	**151**	**0.898**	**0.696**
GTNP	Antelope Flats	–	20	50	**–**	1.000
	Wolf Creek Road	–	8	8	–	1.000
	Total:	**0**	**28**	**58**	**–**	**1.000**

Table 3-2. F_{ST} values based on haplotype frequencies among GYA bison populations.

	LV	**HV-05**	**HV-06**	**MP**	**YNP**
Lamar Valley (LV)	–				
Hayden Valley (HV-05)	0.218**	–			
Hayden Valley (HV-06)	0.367**	0.054*	–		
Mirror Plateau (MP)	0.026	0.103**	0.231**	–	–
GTNP	0.367**	0.054*	0.000	0.231**	0.191**

*$p = < 0.05$, ** $p = < 0.001$

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