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Complete mitochondrial DNA sequence analysis of *Bison bison* and bison–cattle hybrids: Function and phylogeny

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ABSTRACT

Complete mitochondrial DNA (mtDNA) genomes from 43 bison and bison-cattle hybrids were sequenced and compared with other bovids. Selected animals reflect the historical range and current taxonomic structure of bison. This study identified regions of potential nuclear–mitochondrial incompatibilities in hybrids, provided a complete mtDNA phylogenetic tree for this species, and uncovered evidence of bison population substructure. Seventeen bison haplotypes defined by 66 polymorphic sites were discovered, whereas 728 fixed differences and 86 non-synonymous mutations were identified between bison and bison–cattle hybrid sequences. The potential roles of the mtDNA genome in the function of hybrid animals and bison taxonomy are discussed.

1. Introduction

Due to the vital functions of the mitochondria, it is not surprising that many mitochondrial DNA (mtDNA) mutations have been identified that affect fitness, alter athletic performance, and cause a variety of diseases (Bortot et al., 2009; Florentz et al., 2003; Harrison and Burton, 2006; Tanaka et al., 2010; Wallace, 1994). Most mitochondrial studies have been limited to only a few genes or regions of the mitochondrial genome. However, with the advancement of sequencing technologies, it has become possible to sequence whole mtDNA genomes quickly and accurately. Whole mtDNA genome sequencing has recently revealed important insights into cellular metabolism, mitochondrial gene organization, and genome evolution (Boore et al., 2005). Additionally, whole mtDNA genome sequencing has drastically improved the power and resolution of phylogenetic analysis compared with single gene or single region studies (Santamaria et al., 2007; Simon et al., 2006; Zardoya and Meyer, 1996), allowing for more accurate resolution of taxonomic relationships even at deep levels (Cao et al., 2006; Gissi et al., 2008).

It has been estimated that 1500–2000 nuclear proteins are necessary for the numerous activities of the mitochondria, although only about half of these have been identified to date (Elstner et al., 2008; Prokisch et al., 2006). These nuclear proteins interact with mitochondrial proteins to form co-adapted gene complexes which must remain compatible to ensure mitochondrial function. Reduced fitness levels have been observed among the offspring of both interspecific and intraspecific crosses between populations with different mitochondrial types (Barrientos et al., 1998; Burton et al., 1999; Ellison and Burton, 2008; Liepins and Hennen, 1977; Yamaoka et al., 2000). Additionally, it appears that nuclear–mitochondrial incompatibilities may play an important role in reproductive isolation in fish (Bolnick et al., 2008); however, to date, a mammalian model system to study these effects is lacking.

A well-documented example of interspecific hybridization between American bison (*Bison bison*) and domestic cattle (*Bos taurus*) presents a unique opportunity to examine the effects of mtDNA sequence on nuclear–mitochondrial protein interactions, and possibly on hybrid fitness. The two species, which are members of the same Bovinae subfamily, diverged around 1 million years ago and are largely incompatible: hybridization does not occur naturally and most F_1 offspring are sterile (produced from domestic cow by bison bull crosses) (Goodnight, 1914; Hartl et al., 1988; Jones, 1907; Loftus et al.,

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1994). However, a small number of viable bison–cattle hybrids, along with a larger number of purebred bison, were used in the establishment of several bison populations following a dramatic species bottleneck in the late 19th century (Coder, 1975; Garretson, 1938). Unlike first- or second-generation backcrosses, which commonly exhibit morphological characteristics of hybridization, most advanced generation backcrosses are morphologically indistinguishable from purebred bison. As a result, cattle introgression can be found in the nuclear DNA and/or mtDNA genomes of the large majority of bison populations (Halbert and Derr, 2007; Halbert et al., 2005; Vogel et al., 2007; Ward et al., 1999) with around 3.7% of all extant bison harboring cattle mtDNA (Halbert and Derr, unpublished data from more than 10,000 bison in 150 populations).

The relationship between the genera of *Bos* and *Bison* has been a source of debate for decades. In 1758, Linneaus placed bison in the genus *Bos* (*Bos bison*), but bison were subsequently moved to a sister genus (*Bison*) during the 19th century (Wilson and Reeder, 1993). Presently, there are two extant species within the *Bison* genus, the European (*Bison bonasus*) and American bison (*Bison bison*) (McDonald, 1981). The generic distinction of *Bison* has been historically supported by analysis of anatomical distinctiveness (McDonald, 1981; Meagher, 1986; van Zyll de Jong, 1986). However, both species are capable of producing fertile offspring through hybridization with domestic cattle and other members of the genus *Bos* (Boyd, 1908; Goodnight, 1914; Steklenev and Yasinetskaya, 1982) strongly supporting inclusion of *Bison* in the *Bos* genus. Further support for this classification comes from morphological data (Groves, 1981), blood protein analysis (Stormont et al., 1961), phylogenetic analyses of single mitochondrial regions (Burzynska et al., 1999; Janecek et al., 1996; Miyamoto et al., 1989), nuclear ribosomal DNA (Wall et al., 1992), and single nucleotide polymorphism (SNP) analysis (Decker et al., 2009).

Another contentious debate involves the separation of American bison into two subspecies: *B. bison bison* (plains bison) and *B. bison athabascae* (wood bison). The histories of the two lines are similar with extreme population bottlenecks due to environmental and human factors (Isenberg, 2000). In the late 19th century, the wood bison population declined in Canada to an estimated 300 individuals in a single region (now encompassed by Wood Buffalo National Park) (Soper, 1941). Following the enactment of anti-hunting laws, the population increased to 1500–2000 bison. From 1922–1928, approximately 6600 plains bison were imported into the population, leading to a mixture of the two lines (Banfield and Novakowski, 1960; Roe, 1970). Although still somewhat phenotypically distinct, the subspecies designation has been challenged by many (Burton, 1962; Corbet, 1978; van Gelder, 1977; Wilson and Reeder, 1993) and it has been argued that the two are merely ecotypes and not subspecies (Geist, 1991). Furthermore, blood typing, RFLP, and microsatellite DNA analysis have indicated that plains and wood bison are not distinct enough to be considered subspecies (Bork et al., 1991; Peden and Kraay, 1979; Wilson and Strobeck, 1999).

In this study, we sequenced the entire mtDNA genome from 43 American bison and domestic cattle to examine the effects of hybridization between the two species, analyze their phylogenetic relationships, and construct the first whole mtDNA phylogenetic tree of American bison to identify population substructure and subspecific relationships. Bison with native (bison) and non-native (cattle) mtDNA were sequenced to gain insights into differences between the mitochondrial genomes which may contribute to physiological changes in hybrid (bison/cattle) individuals. Synonymous and nonsynonymous differences between bison and hybrid animals, their locations within protein-coding genes, and possible effects on tRNA secondary structure were evaluated. This study is an important step in understanding the mitochondrial sequence diversity found in bison, the role of mitochondrial function in hybridized animals, and the phylogeny of bison in relation to the *Bos* genus.

2. Materials and methods

2.1. Sampling strategy

Whole blood samples were collected between 1997 and 2006. Total genomic DNA was extracted from white blood cells by proteinase K treatment followed by phenol/chloroform extraction (Sambrook et al., 1989). Sample quantity and quality was determined via spectrophotometry (ND-1000; NanoDrop Technologies, Inc., Wilmington, DE). Samples were stored at −80 °C prior to use.

Complete mtDNA sequences were obtained from 43 bison and 3 cattle (Supplemental Materials Table 1) using the following methods described. Each of the bison samples were evaluated for the presence of domestic cattle mtDNA using a PCR-based assay as previously described (Ward et al., 1999). Based on historical records and previous genetic studies, all known extant bison are derived from a handful of foundation herds established in the late 19th and 20th centuries (Coder, 1975; Garretson, 1938; Soper, 1941). To maximize haplotype diversity, efforts were made to include representative haplotypes across 5 of the foundation herds including: Yellowstone National Park (Wyoming, USA), Fort Niobrara National Wildlife Refuge (Nebraska, USA), National Bison Range (Montana, USA), Texas State Bison Herd (Texas, USA), and Elk Island National Park (Alberta, Canada). From these herds, 5 plains bison (*Bison bison bison*) samples and 2 wood bison samples (*Bison bison athabascae*) determined to contain bison mtDNA were selected for sequencing. Additionally, 36 samples from a private bison population recently created from multiple sources were evaluated, including 24 with bison mtDNA and 12 with domestic cattle mtDNA (hereafter referred to as "hybrids") (Supplemental Materials Table 1).

2.2. Sequencing strategy

A rapid method for sequencing both cattle and bison complete mtDNA was developed using the following strategy. Complete mtDNA sequences from human, mouse, and cattle were downloaded from publicly available databases and aligned using Clustal X (Larkin et al., 2007). Primers were designed in highly conserved regions where possible. Pairs of primers were selected across the entire bovine mtDNA genome based on the following specifications: 1) amplified fragment size of 900–1000 base pairs (bp) to allow for direct sequencing using the same (PCR) primers; 2) at least 100 bp of overlap between adjacent pairs to ensure complete sequencing coverage; 3) optimal annealing temperature of 54 °C. These criteria were necessarily adjusted in regions of low conservation. A total of 24 primer pairs were selected (Supplemental Materials Table 2) with an average estimated fragment size of 905 bp (±125.5 bp) and an average overlap of 202 bp (±99.4 bp).

A standard 25 μL PCR mixture was utilized for all primer pairs, including 100 ng template DNA, 1.5 mM $MgCl_2$, 0.025 mM each dNTP, 1× MasterAmp PCR Enhancer with betaine (Epicentre), 0.048 μM each primer, 1× GeneAmp PCR Buffer II and 0.2 μL AmpliTaq® Gold DNA polymerase (Applied Biosystems). Touch-down PCR amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation for 3 min at 96 °C; 3 cycles of 96 °C for 20 s, 57 °C for 30 s, and 72 °C for 1 min with a decrease in annealing temperature of 1 °C during each cycle; 37 cycles of 96 °C for 20 s, 54 °C for 30 s, and 72 °C for 1 min; final extension of 72 °C for 10 min. Resultant amplicons were visualized on 1% agarose gels stained with ethidium bromide and purified with a QIAquick® PCR purification kit following the manufacturer's recommendations (Qiagen).

Bi-directional dye terminator (BigDye® v1.1, Applied Biosystems) sequencing was performed in 10 μL reactions including 90 ng template and 10 pmol primer following the manufacturer's recommendations. Sequence products were purified using sephadex G-50 columns (BioMax) and visualized on a 3130*xl* Genetic Analyzer (Applied Biosystems). Analyzed data was evaluated and assembled into contigs using Sequencher™ 4.9 (Gene Codes Corporation).

2.3. mtDNA genome analysis

The complete mitochondrial sequences of 30 *Bos taurus* (domestic cattle, hereafter "cattle"), 2 *Bos indicus* (zebu), 3 *Bos grunniens* (yak), 1 *Bison bison* (American bison), and 1 *Bubalus bubalis* (water buffalo) were obtained from GenBank (Benson et al., 2005) (Supplemental Materials Table 1). The complete mitochondrial genome sequences were aligned using Clustal X (Larkin et al., 2007) in the MEGA 4.0 phylogenetic package and corrected by hand when necessary. The genomes were parsed into the following data sets for functional and phylogenetic analyses: 1) bison only; 2) bison and hybrids and 3) *Bos*, bison, and hybrids. To investigate the variation among bison mtDNA genomes, an unrooted haplotype network (spring tree) was created using TCS v1.21 (Clement et al., 2000).

2.4. Bison–cattle hybridization analysis parameters

An alignment of bison and hybrid haplotypes was created to assess differences in the two genomes. A previously published cattle mtDNA sequence (AB074966.1) was used to identify putative gene boundaries and overlapping regions. Aligned files were analyzed gene-by-gene in DnaSP v5 (Librado and Rozas, 2009) using the genetic code for mammalian mtDNA to assess the total numbers of polymorphic sites, synonymous changes, and non-synonymous changes. Non-synonymous changes were then coded by amino acid class (non-polar, uncharged polar, positive, negative) to evaluate the number of class-changing mutations between the bison and hybrid groups.

Changes in tRNA structure due to sequence differences between the bison and hybrid groups were evaluated using the online program tRNAscan-SE (Lowe and Eddy, 1997; http://lowelab.ucsc.edu/tRNAscan-SE/). The cove only method (cut-off score = 20 bits) and the mito/chloroplast source definition were used. Locations of changes were recorded in the following categories: D loop/stem, T C loop/stem, central loop, anticodon loop/stem, or acceptor arm stem.

2.5. Phylogenetic analysis

RAxML version 7.0.3 was used to generate unweighted maximum likelihood phylogenetic trees (Stamatakis, 2006). The GTRGAMMA model was utilized, and 1000 replicates were used to generate bootstrap values. To account for different nucleotide substitution rates across the mitochondrial genome, we partitioned the sequence as follows: each of the 13 protein-coding genes and the 2 rDNA genes were treated as independent regions (15 total); the 22 tDNA genes were grouped into one large region; and the D loop was defined as an independent region. For each partition, individual alpha-shape

Table 1
Annotation and gene organization of the *Bison bison* mitochondrial genome. Order, gene, strand (heavy or light), starting and ending nucleotide position, first and last ten bases, and intergenic/overlapping nucleotides ("–" indicates overlapping regions) are based on whole mtDNA genome alignment of all bison haplotypes and comparison with published cattle sequences.

Order	Gene	Strand	Start	End	Length (bp)	First ten bases	Last ten bases	Intergenic nucleotides
	D loop		1	364	362–364	ACTAATGGCT	CCCCCCCCCC	0
1	tRNA-Phe	Heavy	365	431	67	GTTGATGTAG	TCCATAAACA	0
2	12s-rRNA	Heavy	432	1387	956	CATAGGTTTG	TTGGATAAAT	0
3	tRNA-Val	Heavy	1388	1454	67	CAAGATATAG	AATATCTTGA	0
4	16S rRNA	Heavy	1455	3025	1570–1571[d]	ACTAAATCTA	ACAGGGCTTA	0
5	tRNA-Leu	Heavy	3026	3100	75	GTTAAGGTGG	CTCCTTAACA	2 (AA)
6	ND1	Heavy	3103	4058	956	ATGTTCATAA	CGCAAACATA[a]	0
7	tRNA-Ile	Heavy	4059	4127	69	AGAAATATGT	CTTATTTCTA	−3 (CTA)
8	tRNA-Gln	Light	4125	4196	72	CTAGAACTAT	CCAAATTCTA	2 (TT)
9	tRNA-Met	Heavy	4199	4267	69	AGTAAGGTCA	TCCCGTACTA	0
10	ND2	Heavy	4268	5309	1042	ATAAATCCAA	GTATTAGAAT[a]	0
11	tRNA-Trp	Heavy	5310	5376	67	AGGAATTTAG	TTAATTCCTG	1 (C)
12	tRNA-Ala	Light	5378	5446	69	TAAGGATTGC	CTAAATCCTC	1 (A)
13	tRNA-Asn	Light	5448	5521	74[b]	CTAGACTGGT	CTTCAATCTA	0
	L-strand ori. of rep.	Light	5522	5553	32	CTTCTCCCGC	AAGGCGGGAG	0
14	tRNA-Cys	Light	5554	5620	67	AAGCCCCGGC	CCACAGGGCT	0
15	tRNA-Tyr	Light	5621	5688	68	TGGTAAAAAG	CCATTTTACC	1 (C)
16	COI	Heavy	5690	7234	1545	ATGTTCATTA	CCTAAAATAA	−3 (TAA)
17	tRNA-Ser	Light	7232	7304	71–73[d]	TAAGAAAGGA	TCTCTCTCAA	4 (TAAA)
18	tRNA-Asp	Heavy	7309	7377	69	CGAAGTGTTA	GTACACCTCA	1 (T)
19	COII	Heavy	7379	8062	684	ATGGCATACC	AATATTATAA	3 (AAT)
20	tRNA-Lys	Heavy	8066	8132	67	CACCAAGAAG	TCCTTGGTGA	1 (C)
21	ATP8	Heavy	8134	8334	201	ATGCCACAAC	ACCCCTATAA	−40
22	ATP6	Heavy	8295	8975	681	ATGAACGAAA	CAACACATAA	−1 (A)
23	COIII	Heavy	8975	9755	781	ATGACACACC	TGATGAGGCT[a]	3 (CCT)
24	tRNA-Gly	Heavy	9759	9827	69	ATTCTTTTAG	AAAAAGAATA	0
25	ND3	Heavy	9828	10,174	346	ATAAATCTAA	GAACCGAATA[a]	0
26	tRNA-Arg	Heavy	10,175	10,243	69	TGGTACTTAG	TAATTACCAA	0
27	ND4 (L)	Light	10,244	10,540	297	ATGTCTATAG	CCAATGCTAA	−7
28	ND4	Heavy	10,534	11,911	1378	ATGCTAAAAT	CCTCTATACT[a]	0
29	tRNA-His	Heavy	11,912	11,981	70	GTAAATATAG	CTTATTTACC	0
30	tRNA-Ser2	Heavy	11,982	12,041	60	GAAAAAGTAT	GGCTTTTTCG	1 (A)
31	tRNA-Leu2	Heavy	12,043	12,112	70[c]	ACTTTTAAAG	AATAAAAGTA	0
32	ND5	Heavy	12,113	13,933	1821	ATAAACATAT	CCACGAGTAA	−17
33	ND6	Light	13,917	14,444	528	TTAATTTCCA	ATAGTATCAT	0
34	tRNA-Glu	Light	14,445	14,513	69	TATTCTTACA	CTACAAGAAC	4 (ACTA)
35	CYTB	Heavy	14,518	15,657	1140	ATGACTARCC	AAAATGAAGA	4 (CAGG)
36	tRNA-Thr	Heavy	15,662	15,730	69	TCTTTGTAGT	CCCTAAGACT	−1 (T)
37	tRNA-Pro	Light	15,730	15,795	66	TCAAGGAAGA	CTATTCCCTG	0
	D loop		15,796	16,325	528–530	AACGCTATTA	ATCTCGATGG	0

[a] TAA stop codon completed by addition of 3' adenine residues to mRNA.
[b] Region has a fixed insertion in bison as compared to cattle.
[c] Region has a fixed deletion in bison as compared to cattle.
[d] Indel present within gene.

parameters, GTR-rates, and empirical base frequencies were estimated and optimized creating individual nucleotide substitution models. The coordinates for all regions were determined from the multiple alignment and previously published coordinates. The *B. bubalus* sequence was used as the outgroup for the *Bos*/*Bison* phylogenetic tree, and the yak sequences were used as the outgroup for the bison phylogenetic tree.

3. Results

3.1. Description of bison mitochondrial genome

Complete mtDNA genomes from the 43 bison and 3 cattle sequenced in this study were deposited in the GenBank database (accession numbers GU946976–GU947021). The complete *Bison bison*

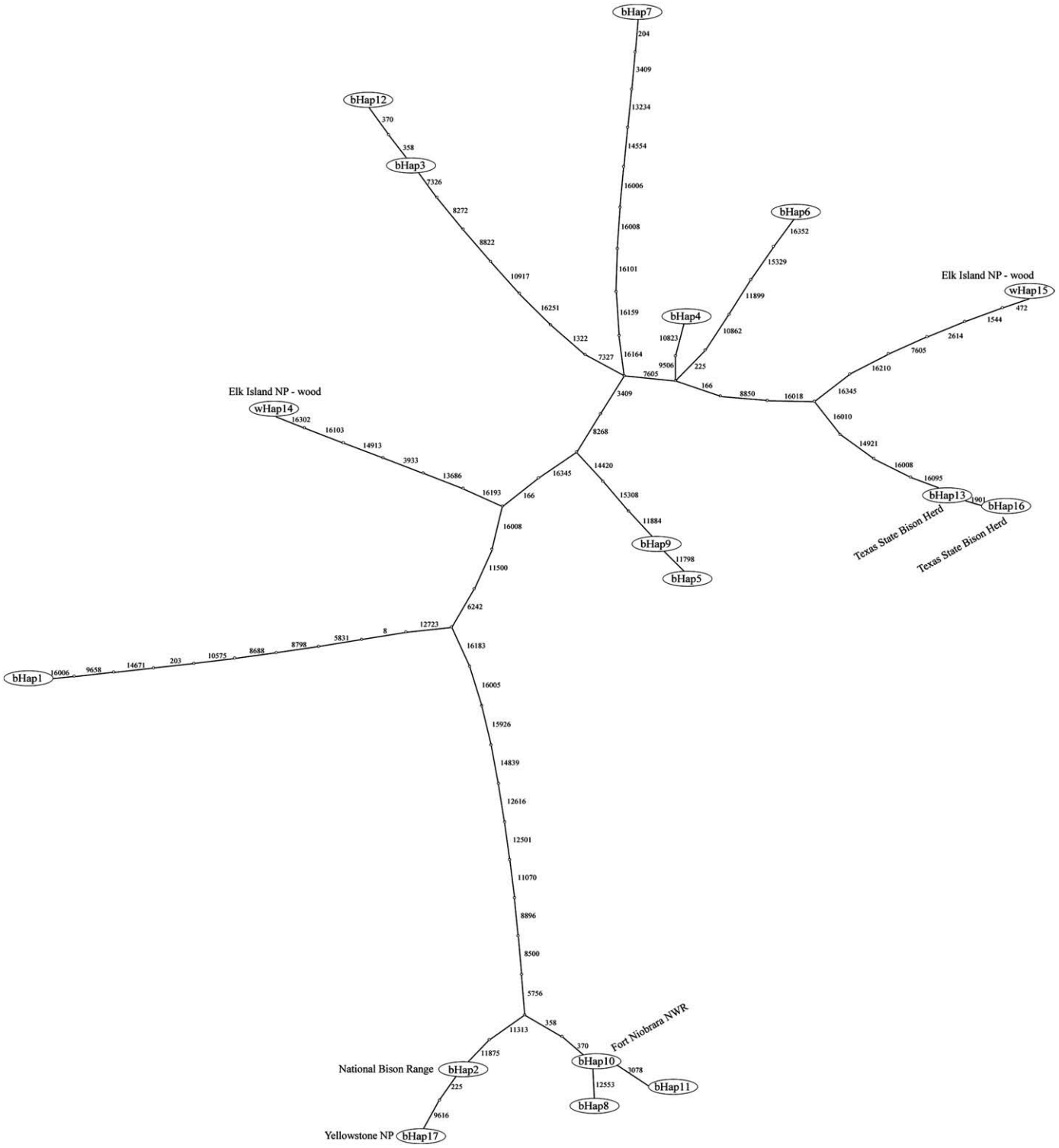

Fig. 1. Bison haplotype network. A spring tree network of 16 bison mtDNA genome haplotypes identified in this study and 1 previously published bison mtDNA genome (bHap1; Achilli et al., 2008) was created in TCS v1.21. Polymorphism locations are indicated by numbers between nodes (see Table 1 for gene location information). Haplotypes representing individuals from historically significant bison herds are indicated alongside the appropriate node (NP, National Park; NWR, National Wildlife Refuge).

mitochondrial genome is 16318–16323 bp long (Supplemental Materials Table 1) consisting of 13 protein-coding genes, 22 tDNA genes, 2 rDNA genes, and the D-Loop region. The gene order, length, and gene coordinates for the mtDNA genome are presented in Table 1. The gene order is conserved between bison and cattle. Including both SNPs and insertion–deletion events (indels), there are 989–995 differences between the bison haplotypes and the cattle genomic reference sequence. The length of the D-loop region varies by 4 bp across bison due to indels. Indels are also present in two genes within bison: 16s rRNA has a 1 bp indel at position 1880 and tRNA serine has a 2-bp variable indel at position 7301–7302. Furthermore, the stop codon for five protein-coding genes is completed by the addition of 3′ adenine residues to the mRNA (Table 1) as previously reported in cattle and other species (Achilli et al., 2008; Boore et al., 2005). There are also several regions in the bison genome where intergenic nucleotides exist or where two genes overlap (Table 1). Whether these regions represent a biological phenomenon or annotation errors is unknown; however, intergenic nucleotides have been reported in other species (Achilli et al., 2008; Boore et al., 2005).

In total, the 17 bison haplotypes included 66 polymorphic sites, 34 singleton variable sites (SNP found in only one animal), and a pair-wise average of 15.3 differences between any two sequences. For comparison, the 39 cattle haplotypes included 426 polymorphic sites, 261 singleton sites, and a pair-wise average of 46.2 differences. These results lead to a 3-fold lower nucleotide diversity (π) value for the analyzed bison haplotypes ($\pi = 0.00094$) than the cattle haplotypes ($\pi = 0.00283$). To illustrate the total number of differences among bison haplotypes, an unrooted haplotype network was created to map the number and position of polymorphisms (Fig. 1).

3.2. Bison–cattle hybridization analysis

Sequence analysis of the 13 protein-coding genes in bison compared to their homologs in hybrid animals revealed a large amount of sequence variation (Table 2). A total of 777 polymorphic sites were identified between bison and hybrid sequences with 728 fixed differences between the two groups. Of the fixed mutations, 642 synonymous and 86 non-synonymous mutations were identified.

To further assess the potential effects of the non-synonymous mutations on protein structure, we identified a total of 40 mutations predicted to cause an amino acid class change. The remaining 46 non-synonymous mutations result in amino acid substitutions within the same class (e.g., non-polar to non-polar).

We also examined the effects of SNPs and indels on tRNA structures between bison and hybrid haplotypes (Table 3). Our analyses indicate that 16 of the 22 tRNA genes annotated have a predicted sequence change in the D loop, T C loop, central loop, or one of the stems of the tRNA and that 9 of these have a sequence change at more than one of the stems and/or loops. A bulge or mis-pairing of nucleotides due to a 1 bp deletion in the hybrid haplotypes was predicted for tRNA-Asn (Fig. 2) and tRNA-Leu. All anticodon sequences were conserved between the bison and hybrid groups.

3.3. Phylogenetic analysis

Phylogenetic analysis of the *Bison* and *Bos* lineages is presented in Fig. 3 (see Supplemental Materials Table 3 for complete SNP table). Two major clades were identified in this analysis: the indicus/taurus clade including *Bos taurus*, *Bos indicus*, and hybrids, and the bison/yak clade including bison (plains and wood) and *Bos grunniens*. Within the indicus/taurus clade, *B. indicus* and *B. taurus* form into separate sub-clades (Fig. 3, I and II, respectively). According to our analysis, cHap56, which was only defined as "'Beef Cattle', Korea" by Achilli et al. (2008), is in the *B. indicus* lineage rather than the *B. taurus* lineage.

Within the taurus sub-clade, strong bootstrap values also support the grouping of cHap51–cHap54 (Chianina, Romagnola, Cinisara, and Agerolese cattle breeds) and cHap49, 50, and 55 (Romagnola, Chianina and Italian Red Pied cattle breeds) (Fig. 3, V and IV respectively). Another statistically significant branching event forms two sub-clades: one including three Angus haplotypes (cHap33, cHap35, and cHap19); and one including the Japanese Black, Angus, and all of the hybrid haplotypes (Fig. 3, III and II respectively). All the hybrid haplotypes group into one sub-clade although this branching pattern is not statistically significant.

We also examined the phylogenetic differences between the mtDNA genome sequences of Bull 86 and its clone, Bull 86^2 (Westhusin et al., 2007). Bull 86^2 harbors a mtDNA haplotype from the recipient egg cell used in the cloning procedure and therefore does not share the same mtDNA haplotype as the original Bull 86. In fact, the two genomes (cHap18 and cHap19 from Bull 86 and Bull 86^2, respectively) differ by 18 SNPs and 1 indel, and fall into two distinct cattle sub-clades (II and III, respectively). To our knowledge, this is the first mtDNA genome sequence and phylogenetic comparison of an animal and its clone. This type of information may prove valuable in understanding phenotypic variability among clones.

The bison/yak clade consists of two sub-clades divided by species: *Bison bison* (plains and wood bison; Fig. 3, VI) and *Bos grunniens* (yak; Fig. 3, VII). The large amount of analyzed sequence and high bootstrap values support the inclusion of the *Bison* genus within the *Bos* genus, with yak being more closely related to bison than to *Bos indicus* or *Bos taurus*.

A more detailed analysis of plains and wood bison lineages reveals significant population substructure with highly significant bootstrap values (Fig. 4; see Supplemental Materials Table 4 for complete SNP table). Using yak as an outgroup, we analyzed the substructure of the representative bison group. A previously published sequence

Table 2
Analysis of protein-coding genes. Sequence variations were recorded between bison and bison–cattle hybrid haplotypes.

Gene name	Abbreviation	Fixed synonymous	Fixed non-synonymous	Fixed total	Amino acid class changes	Total polymorphic sites
ATPase 6	ATP6	28	7	35	3	39
ATPase 8	ATP8	9	4	13	3	15
Cytochrome oxidase I	COI	74	1	75	0	78
Cytochrome oxidase II	COII	43	3	46	2	47
Cytochrome oxidase III	COIII	40	6	46	4	51
Cytochrome *b*	CYTB	67	11	78	4	85
NADH dehydrogenase 1	ND1	56	5	61	3	63
NADH dehydrogenase 2	ND2	64	6	70	3	74
NADH dehydrogenase 3	ND3	17	1	18	1	18
NADH dehydrogenase 4	ND4	85	10	95	3	104
NADH dehydrogenase 4L	ND4(L)	14	2	16	2	17
NADH dehydrogenase 5	ND5	114	25	139	11	147
NADH dehydrogenase 6	ND6(L)	31	5	36	1	39
	Total	642	86	728	40	777

Table 3
Predicted changes in tRNA structure between bison and bison–cattle hybrids. Gray-shaded squares indicate the predicted position of SNPs in the tRNA structure; black square indicates SNPs in both loop and stem structures.

Order from Table 1	Gene	Fixed differences[a]	Polymorphic dites[b]	D loop/stem	TψC loop/Stem	Central loop	Anticodon loop/stem	Acceptor arm stem
1	tRNA-Phe	0	0					
3	tRNA-Val	0	0					
5	tRNA-Leu	1	1					
7	tRNA-Ile	2	0					
8	tRNA-Gln	2	0					
9	tRNA-Met	2	0					
11	tRNA-Trp	0	0					
12	tRNA-Ala	0	1					
13	tRNA-Asn	6	0					
14	tRNA-Cys	1	0					
15	tRNA-Tyr	1	0					
17	tRNA-Ser	0	3					
18	tRNA-Asp	4	0					
20	tRNA-Lys	2	0					
24	tRNA-Gly	3	0					
26	tRNA-Arg	1	0					
29	tRNA-His	0	0					
30	tRNA-Ser2	4	0					
31	tRNA-Leu2	3	0					
34	tRNA-Glu	1	0					
36	tRNA-Thr	3	0					
37	tRNA-Pro	2	0					

[a]Indicates number of fixed differences between bison and hybrid groups.
[b]Indicates number of polymorphic sites within bison.

roots the bison clade (Achilli et al., 2008) and is divergent from the other bison haplotypes analyzed in this study (Fig. 4, III). Unfortunately, this sequence was obtained from a bison at the Antwerp Zoo in Belgium that was originally procured through the University of Utrecht with no data indicating ancestral geographical origins (Antonio Torroni, personal communication). A major sub-clade (Fig. 4, I) is formed from haplotypes from Fort Niobrara National Wildlife Refuge (bHap10), Yellowstone National Park (bHap17), the National Bison Range (bHap2), and the private bison herd. Another major sub-clade (Fig. 4, II) includes sequences from the Texas State

tDNA-asn alignment

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bison              CTAGACTGGTGGGCTCCACCCCCACGAAACTTTAGTTAACAGCTAAATACCCCAATTAATCAGGCTTCAATCTA
bison-cattle_hybrid CTAGACTGGTGGGCTCCACCCCCACGAAACTTTAGTTAACAGCTAAACACCCTAGCTAA-CTGGCTTCAATCTA
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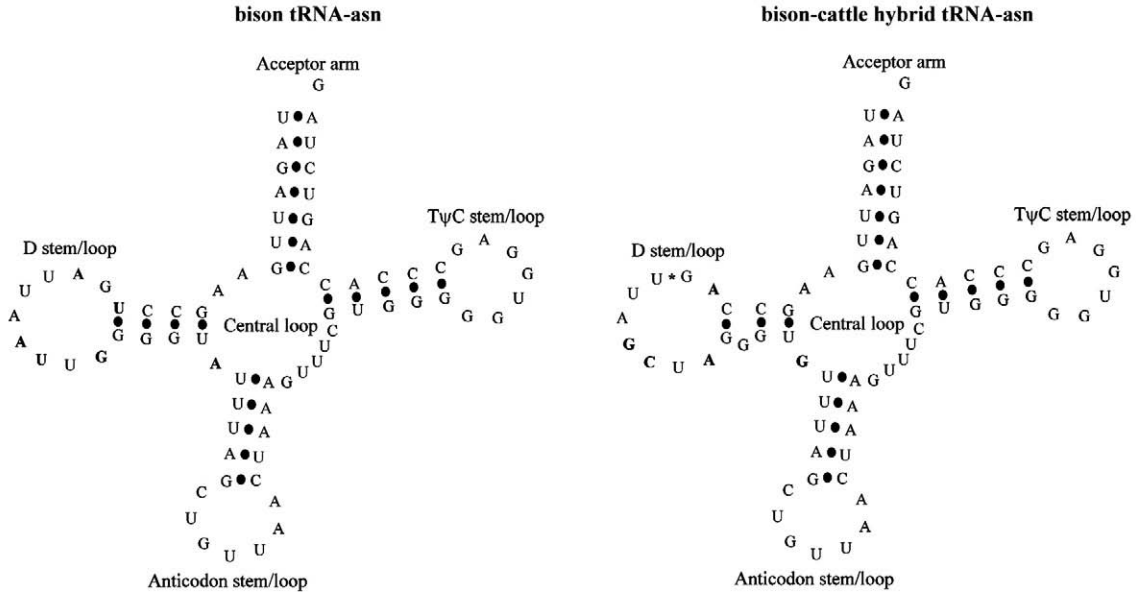

Fig. 2. DNA alignment and predicted tRNA secondary structures of bison and bison–cattle hybrid haplotypes for tRNA-asparagine (asn). The DNA alignment was created in Clustal X (Larkin et al., 2007) and the predicted tRNA secondary structures were created using tRNAscan-SE (Lowe and Eddy, 1997). Differences between the bison and bison–cattle hybrid haplotypes are indicated in bold typeface. An asterisk (*) in the bison–cattle hybrid tRNA structure indicates the location of the indel (missing A nucleotide).

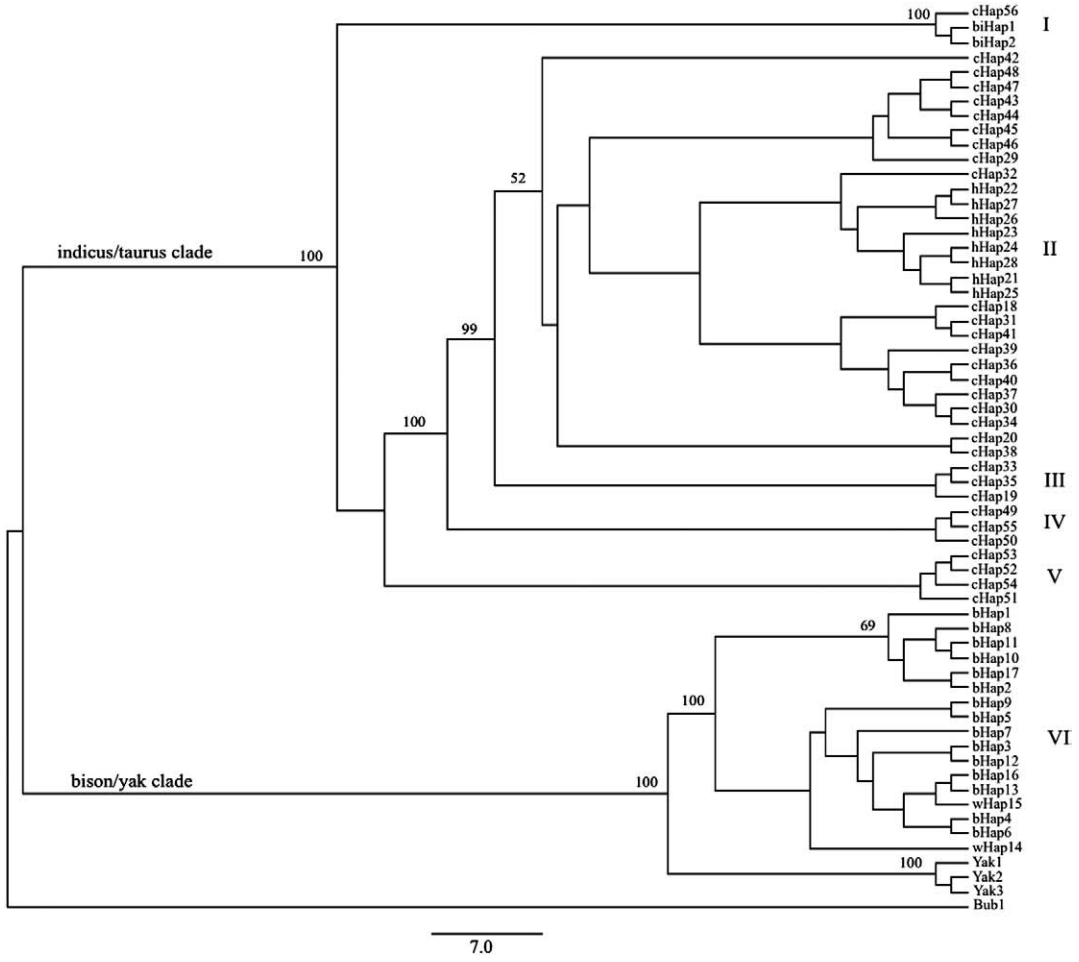

Fig. 3. Maximum likelihood phylogenetic tree of complete mtDNA haplotypes of plains bison *B. bison bison* (indicated with a "b" before haplotype number), wood bison, *B. bison athabascae* (indicated with a "w"), *Bos taurus* (indicated with a "c"), *Bos indicus* (indicated with a "bi"), Hybrid *Bos/Bison* (indicated with a "h"), *Bos grunniens* (Yak 1–3), and *Bubalus bubalis* (Bub1). Clades: I — *Bos indicus*; II — *Bos taurus* and hybrids; III — *Bos taurus* (Angus); IV — *Bos taurus* (Romagnola, Chianina, and Italian Pied); V — *Bos taurus* (Chianina, Romagnola, Cinisara, and Agerolese); VI — bison; and VII — yak. Bootstrap values were determined using 1000 replicates.

Bison Herd (bHap13 and bHap16), the private bison herd, and two wood bison from Elk Island National Park (wHap14 and wHap15), which form unique haplotypes but do not group together.

4. Discussion

Although the bison–cattle hybrids found in nearly all public and private herds are thought to have normal fertility (Halbert and Derr, 2007; Halbert et al., 2005), there are a large number of differences between the mtDNA genomes of the two species (*Bison bison* and *Bos taurus*). By comparing sequences from bison and hybrid animals, we identified at least one non-synonymous mutation in each of the 13 protein-coding genes, with NADH5, cytochrome *b*, and NADH4 harboring the largest number of mutations (25, 11, and 10, respectively; Table 2). In fact, the seven subunits of mitochondrial NADH dehydrogenase, which interact to form one large protein complex, have a total of 54 non-synonymous mutations and 24 amino acid class changes among all 7 subunits. Furthermore, differences between bison and hybrids were identified for 16 of the 22 tRNAs, which may affect tRNA secondary structure and function.

The critical nature of the mitochondria in cellular function and necessary interaction of multiple protein complexes for proper mitochondrial function suggest that the additive effects of such large numbers of non-synonymous mutations will likely affect mitochondrial function and the overall fitness of the organism. It has been demonstrated in species ranging from *Mus musculus* to *Caenorhabditis elegans* that mutations in single protein-coding genes that cause severe mitochondrial disease significantly decrease the fitness of offspring and are often eliminated in the germline. However, less severe mitochondrial gene mutations can be propagated through multiple generations despite negative effects on the organism's health (Fan et al., 2008; Liau et al., 2007). The effects of mutations in multiple protein-coding genes, rDNA genes, and tRNAs as well as their additive biological effects are currently unknown and additional studies are needed to fully understand the implications of these differences on fitness at individual and population levels.

Whereas novel combinations of nuclear alleles have been widely cited as the most likely cause of hybrid vigor (Arnold, 1997; Barton, 2001; Stebbins, 1959), reduced fitness or hybrid breakdown in interspecies hybrids may be explained by disruptions in mitochondrial function due to incompatibilities between the nuclear and mitochondrial genomes (Burton et al., 2006). These effects have been noted in an array of organisms including arthropods, plants, yeast, mice, insects, and birds (Ellison and Burton, 2008; Johansen-Morris et al., 2006; Lee et al., 2008; Nagao et al., 1998; Sackton et al., 2003; Tieleman et al., 2009). Bison–cattle hybrids are an excellent mammalian model to study these issues, since the large number of observed sequence differences — many of which presumably result in functional changes — likely affect nuclear–mitochondrial gene interactions and may also affect fitness.

The data generated in this study is also valuable in understanding the taxonomic classification of American bison, which has been

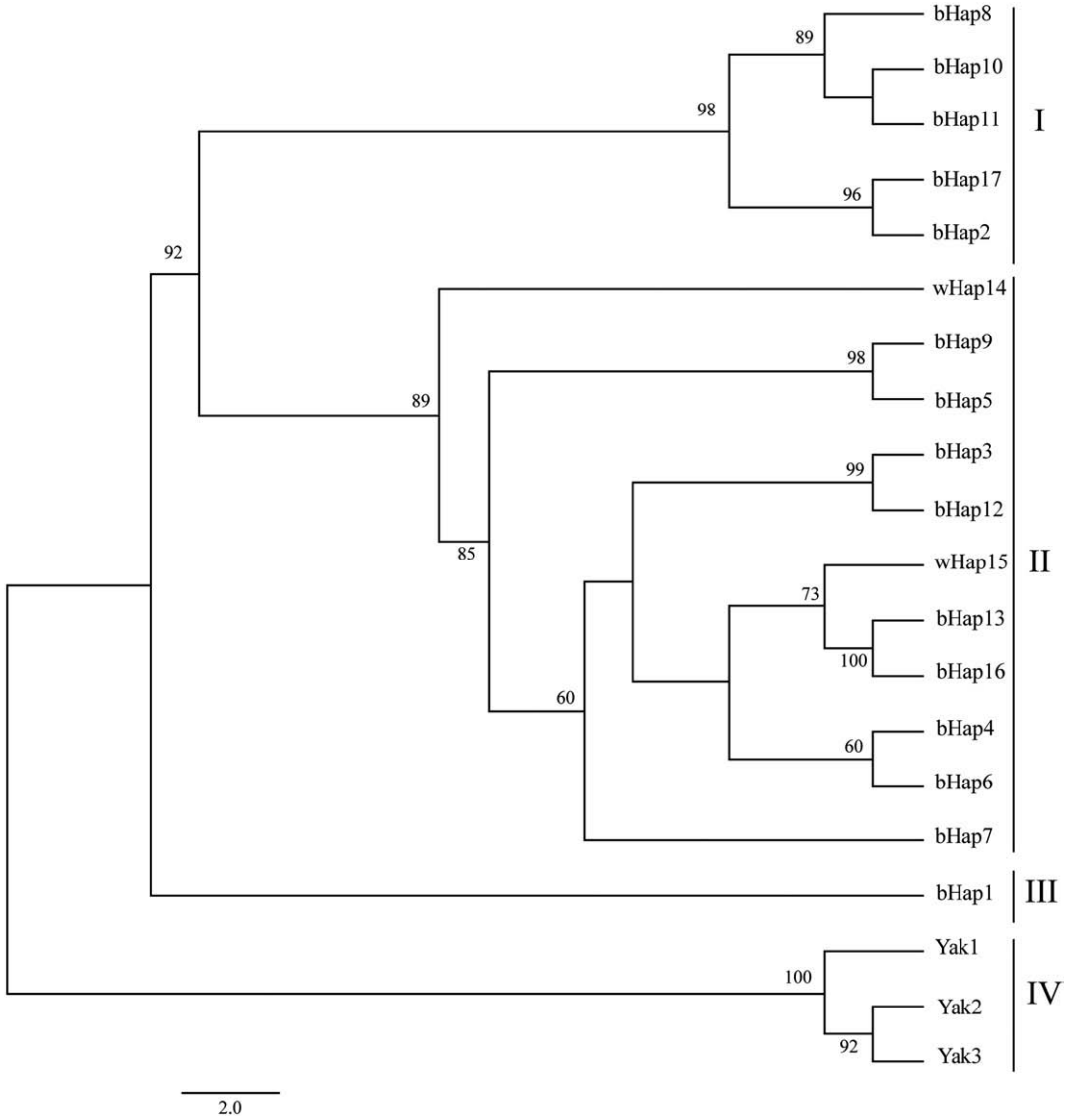

Fig. 4. Maximum likelihood phylogenetic tree of bison complete mtDNA haplotypes rooted by *Bos grunniens* (yak). Clades: I — plains bison (Fort Niobrara NWR, Yellowstone NP, National Bison Range, private herd); II — plains and wood bison (Texas State Bison Herd, private herd, Elk Island NP); III — previously published bison sequence (Achilli et al., 2008); IV — Yak. NP, National Park; NWR, National Wildlife Refuge. Bootstrap values generated by sampling 1000 replicates.

debated at the genus, species, and subspecies level. The paraphyletic nature of the *Bos* and *Bison* lineages has been shown based on both nuclear polymorphisms and analysis of individual mitochondrial regions (Burzynska et al., 1999; Decker et al., 2009; Janecek et al., 1996; Miyamoto et al., 1989; Wall et al., 1992). We also identified paraphyly of the *Bos* genus with respect to *Bison* based on whole mtDNA genome analysis, with bison and *Bos grunniens* forming a distinct clade from *Bos taurus* and *Bos indicus* (Fig. 3). Our data do not support the genus designation of *Bison*. The recent accumulation of molecular data, together with the fact that members of *Bison* can produce viable offspring with several species of *Bos* (van Gelder, 1977), indicate that the *Bison* and *Bos* genera should be reunited.

We identified significant phylogenetic substructure among bison (Fig. 4), which can be used to assess the relationship between the currently recognized subspecies of American bison (wood and plains). The two wood bison haplotypes do not form a single clade (wHap 14, 15) and are mixed with plains bison haplotypes (Figs. 1 and 4). The fact that both of these haplotypes fall into a clade with plains bison suggests that wood bison may never have been a genetically distinct subspecies, although it is also possible that one or both of these sequences are derived from the introduction of plains bison into wood bison herds in the 1920s (Banfield and Novakowski, 1960; Roe, 1970). Regardless of the source of these haplotypes, however, current populations of *B. bison bison* and *B. bison athabascae* are not significantly different with respect to their mitochondrial genomic sequences and should not be considered subspecies. It does appear, however, that the currently listed *B. bison athabascae* are an important source of genetic diversity for the species, since the two wood bison haplotypes were not identified in any of the plains bison populations (also see Wilson and Strobeck, 1999).

Haplotype analysis reveals further insights into the history and population structure of the bison species. Given the severe bottleneck experienced by bison in the late 19th century, when the total number of individuals in the species declined from approximately 30 million (Flores, 1991; McHugh, 1972) to less than 1000 (Coder, 1975; Soper, 1941), it was somewhat unexpected that 16 bison mtDNA haplotypes were identified in this study (Supplemental Materials Table 1; Fig. 1). Even excluding the haplotypes which differ by only 1–2 nucleotides (e.g., bHap12/bHap3), at least 10 distinct bison mtDNA types were identified in this study. Others have also identified high levels of genetic diversity in the bison nuclear genome (Halbert and Derr, 2008; Wilson and Strobeck, 1999), which may be due to the wide-

spread distribution of bison prior to and following the bottleneck, a short bottleneck length, and rapid population expansion following the bottleneck.

In addition, the relationships among bison haplotypes (Fig. 1) are generally reflective of the historical records of population establishment and genetic distances based on nuclear data (Halbert and Derr, 2008). For example, the close relationship of haplotypes identified at the National Bison Range (bHap2) and Yellowstone National Park (bHap17) is explained by the shared history of the herds (Halbert and Derr, 2007) and is also reflected in the nuclear genome (Halbert and Derr, 2008). Additionally, we found that the private bison herd sampled in this study harbors both unique haplotypes and haplotypes representative of several sources including Fort Niobrara National Wildlife Refuge (cHap10), the National Bison Range (cHap2), and the Texas State Bison Herd (cHap13) (Supplemental Materials Table 1). This finding is not surprising given the fact that, like many private herds, this herd was founded with bison from many sources.

5. Conclusion

By using modern sequencing technologies to obtain whole mtDNA genome sequences from several bison and bison–cattle hybrids in this study, we have examined the potential effects of hybridization between American bison and cattle, analyzed the phylogenetic relationship between *Bison* and *Bos*, constructed the first whole mtDNA phylogenetic tree of American bison, and identified population substructure and subspecific relationships among bison populations. Additional studies are now clearly needed to understand the effects of cattle mtDNA in bison on mitochondrial function and physiology, evaluate the potential for fitness differences due to cattle mtDNA in bison, and define the taxonomic relationship of European bison (*Bison bonasus*, the only other extant species in the *Bison* genus) to American bison and the *Bos* genus.

Supplementary materials related to this article can be found online at doi:10.1016/j.mito.2010.09.005.

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