Animal Conservation (1999) **2**, 51–57 © 1999 The Zoological Society of London Printed in the United Kingdom

Identification of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap

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(Received 11 June 1998; accepted 10 September 1998)

Abstract

Many species are currently undergoing reductions in population size due to widespread habitat loss and expanding human activities. Because interspecific hybridization is often a consequence of population decline and fragmentation, identification of individuals or populations with hybrid ancestry is an increasingly important issue in conservation biology. In many wild cattle and bison species, the problem of natural hybridization has been compounded by indiscriminate crossbreeding with domestic cattle for the purpose of improving domesticated stocks. Therefore, a genetic test using the polymerase chain reaction was developed so that wild cattle and bison with domestic cattle mitochondrial DNA (mtDNA) haplotypes could be rapidly identified. Using this genetic test, domestic cattle mtDNA haplotypes were detected in *Bos grunniens* (yak), *Bison bonasus* (European bison), and 6 out of the 15 (40%) *Bison bison* (North American bison) populations tested. In total, 30 out of the 572 (5.2%) North American bison tested, were found to have domestic cattle mtDNA. The hybrid origin of these mtDNA haplotypes was verified in a phylogenetic analysis using the parametric bootstrap. These results are discussed in terms of their implications for the conservation status and future management of wild cattle and bison species.

INTRODUCTION

Within the family *Bovidae*, overhunting and habitat destruction have caused drastic reductions in population size for most wild cattle and bison populations. At present, all populations of *Bos javanicus* (wild banteng) are listed as critical or endangered, *Bos grunniens* (yak) and *Bison bonasus* (European bison) are listed as vulnerable, and some populations of *Bos gaurus* (gaur) are considered critical while others have a low risk of becoming extinct (Heinen & Srikosamatara, 1996). In addition *Bos sauveli* (kouprey) have not been sighted since 1988, and may be extinct (Heinen & Srikosamatara, 1996). While *Bison bison* (North American bison) are not considered threatened, they have recently undergone a major bottleneck event, and the remaining semi-wild populations are largely publicly supported.

In addition to habitat loss and reductions in population size, many wild cattle and bison species are further threatened by hybridization with domestic cattle (*Bos taurus* and *Bos indicus*). Historically, the practice of hybridization with wild species has been used in efforts to improve the genetic characteristics of domesticated stock. For example, banteng were crossed with domestic cattle on the island of Madura nearly 1500 years ago and their descendants are currently distributed across Indonesia (National Research Council, 1983). Hybridization between yaks and domestic cattle has taken place throughout Asia, and Schaller & Wulin (1996) report hybrids in herds of wild yak from the Chang Tang Reserve of the Tibet Autonomous Region. Furthermore, North American bison were crossed with domestic cattle more than 100 years ago (Jones, 1907; M. Boyd, 1914; Goodnight, 1914; Polziehn *et al*., 1995), which may have resulted in the introgression of domestic cattle genes into bison populations that contributed to the founding stocks of some contemporary bison herds.

The problem of interspecific hybridization is not restricted to the wild relatives of domestic cattle. Widespread habitat alteration, population fragmentation, and the introduction of exotic or domestic species into areas inhabited by closely related species have resulted in greater opportunities for interspecific hybridization. Examples include the coyote and gray wolf (Lehman *et al*., 1991), Przewalski's horse and domestic horse (L. Boyd & Houpt, 1994), and various combinations of

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Kemp's ridley, loggerhead, hawksbill and green turtles (Karl, Bowen & Avise, 1995).

For endemic or threatened species, the negative consequences of interspecific hybridization can be both biological and legal. Interspecific hybridization can result in the disintegration of genetic integrity and the loss of native genetic variation or locally adapted gene complexes (genetic swamping). In addition, the presence of hybrid animals in remaining populations of threatened species may result in legal challenges to their protected status (O'Brien & Mayr, 1991; Hill, 1993; Rhymer & Simberloff, 1996). Consequently, interspecific hybridization has become an increasingly important issue in conservation biology.

Careful consideration of interspecific hybridization should be an integral part of developing effective conservation strategies. However, such a strategy requires the development of methods for correctly identifying individuals or populations with hybrid ancestry. Because of their rapid rate of substitution and clonal pattern of inheritance, mitochondrial markers have been effectively used to differentiate between closely related species and identify interspecific hybridization (Carr *et al*., 1986; Spolsky & Uzzell, 1986; Lehman *et al*., 1991; Wayne & Jenks, 1991; Painter, Crozier & Westerman, 1993; Avise *et al*., 1997; Mukai *et al*., 1997). Therefore, a simple genetic test, capable of rapidly identifying domestic cattle mitochondrial DNA (mtDNA) haplotypes, was developed using nucleotide sequence data from the mitochondria of four wild bovid species and 14 breeds of domestic cattle. This genetic test was then used to determine the scope of domestic cattle introgression in a sample of 572 North American bison from 14 public herds and one private herd. In addition, the ability of this test to discriminate between domestic cattle haplotypes, and those of European bison, yak and gaur was demonstrated (Table 1).

Table 1. Wild cattle and bison screened for the presence of domestic cattle mtDNA, using a domestic cattle specific oligonucleotide based genetic test

Species	Location	Sample Size
Bos gaurus	Omaha Zoo, NE	1
Bos grunniens	Brookfield Zoo, IL	1
	Kansas City Zoo, MO	1
	Sunset Zoo, KS	1
Bison bonasus (Czar's Herd)	Puschino Research Station, Russia	4
Bison bison athabascae (wood bison)	Elk Island National Park, Canada	19
	Mackenzie Bison Sanctuary, Canada	23
	Wood Buffalo National Park, Canada	23
Bison bison bison (plains bison)	Antelope Island State Park, UT	95
	Custer State Park, SD	34
	Elk Island National Park, Canada	25
	Finney Game Refuge, KS	26
	Fort Niobrara NWR, NE	34
	Henry Mountains, UT	21
	Maxwell Game Refuge, KS	39
	National Bison Range, MT	113
	Wind Cave National Park, SD	37
	Witchita Mountains NWR, OK	37
	Yellowstone National Park, WY	35
	Williams Ranch, TX	11

NWR, National Wildlife Refuge.

MATERIALS AND METHODS

The nucleotide sequence of a 677 base-pair (bp) segment from the highly variable control region of the mitochondrial genome was determined for *B. bison* (53 animals), *B. bonasus* (3 animals), *B. grunniens* (2 animals), *B. gaurus* (1 animal), *B. taurus* (Longhorn breed, 3 animals) and *B. indicus* (Nellore breed, 1 animal) (AF083353–AF083371). In addition, a nucleotide sequence from a single representative of the Charolais breed (*B. taurus*), two individuals from each of six additional *B. taurus* breeds (Angus, Hereford, Jersey, Friesian, N'Dama and Simmental), and two individuals from each of five additional *B. indicus* breeds (Butana, Kenana, Hariana, Sahiwal and Tharparker) was kindly provided by D. Bradley (Loftus *et al*., 1994). The region examined corresponds to that between nucleotides 15 854 and 188 in the domestic cow genome (Anderson *et al*., 1982).

Total genomic DNA was isolated from white blood cells by proteinase K treatment followed by phenol chloroform extraction (Sambrook, Fritsch & Maniatas, 1989). The entire control region was amplified using oligo-nucleotide primers located in the flanking $tRNA^{THR}$ (5'-AGAGAAGGAGAACAACTAACCTCC-3', 15695) and 12S rRNA (5'-AACAGGAAGGCTGGGACC-3', 457) genes using the polymerase chain reaction (PCR) (Saiki *et al*., 1988). The numbers after the primer sequences indicate the position of the 5' nucleotide in the domestic cow mitochondrial genome (Anderson *et al*., 1982). Amplification was performed using a modified version of the touchdown PCR method described by Don *et al*. (1991), and consisted of 45 s at 94° C (denaturation), 45 s at 60° C (annealing), and 45 s at 74° C (extension) in the presence of 2.5 mM $MgCl_2$ and 2.5 U of Taq polymerase (Promega). The annealing temperature was lowered 1° C every 2 cycles until it reached 55° C, which was used for 5 cycles. After this, the annealing temperature was dropped to 52° C for the remaining 20 cycles of a 35 cycle complete run. Amplification products were resolved on 1.5% (w/v) agarose gels and purified with QIAquick gel extraction columns (Qiagen).

Nucleotide sequences were determined using a dye terminator cycle sequencing kit with Amplitaq DNA polymerase FS and an ABI PRISM 377 automated DNA sequencer (Applied-Biosystems; Perkin Elmer). Several internal sequencing primers designed using MacVector 5.0 (International Biotechnologies Inc.) were used to determine the sequence of the 677 bp control region segment for the species examined, and these primer sequences will be made available upon request. Sequences were aligned using Clustal V (Higgins & Sharp, 1989) and manually inspected.

Estimates of nucleotide divergence were calculated using the program MEGA (Kumar, Tamura & Nei, 1993), using the Tamura & Nei (1993) correction, which was specifically developed for mitochondrial control

region sequences. Because the rate of nucleotide substitution is known to vary extensively from site to site in mammalian control region sequences (Kumar *et al*., 1993), a correction for among-site rate heterogeneity was incorporated using a continuous gamma distribution. The maximum likelihood estimate of the shape parameter (α) was determined using the method described by Yang (1994) as implemented in PAUP* version 4.0d63 (PAUP*) (Swofford, 1998). In order to simplify this analysis, only one individual from each domestic cattle breed was included in these comparisons. In addition, individuals from the two African *B. indicus* breeds (Butana and Kenana) were excluded from this analysis because of their problematic taxonomic placement (Loftus *et al*., 1994).

MacVector 5.0 (International Biotechnologies Inc.) was used to examine nucleotide sequences for primer sites that were conserved across all 14 breeds of domestic cattle and that were highly degenerate in wild cattle and bison species. The resulting oligonucleotide primers (5'-AGCTAACATAACACGCCCATAC-3', 15907 and 5'-CCTGAAGAAAGAACCAGATGC-3', 16264) were used in multiplex PCR reactions with highly conserved primers located in the 16S rRNA gene (5'-CCCGCCT-GTTTATCAAAAACAT-3', 2284 and 5'-CCCTCCG-GTTTGAACTCAGATC-3', 2878) (Derr *et al*., 1992). Samples for which the control region product amplified were considered to have domestic cattle mtDNA haplotypes, while the presence of a 590 bp 16S rRNA product served as an internal positive control for each individual reaction (Fig. 1). Amplifications consisted of 35 cycles of 30 s at 94° C (denaturation), 30 s at 55° C (annealing), and 1 min at 74° C (elongation), in the presence of 2.0 mM $MgCl_2$ and 2.5 U of Taq polymerase (Promega). However, efficient amplification of some less well preserved samples required slightly less stringent reaction conditions (54° C annealing temperature and 2.5 mM $MgCl_2$).

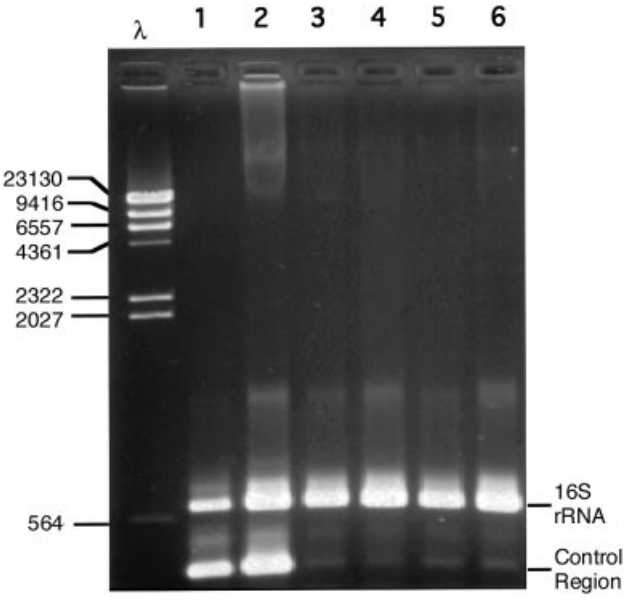

Fig. 1. Results of duplex PCR amplification of DNA from (1) *Bos taurus*, (2), *Bos indicus*, (3), *Bison bison*, (4) *Bison bonasus* (5) *Bos grunniens* and (6) *Bos gaurus* using mtDNA control region and 16S rRNA primers. Samples that produced the 357 bp mtDNA control region band possess domestic cattle mtDNA haplotypes.

In order to verify the results of the genetic test, mtDNA control region sequences were determined for all individuals identified as domestic cattle hybrids. Maximum parsimony analysis of all cattle and bison haplotypes identified, was conducted using the tree-bisection and reconnection (TBR) method of branch swapping and the heuristic search algorithm of PAUP* (Swofford, 1998). Maximum likelihood analysis was also conducted using the TBR method of branch swapping and the heuristic search algorithm of PAUP* (Swofford, 1998). However, due to the computational complexity of maximum likelihood analysis, a reduced data set of representative haplotypes sampled from the various clades of the parsimony tree was used. The model of Tamura & Nei (1993) was chosen for the maximum likelihood analysis, and a discrete approximation to the gamma distribution (TN+ dG_n model) with the number of rate categories determined by the method described by Yang (1994), was applied to account for among-site rate heterogeneity. The explanatory power of this model was assessed using the generalized likelihood ratio test of Goldman (1993) in order to determine if it provided an adequate representation of the pattern of molecular evolution for the sampled cattle and bison sequences.

The presence of haplotypes from wild cattle and bison species within domestic cattle clades of optimal parsimony or likelihood trees was considered as evidence of interspecific hybridization. Each of the hybridization events indicated by the phylogenetic trees (paraphyly) was treated as a separate hypothesis and was tested against a corresponding null hypothesis of monophyly, using the parametric bootstrap method as described by Hillis & Huelsenbeck (1994) and by Huelsenbeck, Hillis & Jones (1996). The optimal tree and model parameter estimates under each hypothesis of monophyly were obtained using maximum likelihood by constraining the presumably hybrid and native haplotypes identified in a given species to form a monophyletic group. The maximum likelihood estimates of the constraint tree topology, branch lengths and substitution parameters were then used to simulate 100 data sets with the program Seq-Gen 1.04 (Rambaut & Grassly, 1997). By analyzing each simulated data set using maximum likelihood and computing the difference in likelihood scores between the optimal tree for that data set and the best tree consistent with the null hypothesis of monophyly, the expected distribution of difference scores was obtained. This distribution was used to test the possibility that the non-monophyletic clades (consistent with hybridization) obtained from phylogenetic analyses resulted from systematic error when monophyly was true.

RESULTS

Observed levels of intraspecific variation were insufficient to negatively impact the discriminatory power of the genetic test. Mean intraspecific divergence was 0.89 (± 0.54)% and 0.71 (± 0.45)% respectively among the eight European and four Indian cattle breeds examined. The average genetic distance between North American bison haplotypes was 0.69 (± 0.27)%, while no variation was found among the three European bison or two yak sequences examined. Alternatively, large estimates of interspecific divergence between domestic cattle and wild cattle or bison species were obtained, and ranged from 12.28 (± 0.41)% to 29.53 (± 1.37)% (Table 2). Interestingly, the divergence between North American bison and yak (12.28 (± 0.41)%) was lower than expected, relative to that between the two bison species (17.73 (± 0.57)%), which suggested that an ancient hybridization event or phylogenetic lineage sorting may have taken place (Janecek *et al*., 1996). However, these estimates of genetic divergence, as well as those shown in Table 2, indicated that the haplotypes used to design domestic cattle specific oligonucleotides were not present in a given species due to recent interspecific hybridization.

The development of domestic cattle specific oligonucleotide primers provided a rapid method of differentiating between domestic cattle and wild cattle or bison mitochondrial haplotypes. As a result, one of the four European bison from Russia, and one of the three yak (Kansas City Zoo) were identified as having domestic cattle mtDNA. In addition, domestic cattle mtDNA haplotypes were found in 6 out of the 15 (40.0%) North American bison populations and 30 out of the 572 (5.2%) bison tested.

Animals identified using the genetic test as having domestic cattle mtDNA were further examined by determining the nucleotide sequence of the 677 bp control region segment as described above. Based on these sequences, four distinct haplotypes were identified among the 30 North American bison found to have domestic cattle mtDNA (Table 3). One of these haplotypes was unique to Custer State Park in South Dakota, where 7 out of the 34 (20.6%) bison tested had domestic cattle mtDNA. Another domestic cattle haplotype was found only in bison from the Williams ranch in west Texas, where all 11 of the animals tested had domestic cattle mtDNA. This haplotype was identical to a haplotype found in both Friesian and Hereford breeds of domestic cattle. A third haplotype was shared among bison from Custer State Park and the Williams Ranch. This haplotype was identical to a haplotype found in Friesian and Simmental breeds of domestic cattle. The fourth haplotype was present in all bison with domestic cattle mtDNA from the Maxwell (7 out of 39) and Finney (1 out of 26) State Game Refuges in Kansas as well as those from the National Bison Range (3 out of 113) in Montana and Antelope Island State Park (1 out of 95) in Utah.

The four mtDNA haplotypes from North American

Table 2. Percentage nucleotide divergence for 677 bp of the mtDNA control region, corrected using the Tamura–Nei model (above diagonal) with between-site rate heterogeneity (below diagonal, $\alpha = 0.15$)

	Bos taurus	*Bos indicus*	*Bos grunniens*	*Bos gaurus*	*Bison bonasus*	*Bison bison*
Bos taurus	-	5.42 ± 0.28	12.10 ± 0.26	11.53 ± 0.25	9.77 ± 0.34	9.91 ± 0.24
Bos indicus	8.18 ± 0.63	-	11.17 ± 0.14	12.25 ± 0.18	10.25 ± 0.35	10.47 ± 0.43
Bos grunniens	29.53 ± 1.37	24.23 ± 0.56	-	10.25 ± 0.00	8.82 ± 0.00	7.36 ± 0.13
Bos gaurus	25.81 ± 1.04	28.20 ± 1.00	21.60 ± 0.00	-	12.83 ± 0.00	10.47 ± 0.28
Bison bonasus	19.00 ± 1.18	21.06 ± 1.20	16.59 ± 0.00	28.70 ± 0.00	-	9.44 ± 0.17
Bison bison	19.72 ± 0.85	24.73 ± 1.22	12.28 ± 0.41	21.16 ± 1.14	17.73 ± 0.57	-

Table 3. Haplotypic frequency data for 83 North American bison control region sequences. Haplotypes identified as having a domestic cattle origin are marked with an asterisk (*). Wood bison populations are denoted with a (W)

	1	2	3	4	5	6	7	8	9*	10*	11*	12*
Elk Island National Park (W)				3				1				
Mackenzie Bison Sanctuary (W)			1	2				2				
Wood Buffalo National Park (W)			2	1	1							
Antelope Island State Park					4				1			
Custer State Park						1		1			4	3
Elk Island National Park	1					2	1	1				
Finney Game Refuge									1			
Fort Niobrara NWR						5						
Henry Mountains						5						
Maxwell Game Refuge									7			
National Bison Range						1	2	2	3			
Wind Cave National Park						5						
Witchita Mountains NWR		1				3						
Yellowstone National Park						3		2				
Williams Ranch										2	9	
Total Number of Individuals	1	1	3	6	5	25	3	9	12	2	13	3

bison identified as originating from domestic cattle had an average genetic distance of 19.36 (± 1.04)% from the eight native North American bison haplotypes identified, and a difference of only 0.77 (± 1.10)% from the eight European cattle breeds examined. Similarly, the European bison identified by the genetic test as having domestic cattle mtDNA differed by 16.91 (± 0.00)% from the other European bison that were sequenced, but only by 0.87 (± 0.44)% from the eight European cattle breeds examined. Finally, the single yak identified as having domestic cattle mtDNA differed by 26.85 (± 0.00)% from the other yaks examined, but only by 0.69 (± 0.45)% from the four Indian cattle breeds.

The results of phylogenetic analysis corroborated the conclusion that mtDNA haplotypes from the North American bison, European bison and yak, identified using the genetic test as having domestic cattle mtDNA, were of domestic cattle origin. In the maximum parsimony result, the domestic cattle haplotypes identified in North American bison and European bison were located within a largely unresolved clade of European and African cattle breeds, while the domestic cattle haplotype identified in yak was located within a clade of Indian cattle breeds (Fig. 2). Maximum likelihood analysis was conducted using the TN+ dG_4 model of molecular evolution, and resulted in a single tree (Fig. 3) which was completely congruent with the results from maximum parsimony analysis. The TN+ dG_4 model was accepted as having adequate explanatory power because it did not provide a significantly worse fit to the data than the theoretically perfect fit of the multinomial ($P > 0.40$).

Constraining the maximum likelihood topology so that European bison, North American bison, or yak haplotypes were monophyletic resulted in likelihood scores that were greater (less optimal) than that of the global maximum likelihood topology (Fig. 3) by 17, 28 and 34 units respectively. Using the parametric bootstrap method, it was determined that the probability of obtaining likelihood score differences as great as those observed, when the null hypothesis of monophyly was true, was $P < 0.05$ for all three hypotheses of monophyly. Therefore, the hypotheses of monophyly were rejected and the hypotheses that domestic cattle introgression has occurred in North American bison, European bison and yak were accepted as significantly greater explanations of the observed data.

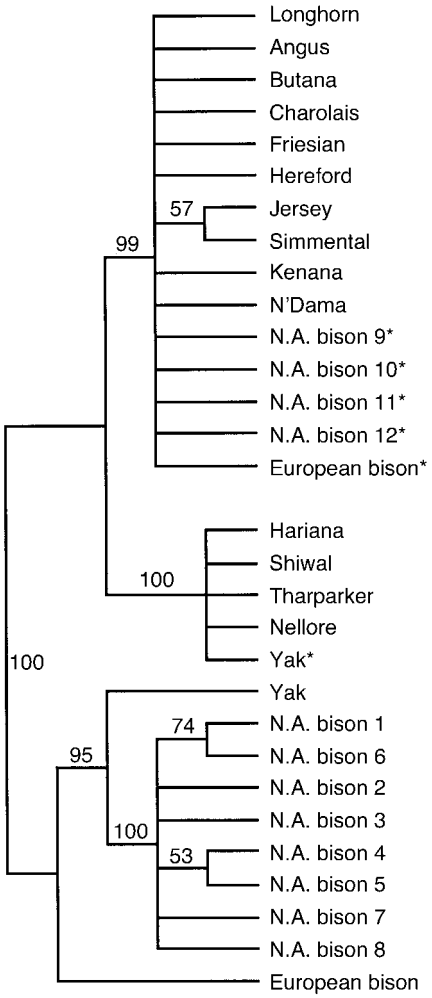

Fig. 2. Majority rule bootstrap consensus tree from maximum parsimony. The tree score = 193 steps, the consistency index (excluding uninformative characters) = 0.62, and the retention index = 0.91. The frequency (%) with which a given branch was recovered in 2000 bootstrap replications is shown above all branches recovered in more than 50% of bootstrap replicates. Haplotypes from wild cattle and bison species which were determined by the genetic test to have a domestic cattle origin are marked with an asterisk (*). N.A., North American.

DISCUSSION

The results of screening wild cattle and bison species with the PCR-based genetic test indicated that hybridization between domestic cattle and North American bison, European bison and yak had occurred. This conclusion was supported by phylogenetic analyses in which parametric bootstrap results demonstrated that a topology consistent with hypotheses of interspecific hybridization had significantly ($P < 0.05$) greater support than did topologies in which North American bison, European bison, or yak haplotypes were monophyletic. While the results of these phylogenetic analyses were consistent with interspecific hybridization, such results could also be produced by non-phylogenetic lineage sorting. However, the fast rate of reciprocal monophyly for mtDNA markers (Moore, 1995) and the extremely small genetic distances between presumably hybrid haplotypes and those of domestic cattle make the hypothesis of lineage sorting implausible. In addition, the observation that domestic cattle introgression in the two bison species involved breeds from the European and African domestic cattle lineage, while Indian cattle breeds were involved in hybridizations with yak, is consistent with historical accounts of hybridization events (Jones, 1907; M. Boyd, 1914; Goodnight, 1914; National Research Council, 1983), and with the geographic distributions of

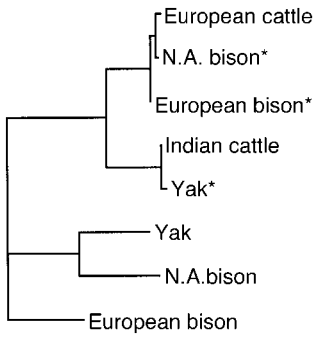

Fig. 3. Maximum likelihood topology constructed using the TN+ dG_4 model of molecular evolution. The –ln likelihood for this tree = 1550.94951. Haplotypes from wild cattle and bison species which were determined by the genetic test to have a domestic cattle origin are marked with an asterisk (*). N.A., North American.

the two bison species, yak, and the two domestic cattle species (Macdonald, 1984; Loftus *et al.*, 1994). Therefore, the results of this study indicate that independent hybridization events have occurred between domestic cattle and North American bison, European bison and yak.

For threatened or publicly supported species, the identification of populations with hybrid ancestry leads to the question of how to deal with these populations in the future management of the species. The simplest approach to dealing with interspecific hybridization is to simply ignore it, thus accepting some level of genetic introgression as inevitable. This may be the only real option for endemic or highly threatened species that consist of only a few remaining individuals or a single small population. However, this would be an unsatisfactory approach for species with less extreme circumstances, and could result in the introduction of hybrid individuals into genetically pure populations. This appears to have been the case for the National Bison Range and Antelope Island State Park bison herds, where park records (D. Wiseman, pers. comm.; K. Sherman, pers. comm.) and the results of the genetic analyses presented here indicate that animals with domestic cattle mtDNA haplotypes were present as a result of transfers from the Maxwell State Game Refuge bison herd.

For many species, the most practical approach for dealing with introgression is to use genetic markers, such as those developed here, to identify populations with hybrid ancestry and eliminate them from conservation efforts such as captive breeding and reintroduction programs. This would prevent the problem of interspecific hybridization from being exacerbated by the introduction of hybrid individuals into genetically pure populations. However, this strategy is only possible when multiple populations exist, and when remaining populations have large enough effective population sizes to prevent the erosion of genetic diversity. Therefore, accurate ecological and population genetic information will be required in order to determine if this approach is appropriate for a given species.

Full assessment of the impact of domestic cattle introgression on North American bison, European bison and yak populations will require information from multiple independent nuclear loci, in addition to mtDNA data, so that all populations with hybrid individuals can be identified, and the frequency of domestic cattle genes within these populations can be determined. In addition, because the number of European bison and yak examined was limited by the difficulty in obtaining samples from these relatively rare and exotic animals, additional genetic and demographic information will be required to determine the extent of domestic cattle introgression in these species and the appropriate measures for dealing with this issue in future conservation efforts. In the case of North American bison however, there are a number of fairly large populations for which there is no evidence of domestic cattle introgression. Therefore, it seems prudent to maintain these populations in isolation from those with hybrid ancestry until a more complete picture of the impact of this hybridization can be developed. Even if the expected levels of introgression are low, it seems unwise to allow animals with hybrid ancestry to be transferred into presumably pure populations if other options are available.

As indicated by the wild cattle and bison examples, human activities may be increasing the opportunities for interspecific hybridization in many species. Attempts to deal with interspecific hybridization can be confounded by other problems faced by threatened species, such as the loss of genetic variability and localized population extinction. Therefore, because the consideration of genetic purity in conservation plans will generally involve trade-offs with other aspects of species conservation, the application of information gained about hybrid animals is necessarily dependent on the individual circumstances of the species examined. However, by ignoring the issue of interspecific hybridization, negative consequences could be produced by otherwise well intentioned conservation action. Therefore, generally applicable approaches for detecting hybrid individuals, and rigorous statistical methods of documenting introgression, such as those presented here, are required for making responsible conservation decisions.

Acknowledgments

We are very grateful to the zoos, park managers and landowners who allowed us access to their animals. We are also grateful to Mark Drew and Julie Schneider for assistance in collecting samples, and to Jim Womack and Rodney Honeycutt for providing additional samples. Dan Bradley kindly provided sequence data for several domestic cattle breeds. Claire Kolenda, Doug Melendy, Heather Streppa and Natalie Rayford provided technical assistance and Don Davis, Rodney Honeycutt, Lynn Lamoreux, Alex Rooney, Bob Schnabel and Jim Womack provided helpful comments and discussion. This work was supported by a National Science

Foundation grant DEB-9622126 (J.N.D., J.W.T.) and was conducted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Genetics at Texas A & M University (T.J.W.).

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