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SHORT COMMUNICATION

Genetic diversity of the captive Asian tapir population in Thailand, based on mitochondrial control region sequence data and the comparison of its nucleotide structure with Brazilian tapir

Yuttamol Muangkram[a,b,c], Akira Amano[a], Worawidh Wajjwalku[c], Tanu Pinyopummintr[c], Nikorn Thongtip[c], Nongnid Kaolim[c], Manakorn Sukmak[c], Sumate Kamolnorranath[d], Boripat Siriaroonrat[d], Wanlaya Tipkantha[d], Umaporn Maikaew[e], Warisara Thomas[f], Kanda Polsrila[g], Kwanreaun Dongsaard[h], Saowaphang Sanannu[i] and Anuwat Wattananorrasate[j]

[a]Graduate School of Life Sciences, Ritsumeikan University, Kusatsu, Shiga Japan; [b]The Graduate School, Kasetsart University, Jatujak, Bangkok, Thailand; [c]Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand; [d]Bureau of Conservation, Research and Education, Zoological Park Organization, Dusit, Bangkok, Thailand; [e]Khao Kheow Open Zoo, Sriracha, Chonburi, Thailand; [f]Nakhon Ratchasima Zoo, Muang, Nakhon Ratchasima, Thailand; [g]Song Khla Zoo, Muang, Song Khla, Thailand; [h]Chiang Mai Zoo, Muang, Chiang Mai, Thailand; [i]Dusit Zoo, Dusit, Bangkok, Thailand; [j]Safari World Public Company Limited, Klongsamwa, Bangkok, Thailand

ABSTRACT

The Asian tapir (*Tapirus indicus*) has been classified as Endangered on the IUCN Red List of Threatened Species (2008). Genetic diversity data provide important information for the management of captive breeding and conservation of this species. We analyzed mitochondrial control region (CR) sequences from 37 captive Asian tapirs in Thailand. Multiple alignments of the full-length CR sequences sized 1268 bp comprised three domains as described in other mammal species. Analysis of 16 parsimony-informative variable sites revealed 11 haplotypes. Furthermore, the phylogenetic analysis using median-joining network clearly showed three clades correlated with our earlier cytochrome *b* gene study in this endangered species. The repetitive motif is located between first and second conserved sequence blocks, similar to the Brazilian tapir. The highest polymorphic site was located in the extended termination associated sequences domain. The results could be applied for future genetic management based in captivity and wild that shows stable populations.

Introduction

The Asian or Malayan tapir (*Tapirus indicus,* Desmarest 1819) is scientifically classified in the family Tapiridae that has four extant species (Carter 1984). The Asian tapir is only found in Southeast Asia and it is isolated from the other three species, which are found in the Neotropical Americas. The recent geographic distribution of the Asian tapir includes Thailand, Myanmar, Malaysia, and the island of Sumatra in Indonesia (Corbet & Hill 1992). The population is now categorized as Endangered on the 2008 IUCN red list of threatened species (Lynam et al. 2008) resulting from the main threat, i.e. habitat loss (Medici et al. 2003), although it is now showing signs of recovery following wide-scale protection of their preferred habitat (Lynam et al. 2012).

Little genetic information is known about the wild and captive Asian tapir populations (Rovie-Ryan et al. 2008; Ogata et al. 2009; Muangkram et al. 2013b). Recently, the full length of complete mitochondrial genome is elucidated by our previous study (Muangkram et al. 2016). The genetic studies in this endangered species provide the valuable tools for the management of threatened species (Schwartz et al. 2007). The rapidly evolving mitochondrial DNA (mtDNA) genome serves as a powerful tool for studying evolutionary relationships, both intra-species and inter-species (Brown et al. 1979; Kocher et al. 1989). The mtDNA is commonly used in genetics studies because of the effects of maternal inheritance (Hutchison et al. 1974; Giles et al. 1980). Earlier genetic diversity studies in the Asian tapir focused primarily on the *cytochrome b* (*Cytb*) gene sequence (Rovie-Ryan et al. 2008; Ogata et al. 2009; Muangkram et al. 2013b). For example, in a recent study, we describe seven haplotypes of the Asian tapir based on cytochrome *b* sequences (Muangkram et al. 2013b). Three of these are present in the Thai captive population including Ti-1 (48%), Ti-2 (36%), and Ti-3 (16%). The results could predict that three of those originated from different geographic range. This genetic information could provide the strategic plan for the future breeding program in Thai zoos and also encourages the conservation genetic of their diversity both in captivity and wild.

The most variable segments of the mtDNA genome are located in the control region (CR) or displacement loop (D-loop) in most mammal species (Cann et al. 1984), including

Asian tapirs (Muangkram et al. 2016). The mitochondrial control region contains the major regulatory elements for replication and expression of the mitochondrial genome and it is commonly used to determine the intra-specific level of genetic diversity among populations (Sbisà et al. 1997). The composition of the CR includes three domains: extended termination associated sequences (ETAS), the central domain, and conserved sequence blocks (CSB) (Sbisà et al. 1997). The ETAS contains two blocks that are conserved in most mammal species (ETAS1 and ETAS2), and the CSB is also strongly conserved with three blocks (CSB1, CSB2, and CSB3). CSB1 in particular is an essential element of the mammalian mtDNA genome (Saccone et al. 1987; Sbisà et al. 1997). To date, the CR sequences of two Tapiridae species have been deposited in GenBank including one haplotype of Brazilian tapir (accession number AJ428947) (Arnason et al. 2008) and three haplotypes of Asian tapir (accession number NC023838, KJ417808, and KJ417810) from our previous study (Muangkram et al. 2016).

In this study, we described the first phylogenetic analysis of the CR sequence of captive Asian tapirs in Thailand. The sample analysis was also including the DNA representatives from our previous study (Muangkram et al. 2013b) and additional specimens. The results give the genetic information for matching of this endangered species in conservation breeding program to maintain the genetic diversity. Furthermore, we also comparatively analyzed the nucleotide structure between Asian tapir and Brazilian tapir to provide the basic knowledge of genetic relationship within Tapiridae species.

Methods

We collected 37 blood and hair samples from captive Asian tapirs held in zoos under the Zoological Park Organization of Thailand (Dusit Zoo, Khao Kheow Open Zoo, Chiang Mai Zoo, Nakhon Ratchasima Zoo, and Song Khla Zoo) and one private zoo. Blood samples were collected using our previous method (Muangkram et al. 2013a) and hair samples were kept in zip bags. DNA was extracted using a modified phenol/chloroform technique with ethanol precipitation (Nelson & Krawetz 1992). The full-length of the CR was amplified using DreamTaq® DNA

Polymerase (Fermentas International, Saskatchewan, Canada) and one primer pair: the forward primer on $tRNA^{Pro}$ 5′-CAC CCA TCA ACA CCC AAA GCT-3′ (Muangkram et al. 2016) and the reverse primer on $tRNA^{Phe}$ 5′-GGG CAT CTT CAG TGC CTC GCT T-3′ (Ward et al. 1999). The total reaction volume for each PCR was 50 μl, which included 5 μl of 10× Taq buffer with $(NH_4)_2SO_4$, 5 μl of dNTP (0.2 mM of each), 1 μM of each primer, 4 mM of $MgCl_2$, 1.25 U Taq polymerase, 1 μg of template DNA, and nuclease-free water. PCRs were performed using an initial denaturation step of 3 min at 95 °C followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 54 °C, and extension for 60 s at 72 °C, and final incubation at 72 °C for 10 min. The desired PCR product sized 1355 bp in length was first confirmed using 1.5% agarose gel electrophoresis and then purified using the GeneMark Gel Elution kit (GeneMark Inc., Taipei, Taiwan) according to the manufacturer's instructions. All confirmed PCR products were sent to 1st BASE Laboratories in Malaysia for DNA sequencing. The complete CR sequences from this study have been deposited in GenBank under accession numbers KM100153–KM100189.

The 37 CR sequences were aligned using CLUSTALW (Thompson et al. 1994) via MEGA6 (Tamura et al. 2013). The sequence was located using the complete mitochondrial genome of Asian tapir accessed from GenBank accession number NC023838 (Muangkram et al. 2013b). The nomenclature of the haplotype followed an earlier $Cytb$ gene study of captive Asian tapir in Thailand by grouping into Ti-1, Ti-2, and Ti-3 (Muangkram et al. 2013b). Polymorphism and nucleotide diversity were calculated using DnaSP v5 (Librado & Rozas 2009). Finally, a phylogenetic analysis was constructed using a median-joining (MJ) network illustrated via NETWORK 4.6.1.2 (Bandelt et al. 1999) to estimate the intra-specific relationship among populations.

Results

The CR sequences were represented on the heavy chain and bound by $tRNA^{Pro}$ and $tRNA^{Phe}$ as in other mammal species. Our samples ($N=37$) were divided into 11 maternal lineages (Table 1). The complete CR was 1268 bp in length comprising three typical

Table 1. Our results showed 11 haplotypes with 16 polymorphisms based on 1268 bp in length of the control region of captive Asian tapirs.

	ETAS													Central	CSB		
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	6	
	5	5	6	6	6	6	6	6	6	6	7	7	7	7	0	1	
	7	9	0	0	2	3	4	4	4	6	9	5	5	8	9	2	
Haplotype	8	3	0	9	0	7	3	4	5	5	9	3	6	2	7	8	N
Ti-1A	G	G	A	C	G	G	T	A	T	C	A	A	C	C	T	A	10
Ti-1B	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	.	1
Ti-1C	.	A	G	.	.	.	.	.	.	.	.	G	.	.	C	.	4
Ti-2A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C	.	2
Ti-2B	.	.	.	.	.	.	.	G	.	.	.	.	.	T	C	.	3
Ti-2C	.	.	.	.	.	.	.	.	.	T	.	.	.	T	C	.	1
Ti-2D	.	.	G	.	A	.	.	.	.	T	.	.	.	T	C	.	1
Ti-2E	.	.	.	T	A	.	.	.	.	.	.	.	.	T	C	.	5
Ti-3A	A	.	.	.	.	.	.	.	C	.	G	.	.	T	C	.	2
Ti-3B	A	.	.	.	.	.	C	.	C	.	G	.	T	T	C	.	6
Ti-3C	A	.	.	.	A	A	C	.	C	.	G	.	.	T	C	G	2
															Total		37

The highest variation was on extended termination associated sequences (ETAS). Base numbers of the sequences correspond to the complete mitochondrial DNA genome of the Asian tapir (NC023838). Dots indicate nucleotides identical to *Tapirus indicus* haplotype Ti-1A.

domains: ETAS (24.13%), central domain (24.84%), and CSB (51.03%). Two blocks of ETAS (ETAS1 and ETAS2) and three blocks of CSB (CSB1, CSB2, and CSB3) were conserved as in other mammal species. The comparison of nucleotide structure found that the total CR length of the Asian tapir was 58 bp shorter than the Brazilian tapir as a result of shorter lengths of the ETAS, central domain, and CSB by 11, 1, and 46 bp, respectively. However, the nucleotide composition between these two species was similar in each domain. In addition, the analysis of polymorphism based on CR sequences between the Asian tapir and Brazilian tapir found 303 polymorphic sites (22.9%).

Sixteen parsimony-informative variable sites (all transition substitutions) revealed 11 haplotypes across our captive Asian tapir samples originated from Thailand (Table 1). The variable site was 1.26% of CR region length. The highest polymorphic site was located in the ETAS domain with thirteen variable sites. The nucleotide diversity (π) was 0.00353 ± 0.0028 and the sequence conservation was 0.987. Haplotype diversity (Hd) was 0.877 ± 0.031. The most common nucleotide within the ETAS and CSB domains was adenine, and the most common within the central domain was thymine. Overall, the $G+C$ (0.3914) content was lower than the $A+T$ content as has been observed in other organisms. As expected, the long repetitive sequence 5'-(CACACGTATACGCATA)$_{18}$-3' was located between CSB1 and CSB2. Comparing the repetitive sequences of the Asian tapir with Brazilian tapir showed it was also located between CSB1 and CSB2, but the pattern in the Brazilian tapir was different with 5'-(TACRCAYACGYW)$_{27}$-3'.

The most common haplotype was Ti-1A (27.0%). The other populations mostly belonged to haplotype Ti-3B, Ti-2E, Ti-1C, and Ti-2B by 16.2%, 13.5%, 10.8% and 8.1%, respectively. Only two individuals were found in haplotype Ti-2A, Ti-3A, and Ti-3C. Haplotype group of Ti-1B, Ti-2C, and Ti-2D were only represented by a single individual (Table 1). The intra-specific level among our samples was determined using a MJ network based on 1268 bp sequence (Figure 1). Three clades in the captive Thai population could be divided into three haplotypes of clade Ti-1 (40.54%) (including Ti-1A, Ti-1B, and Ti-1C), five haplotypes of clade Ti-2 (32.43%) (including Ti-2A, Ti-2B, Ti-2C, Ti-2D, and Ti-2E), and three haplotypes of clade Ti-3 (27.03%) (including Ti-3A, Ti-3B, and Ti-3C).

Discussion

The organization of CR elements and the repetitive sequences between CSB1 and CSB2 of Asian tapir was similar to the dominant populations of Perissodactyla species such as Brazilian tapir, white rhinoceros, black rhinoceros, Indian rhinoceros, donkey, and horse (Jama et al. 1993; Xu et al. 1996; Sbisà et al. 1997; Xu & Arnason 1997; Arnason et al. 2008). These typical patterns have also been observed in some Carnivora species such as gray seal, harbor seal, and elephant seal, although they differ from domestic cat, Artiodactyla species, and Cetaceans (Hoelzel et al. 1994; Sbisà et al. 1997). The identified repeat motif in the Asian tapir differed markedly from the motif identified in the single Brazilian tapir sequence currently deposited in GenBank. The Brazilian tapir motif was shorter 4 bp, but occurred at a higher copy number (27 copies). Overall, this resulted in a longer CR region in Brazilian tapirs
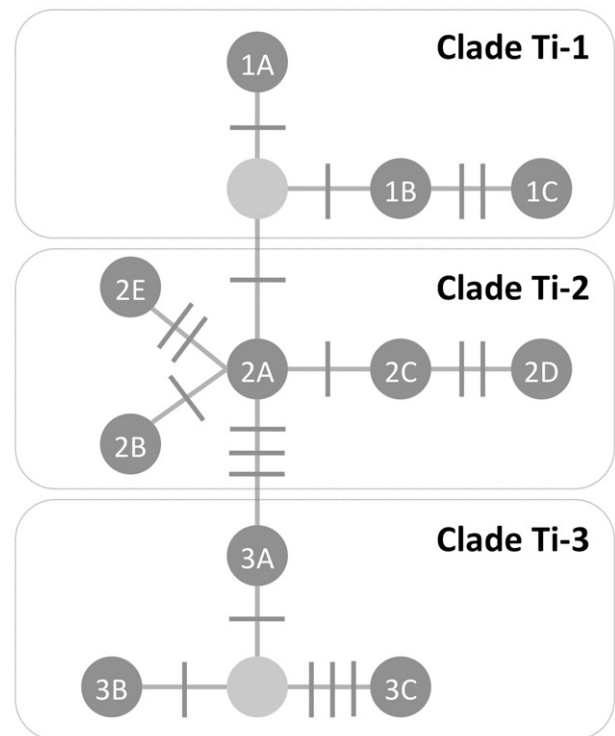

Figure 1. MJ network analysis based on 1268 bp in length of the control region divided the eleven haplotypes of captive *Tapirus indicus* populations into three major lineages: clade Ti-1 (three haplotypes: 1A, 1B, and 1C), clade Ti-2 (five haplotypes: 2A, 2B, 2C, 2D, and 2E), and clade Ti-3 (three haplotypes: 3A, 3B, and 3C). The light gray circles refer to the mutated position. The gray crossbars refer to the number of nucleotide substitutions.

when compared with Asian tapirs. It is of concern that we did not find any variation in number of repeats across the identified haplotypes that could infer the intra-specific relationship. Such variation is typical in other species (Xu et al. 1996; Xu & Arnason 1997). It remains to be determined if the lack of variation we observe is a result of low genetic diversity currently represented in captivity, or a result of low genetic diversity in the species due to low population sizes (human-induced or otherwise), or a genuine feature of the mtDNA makeup of this species (Lynam et al. 2012). Analyzing samples from wild-caught individuals across the range will help us determine which hypothesis is most likely.

Our suggestion in genetic management, the calculation results of Hd described the high score (0.877), meaning that the maternal lineage of this species is rapidly evolving with high level of genetic diversity (11 haplotypes). The phylogenetic relationship of the three clades in this study correlated well with the results from our previous work based on the Cyt*b* gene presented three groups of captive Asian tapir populations originated from Thailand geographic range (Muangkram et al. 2013b). Transmission of genetic diversity based on the maternal lineage is limited from mother to offspring (Hutchison et al. 1974; Giles et al. 1980; Harrison 1989). Haplotype Ti-1A is the most dominant (27.0%), and in populations that contain more than five females this haplotype could be successfully transmitted to future generations. Haplotypes Ti-1C and Ti-3B could also be maintained by breeding from two healthy females in the population. The situation is more critical for

haplotypes Ti-2C, Ti-2E, Ti-3A, and Ti-3C that were represented by only one female each. They are at a much higher risk of extinction if these females are unable to breed. Haplotypes Ti-1B, Ti-2A, Ti-2B, and Ti-2D may already functionally extinct as only non-breeding females remain within the captive population. Overall, this represents a huge loss of diversity as less than five haplotypes are expected to carry on into the next generation.

Genetic diversity has recently been recognized as critical for the management of wild tapir populations that are now starting to show some population growth (Lynam et al. 2012). The risk of genetic drift in poorly managed *in situ* and *ex situ* breeding programs could lead to loss of important genetic variation (Lacy 1987). In addition, coordinating management efforts between the captive populations in zoos and wild populations is crucial to maintain critical levels of gene flow. A further extension of our work is to use the small region containing highly variable sites on the 5′ proximal part of CR sized 476 bp in length using the forward primer on tRNA^{Pro} (Muangkram et al. 2013b) and the reverse primer on the central domain of the CR (Ward et al. 1999). This fragment could be used to manage the genetic diversity of new Asian tapir specimens.

Our study focused mainly on the evolutionary relationship between the Asian and the Brazilian tapir. More work is needed to resolve the relationship with Mountain and Baird's tapirs. Furthermore, combining information from analysis of Cyt*b* gene (Ogata et al. 2009; Muangkram et al. 2013b) and non-coding region of the CR (this study) could help researchers to better understand and maintain genetic diversity within the Tapiridae for improved genetic management of this endangered species. Such genetic monitoring and health assessment could be incorporated into population viability assessments and could be used to identify those haplotypes most at risk of extinction (Muangkram et al. 2013a). Moreover, the use of other polymorphic markers such as the Y-chromosome and genetic diversity in parental lineages including microsatellite markers could also provide more powerful tools for captive breeding and reintroduction programs for Asian tapirs.

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Disclosure statement

References

Arnason U, Adegoke JA, Gullberg A, Harley EH, Janke A, Kullberg M. 2008. Mitogenomic relationships of placental mammals and molecular estimates of their divergences. Gene. 421:37–51.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 16:37–48.

Brown WM, George M Jr, Wilson AC. 1979. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA. 76:1967–1971.

Cann RL, Brown WM, Wilson AC. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics. 106:479–499.

Carter DC. 1984. Perissodactyls. In: Anderson S, Jones JK Jr, editors. Order and families of recent mammals of world. New York: John Wiley and Sons. p. 519–562.

Corbet GB, Hill JE. 1992. The mammals of the Indomalayan region: a systematic review. New York: Oxford University Press.

Desmarest AG. 1819. Nouveau dictionnaired'histoirenaturelle. Paris: Chez Deterville.

Giles RE, Blanc H, Cann HM, Wallace DC. 1980. Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci USA. 77:6715–6719.

Harrison RG 1989. Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. Trends Ecol Evol. 4:6–11.

Hoelzel AR, Lopez JV, Dover GA, O'Brien SJ. 1994. Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. J Mol Evol. 39:191–199.

Hutchison CA, Newbold JE, Potter SS, Edgell MH. 1974. Maternal inheritance of mammalian mitochondrial DNA. Nature. 251:536–538.

Jama M, Zhang Y, Aman RA, Ryder OA. 1993. Sequence of the mitochondrial control region, tRNA(Thr), tRNA(Pro) and tRNA(Phe) genes from the black rhinoceros, *Diceros bicornis*. Nucleic Acids Res. 21:4392.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA. 86:6196–6200.

Lacy RC. 1987. Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. Conserv Biol. 1:143–158.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25:1451–1452.

Lynam A, Traeholt C, Martyr D, Holden J, Kawanishi K, van Strien NJ, Novarino W. 2008. *Tapirus indicus*. In: IUCN 2013. IUCN Red List of Threatened Species: Version 2013.2 [Online] [cited 2015 Aug 30]. Available from: http://www.iucnredlist.org.

Lynam AJ, Tantipisanuh N, Chutipong W, Ngoprasert D, Baker MC, Cutter P, Gale G, Kitamura S, Steinmetz R, Sukmasuang R, et al. 2012. Comparative sensitivity to environmental variation and human disturbance of Asian tapirs (*Tapirus indicus*) and other wild ungulates in Thailand. Integr Zool. 7:389–399.

Medici EP, Lynam A, Boonratana R, Kawanishi K, Hawa Yatim S, Traeholt C, Holst B, Miller PS. 2003. Malay tapir conservation workshop. Final report. IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN.

Muangkram Y, Salakij C, Siriaroonrut B, Tipkantha W, Narkkong NA, Rotphai A, Wajjwalku W. 2013a. Haematological, cytochemical and ultrastructural characteristics of blood cells and serum chemistry in captive Malayan tapir (*Tapirus indicus*) in Thailand. Comp Clin Pathol. 22:1015–1024.

Muangkram Y, Wajjwalku W, Kaolim N, Buddhakosai W, Kamolnorranath S, Siriaroonrat B, Tipkantha W, Dongsaard K, Maikaew U, Sanannu S. 2016. The complete mitochondrial genome of the Asian tapirs(*Tapirus indicus*): the only extant Tapiridae species in the old world. Mitochondrial DNA. 27:413–415.

Muangkram Y, Wajjwalku W, Salakij C, Kaolim N, Siriaroonrut B, Kamolnorranath S, Tipkantha W, Maikaew U, Thomas W, Polsrila K, et al. 2013b. Use of mitochondrial cytochrome *b* sequences to determine the origin of captive Asian tapirs *Tapirus indicus*: implications for conservation. Endang Species Res. 21:97–103.

Nelson JE, Krawetz SA. 1992. Purification of cloned and genomic DNA by guanidine thiocyanate/isobutyl alcohol fractionation. Analyt Biochem. 207:197–201.

Ogata M, Watanabe S, Ogawa H. 2009. Genetic variation of Asian tapir. Jpn J Zoo Wildl Med. 14:73–76.

Rovie-Ryan JJ, Traeholt C, Marilyn-Jaoi E, Zainuddin ZZ, Shariff KM, Elagupillay S, Farouk MYM, Abdullah AA, Cornelia CS. 2008. Sequence variation in Malayan tapir (*Tapirus indicus*) inferred using partial sequences of the cytochrome *b* segment of the mitochondrial DNA. J Wildl Parks. 25:16–18.

Saccone C, Attimonelli M, Sbisà E. 1987. Structural elements highly preserved during the evolution of the Dloop-containing region in vertebrate mitochondrial DNA. J Mol Evol. 26:205–211.

Sbisà E, Tanzariello F, Reyes A, Pesole G, Saccone C. 1997. Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. Gene. 205:125–140.

Schwartz MK, Luikart G, Waples RS. 2007. Genetic monitoring as a promising tool for conservation and management. Trends Ecol Evol. 22:25–33.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30:2725–2729.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Ward TJ, Bielawski JP, David SK, Templeton JW, Derr JN. 1999. Identification of domestic cattle hybrids in wild cattle and bison species: a general approach using mtDNA markers and the parametric bootstrap. Anim Conserv. 2:51–57.

Xu X, Arnason U. 1997. The complete mitochondrial DNA sequence of the white rhinoceros, *Ceratotherium simum*, and comparison with the mtDNA sequence of the Indian rhinoceros, *Rhinoceros unicornis*. Mol Phylogenet Evol. 7:189–194.

Xu X, Gullberg A, Arnason U. 1996. The complete mitochondrial DNA (mtDNA) of the donkey and mtDNA comparisons among four closely related mammalian species-pairs. J Mol Evol. 43:438–446.