Bovine mtDNA Discovered in North American Bison Populations

RENEE O. POLZIEHN,* CURTIS STROBECK,* JANE SHERATON,† AND ROBIN BEECH‡

*Department of Biological Sciences, BioSciences Building, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

†Department of Biology, McGill University, 1205 Dr. Penfield Avenue, Montreal, Quebec H3A 1B1, Canada

‡Macdonald Campus, McGill University, Ste. Anne de Bellevue 21, 111 Lakeshore Road, Montreal, Quebec H9X 1C0, Canada

North American bison (*Bison bison*), which once numbered in the millions, were nearly extirpated by the late 1800s. The present recovery of the bison is owed to ranchers who collected bison scattered across the prairies for the purpose of establishing founding stocks (Allen 1877; Garretson 1934; Rorabacher 1970; Dary 1974; Ogilvie 1979). By 1889 there were an estimated 200 bison in Yellowstone National Park, Wyoming, 550 bison in Wood Buffalo National Park (Alberta and Northwest Territories; WBNP), and only 340 bison held on private ranches and wild herds (Jenning & Hebbing 1983). By the 1900s the bison population in Yellowstone and WBNP had dropped to 22 and 250 animals, respectively, because of poaching. Bison used to restock parks in both the United States and Canada were selected from the small surviving nucleus of animals.

Today bison populations are flourishing, and more than 18,696 bison can be found in the North American parks alone (Walker 1993). Consequently, conservation efforts have moved from increasing bison numbers to accumulating information on the genetic diversity and relatedness of populations. In the process of constructing a phylogeny for North American bison from mitochondrial DNA (mtDNA), we discovered a bovine (*Bos taurus*) haplotype (mitochondrial genotype) in the Custer State Park (CSP) herd of South Dakota. The discovery of the bovine haplotype prompted a survey for bovine haplotypes among eight other park populations of bison. Because bison were exchanged between several established park populations, the possibility exists that bovine genes have spread throughout North American bison populations. Park populations that did not exchange animals might also contain bovine genes, because most founding populations began on ranches that also crossed bison with cattle (Garretson 1934; Rorabacher 1970; Dary 1974).

In addition to surveying populations for bovine haplotypes, we used recorded observations of cross-breeding between bison and cattle, the history of CSP, the maternal expression of mtDNA, and a model of chromosomal inheritance to explain the presence and probability of bovine DNA in CSP.

Materials and Methods

Sampling and DNA Purification

Whole blood was collected in 10 ml EDTA or heparinized Vacutainers™ from 269 bison by Parks Canada and the United States Fish and Wildlife Services. The number of bison sampled in each park included 12 bison from Yellowstone; 22 from the National Bison Range, Montana; 20 from the Fort Niobrara Wildlife Refuge, Nebraska; 22 from the Mackenzie Bison Sanctuary, Northwest Territories; 20 from the Wichita Mountains Wildlife Reserve, Oklahoma; 30 from CSP; 45 from Elk Island National Park (wood bison), Alberta; 40 from Elk Island National Park (plains bison), Alberta; and 58 bison at WBNP. The blood samples were shipped on ice and stored at 4°C or −20°C until processed.

DNA was isolated from whole blood by isolating and then lysing the white blood cells. First, blood was transferred to a 30-ml Corex tube and combined with an equal volume of ACK (0.15 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM disodium EDTA) at 0°C. The sample was gently shaken for 2 minutes and centrifuged at 400 G for 5 minutes. The supernatant was

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removed by aspiration, and the shaking and centrifugation was repeated. The pellet was washed in 15 ml of PBS (Sambrook et al. 1989; Bork et al. 1991) and centrifuged at 400 G for 5 minutes. Again the supernatant was removed by aspiration, and the pellet was resuspended in 5 ml of PBS. Cells were lysed as described by Bork et al. (1991). DNA was purified by organic extraction and precipitated as described by Sambrook et al. (1989).

Isolation of Control Region

The control region of mtDNA was enzymatically amplified in 100 μl volume containing 0.2 mM each of dATP, dTTP, dCTP, and dGTP, 1 × *Thermus aquaticus (Taq)* magnesium-free polymerase buffer (Promega, Madison, WI), 2.0 μM magnesium chloride, 20 pM of the primers CST 1 or 39 and CST 2, genomic DNA (10–1000 ng), and 1 unit of *Taq* polymerase. About 50 μl of mineral oil was added to each sample prior to amplification. With a Perkin-Elmer Cetus 480 Thermocycler, amplification cycles included denaturation at 94°C for 5 minutes, annealing at 54°C for 30 seconds, synthesis at 72°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, 54°C for 30 seconds, 72°C for 2 minutes, ending with 94°C for 15 seconds, 54°C for 30 seconds, and 72°C for 10 minutes. Primer sequences CST 1 (5′GGAAGGCTGGGACCAAACCT 3′) and CST 2 (5′ TAATATACTGGTCTTGTAAACC 3′) selected for amplification were based on a published control consensus sequence (Kocher et al. 1989); later, CST 39 (5′ GGGTCGGAAGGCTGGGACCAAACC 3′) replaced CST 1.

Sequencing

Bison control-region sequences were aligned to the bovine control sequence provided by Anderson et al. (1981). Because of expense and time constraints, sequence analysis was restricted to 32 individuals representing bison from Elk Island Wood bison (6), Elk Island plains bison (6), Mackenzie bison (4), CSP bison (6), Wichita Mountains bison (2), and WBNP (8). DNA was amplified for 40 cycles, from which 30 μl was electrophoresed on a 1% LMP gel and the PCR product excised. The slice was heated to 65°C and diluted with water to 1/50. This dilution of DNA served as the template for an asymmetric PCR, whereby the concentration of one primer was 2 μM and the other 20 μM. The PCR products were pooled from two reactions, extracted with 100 μl of chloroform, precipitated with 500 μl of ethanol:ammonium acetate (4:1), and centifuged for 30 minutes. All traces of the ethanol were removed, and the pellet was resuspended in 10 μl water. Sequencing reactions were performed as described in the T7 Sequencing Kit™ (Pharmacia), and 5 μl of each reaction was loaded on an 8% polyacrylamide gel. The gels were quenched in 5% acetic acid and 5% methanol, transferred to Whatman paper, and autoradiographed overnight at ambient temperature on Kodak GBX-2 film.

Restriction Analysis

To increase the number of animals surveyed for the bovine haplotypes, nine restriction enzymes were employed (*Alu I, Cfo I, Dde I, Hae III, Hinf I, Sau 3A, Taq 1, Hpa II,* and *Sty I.*). Only *Hinf I* and *Sty I* detected polymorphisms of restriction-fragment length. Approximately 100–200 ng amplified DNA was digested with 2 units *Hinf I* or *Sty I* during incubation at 37°C overnight in a 1/10 dilution of buffer. The salt concentrations of the buffer were reduced to compensate for salt residues already present in the amplified products. The digested DNA was separated using a BioRad vertical gel apparatus on a 4 or 6% acrylamide TBE (0.090 M Tris-borate/0.002 M EDTA) gel buffered for 2–2.5 hours in 0.5 × TBE at 25 mA per gel to a maximum of 300 volts. The gels were stained with ethidium bromide, and the DNA fragment lengths were estimated by including a 123 base-pair ladder (BRL) in each gel.

Results and Discussion

As part of a phylogenetic study to determine the relationship of North American bison, the control region of mtDNA was initially sequenced from six wood and six plains bison from Elk Island National Park and was later expanded to include bison from CSP, Wichita Mountains Wildlife Refuge, WBNP, and the Mackenzie Bison Sanctuary. The sequence information revealed site 429 as a *Sty I* restriction site and confirmed site 607 as a variable *Hinf I* site. With reference to these sites only two genotypes were found in the Elk Island populations (Table 1). They are described as the variants with a cytosine at site 429 and adenine at site 607 ($C_{429}A_{607}$) or a cytosine at site 429 and a guanine at site 607 ($C_{429}G_{607}$) (Fig. 1).

When bison at CSP were surveyed for these variants using the restriction enzymes *Sty I* and *Hinf I*, two additional gentoypes were identified. To correctly identify the variation at these sites, the control regions of six CSP bison, representing all four genotypes, were sequenced (Fig. 1). Two bison were homologous to the genotypes at Elk Island, three had a thymine at site 429 and a guanine at site 607 ($T_{429}G_{607}$), and one had a thymine at site 429 and an adenine at site 607 ($T_{429}A_{607}$).

The sequences from the CSP bison were compared to both bison and bovine consensus sequences. The three bison with gentoype $T_{429}G_{607}$ were specific to CSP and did not appear in any of the remaining parks. The unique CSP genotype ($T_{429}A_{607}$) was homologous to the bovine genotype. The $T_{429}A_{607}$ genotype differed by 80 nucleotides from the bison genotypes and by only one from the bovine genotype. Excluding the $T_{429}A_{607}$ geno-

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                                                                                295
BISON(CG)   CC--------  -CCAAA--AT  GCATTACC--  CAAAC-GGGG  GA-ATATACA  TAACATTAAT
BISON(CA)   ..G.......  ..........  ..........  .......A..  ..T.......  ...T......
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ..ATACACAG  A...C.GA..  .A......TA  .GC.A.....  T.........  ..........
BOVINE      ..ATACACAG  A...C.GA..  .A......TA  .GC.A.....  T.........  ..........

                                                                                355
BISON(CG)   GTAATAAAAA  CATATTATGT  ATATAGTACA  TTAAATTATA  TGCCCCATGC  ATATAAGCAA
BISON(CA)   ..........  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ........G.  ....A.....  ..........  ..........  ..........  ..........
BOVINE      ........G.  ....A.....  ..........  ..........  ..........  ..........

                                                                                415
BISON(CG)   GTACTTATCC  TCTATTGACAG TACATAGTA   CATAAAGTTA  TTAATTGTAC  ATAGCACATT
BISON(CA)   .........T  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ......A...  ..........  ..........  ..........  ..........
CSP (TA)    ....A.GA..  .....-AG..  .......A..  ....T.A...  ..G.C.....  ....T.....
BOVINE      ....A.GA..  .....-AG..  .......A..  ....T.A...  ..G.C.....  ....T.....

                 429 Sty I site                                                 467
BISON(CG)   ATGTCAAATC  TACCCTTGGC  AACATGCATA  -------TCC  CT-TCCATTA  GATCACGAGC
BISON(CA)   ..........  .C........  ..........  .......C..  ..........  ..........
CSP (TG)    ..........  ...T......  ..........  -------...  ..........  ..........
CSP (TA)    .........T  C.TT....AT  .GT..ATC..  TTATATA.T.  ..TA......  ..........
BOVINE      .........T  C.TT....AT  .GT..ATC..  TTATATA.T.  ..TA......  ..........

                                                                                527
BISON(CG)   TTAATTACCA  TGCCGCGTGA  AACCAGCAAC  CCGCTAGGCA  GAGGATCCCT  CTTCTCGCTC
BISON(CA)   ....C.....  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ..........  ..........  ..........  ..........  .-........  ..........
BOVINE      ..........  ..........  ..........  ..........  .-........  ..........

                                                                                587
BISON(CG)   CGGGCCCATG  AACCGTGGGG  GTCGCTATTT  AATGAACTTT  ATCAGACATC  TGGTTCTTTC
BISON(CA)   ..........  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ......T...  ..........  ..........
CSP (TA)    .........A  ..........  ........CC  ......T...  .C...G....  ..........
BOVINE      .........A  ..........  ........CC  ......T...  .C...G....  ..........

                 607 Hinf I site                                                647
BISON(CG)   TTCAGGGCCA  TCTCACCTAG  AATCGCCCAT  TCTTTCCTCT  TAAATAAGAC  ATCTCGATGG
BISON(CA)   ..........  .........A  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ..........  .....T...A  ..CG.T....  ..........  ..........  ..........
BOVINE      ..........  .....T...A  ..CG.T....  ..........  ..........  ..........

                                                                                807
BISON(CG)   ACTAATGGCT  AATCAGCCCA  TGCTCACACA  TAACTGTGCT  GTCATACATT  TGGTATTTTT
BISON(CA)   ..........  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ..........  ..........  ..........  ..........  ..........  ..........
BOVINE      ..........  ..........  ..........  ..........  ..........  ..........

                                                                                767
BISON(CG)   TTATTTTGGG  GGATGCTTGG  ACTCAGCTAT  GGCCGTCAAA  GGCCCTGACC  CGGAGCATCT
BISON(CA)   ..........  ..........  ..........  ..........  ..........  ..........
CSP (TG)    ..........  ..........  ..........  ..........  ..........  ..........
CSP (TA)    ..........  ..........  ..........  ..........  ..........  ..........
BOVINE      ..........  ..........  ..........  ..........  ..........  ..........

                                                                                826
BISON(CG)   ATTGTAGCTG  GACTTAACTG  CACCTTGAGC  ACCAGCATAA  TGGTAAGCAT  GGACA-TATA
BISON(CA)   ..........  ..........  ..........  ..........  ......G...  ..........
CSP (TG)    ..........  ..........  ..........  ..........  .....G....  ..........
CSP (TA)    ..........  ..........  ..T.......  ..........  ..A.......  .....T..C.
BOVINE      ..........  ..........  ..T.......  ..........  ..A.......  .....T..C.

                                                                                885
BISON(CG)   GTCAATGGTT  ACAGGACATA  AACTGTATTA  TATAT-CCCC  CCCTCCATAA  AAATTCCCCC
BISON(CA)   ..........  .........-  ..........  .....C....  ..........  ..........
CSP (TG)    ..........  ..........  ..T.......  ..........  ..........  ..........
CSP (TA)    .........C  ..........  ..T.A.....  .....C....  ..T..ATA..  ..T.......
BOVINE      .........C  ..........  ..T.A.....  ..........  ..T..ATA..  ..T.......

                                                            930
BISON(CG)   -TTAAATATT  TACCACTGCT  TTTAACAGAT  TTTTCCCTAG  TTACCT
BISON(CA)   ..........  ..........  ..........  ..........  ......
CSP (TG)    ..........  ..........  ..........  ..........  ......
CSP (TA)    C........C  ......CA..  .........C  ..........  A...T.
BOVINE      C........C  ......CA..  .........C  ..........  A...T.
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Figure 1. Bison control sequence. Base substitutions for the bovine sequence and the two additional unique Custer State Park bison are shown below the bison sequences ($C_{429}G_{607}$) and $C_{429}A_{607}$). Deletions are indicated with a (−).

Table 1. Mitochondrial haplotype variants observed in the control region of North American bison populations.

Park	Sample Size	Haplotype Variants			
		$C_{429}A_{607}$	$C_{429}G_{607}$	$T_{429}A_{607}$	$T_{429}G_{607}$
Elk Island National (wood) Park	45	—	45	—	—
Elk Island National (plains) Park	40	20	20	—	—
Yellowstone National Park	12	9	3	—	—
Wichita Mts. Wildlife Refuge	20	19	3	—	—
Ft. Niobrara Wildlife Refuge	20	20	—	—	—
Mackenzie Bison Sanctuary	22	9	13	—	—
National Bison Range	22	7	15	—	—
Custer State Park	30	11	8	2	9
Wood Buffalo National Park	58	16	42	—	—

type, a maximum nucleotide difference of 2.2% was observed among bison genotypes and 11.8% between bison and bovine genotypes. Subsequently, six more populations were screened for the bovine genotype. Although a small number of bison were sequenced (32), a total of 269 bison were surveyed for the restriction-fragment-length polymorphisms (Table 1). The bovine mtDNA was restricted to the two individuals from the CSP population. Other bovine haplotypes may exist in the bison populations, but screening the populations with the seven additional restriction enzymes would likely have detected their presence.

Because the CSP bison were never exposed to cattle in the park, the bovine mtDNA must have been present in the bison used to establish the CSP population in 1914. Bison in the founding populations were often encouraged to breed with domestic cattle, and although bison bulls would freely mate with either domestic or bison cows the domestic bulls were reluctant to breed the bison cows (Rorabacher 1970). This breeding behavior of bison, and the fact that mtDNA is maternally inherited (Brown et al. 1979), supports the conclusion that bovine mtDNA was introduced into CSP through a mating between a bison bull and a domestic cow.

The outcome of cross-breeding bison and cattle, however, has been debated for years. In 1843 Wickliffe was the first to record that domestic cattle bred to bison usually produced sterile hybrid bulls and fertile hybrid cows (Garretson 1934). Numerous hybridization experiments were conducted at Manyberries Range Experimental Substation, Alberta, during 1916–1964. In one experiment, in a cross of 82 domestic cows with bison bulls, 36 calves were successfully born, 20 cows died, 14 calves were stillborn, and 12 fetuses were aborted (Rorabacher 1970). Ranchers also experienced high male sterility and many cow/calf losses or abortions (Garretson 1934; Rorabacher 1970), but others claimed to have had few problems (Allen 1877; Garretson 1934).

The founding bison of the CSP can be traced to six bulls, 18 cows, and 12 calves purchased from rancher James Philip (Dary 1974). Philip's animals were descendants of five calves originally captured in 1881 near Fort

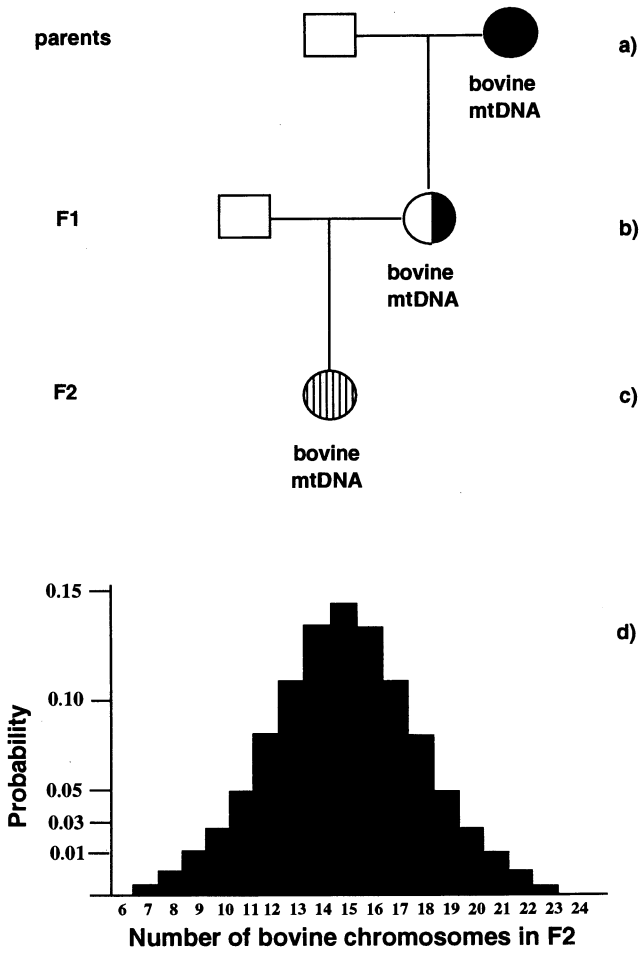

Figure 2. Hypothetical composition of the bison gene pool at Custer State Park: a bison bull (2N = 60) and a domestic cow (2N = 60) were crossed to produce (a) an F1 female with 30:30 bison-to-bovine chromosomes and (b) the F1 female was backcrossed to a bison bull to produce (c) offspring with the number of bovine chromosomes ranging from 0 to 30. Also given is the probability of the number of bovine chromosomes present in the F2 female used to found Custer State Park (d).

Bennett, North Dakota, by Pete Dupree. By 1888 Dupree had four bulls, five cows, and seven hybrids (Jenning & Hebbing 1983). Dupree sold his bison to James Philip when the population had grown to 83 bison. Philip had the animals moved to a pasture near Fort Pierre, South Dakota, where they multiplied both naturally and through contributions made by ranchers who found bison scattered among their cattle herds during round-ups. Philip, who did not like bison hybrids (Dary 1974), believed he had removed all hybrids from his herd. But detecting hybrids may have been difficult because backbreeding of hybrids with bison increases the hybrid's fertility (Rorabacher 1970) and removes bovine characteristics (Allen 1877). Because Philip took great care to remove obvious hybrids from his herd, the bison introduced to CSP likely included a single (F2) female who was, at most, the offspring of a first-generation hybrid female (F1) backcrossed to a bison bull.

The presence of bovine mtDNA in the CSP bison raises the question of how many bovine nuclear genes (nDNA) have been integrated into this population. The frequency of bovine mtDNA in the population and the presumed genotype of the hybrid F2 founding female were used to provide such an estimate. Assuming that the female bison in Custer State Park have an equal number of offspring over their lifetime, the frequency of bovine mtDNA would not change over the years. The frequency of bovine mtDNA observed in the population was 0.0667 (2/30), which would be equivalent to the frequency of bovine mtDNA in the founding female population. Assuming that half of the F2 hybrid founding female's chromosomes came from a F1 female hybrid and half from a bison bull, the frequency of F1 hybrid genes in the founding population is 0.0333. If it is also assumed that there were no hybrids in the founding male population, then the frequency of F1 hybrid genes in the offspring of the founding males and females is 0.0167. On average, only half of the F1 hybrid genes inherited in the F2 hybrid founding female would have been bovid (see distribution in Fig. 2). Therefore, the expected proportion of bovine nDNA in the CSP population, given equal representation of the founding lineages in the extant population, would be 0.0083. Thus, the proportion of bovine nDNA in the CSP bison herd and in each individual will be quite low. Although the presence of bovine DNA is not detected in the appearance of the CSP bison and does not diminish the bison's ability to survive, it stresses the importance of knowing a species' gene pool before relocating or exchanging individuals.

We have shown evidence for hybridization in North America between domestic and wild species. Examples of hybridization between wild species include the gray wolf and coyote (Wayne et al. 1992) and the mule deer and white-tailed deer (Cronin 1988). Although hybridization may occur naturally, habitat perturbation and importation of nonnative species are subjecting wild, native North American species to ever-increasing hybridization pressures. As long as hybridization between species was minimal and population numbers remained high, little thought was given to the presence of hybrids.

Integration of hybrids into conservation policies has become a difficult and complex issue. Hybrids may exist in an area for reasons such as dispersal patterns bringing species together and hybrids being better suited to an area. Whether or not hybridization has serious implications for species survival, management, or legislation depends on the species (see Dowling et al. 1992). As a species' population decreases, the impact of hybridization increases. Therefore, species with large populations will be the least affected by hybridization and small populations the most affected. Once a species' population drops below a sustainable level, options for species survival may be significantly reduced, and it may be necessary to protect all individuals, even hybrids, to preserve the remaining genetic diversity (Echelle 1991). Given the number of bison available to establish new herds, preference will most likely be given to animals with no evidence of hybridization. Thus, despite the presence of bovine haplotypes, the unique bison haplotype found in Custer State Park, absent from any other population, makes the Custer State Park population worthy of preservation.

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