Original Article

Genetic characterization of banteng (*Bos javanicus*) populations in Thailand for conservation

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Abstract

Banteng (*Bos javanicus*), an endangered, wild ungulate, plays a major role in seed dispersal and as a prey animal in Thailand. The population of wild banteng is threatened by poaching and habitat losses. Captive breeding management of banteng has been established and reintroduction of banteng has been successful in some areas. This study investigated the genetic variation of wild and captive banteng, based on mitochondrial DNA (mtDNA) and the Y-chromosome. The mtDNA analysis revealed three novel maternal haplotypes. The Y-chromosome analysis showed two Y-chromosome haplotypes based on the SRY region in the Thai population. This region may be useful as a Y-chromosome marker for genetic management. The phylogenetic analysis using mtDNA and the Y-chromosome demonstrated that the studied banteng were clustered with the sequence of *Bos javanicus* available in Genbank. Based on our data, no hybridization between banteng and domestic cattle was observed.

Keywords: Banteng, mtDNA, Y-chromosome

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Introduction

Large herbivores have played an important role in maintaining ecosystem function and biodiversity conservation. Many herbivores have been known as ecosystem engineers by shaping the structure along the trophic cascade and function of landscapes. Through their size, feeding choice, metabolic requirements, social behavior and movement patterns, the large herbivores have direct and indirect effects on nutrient cycling, seed dispersal and in the food chain for predators and scavengers (Bakker *et al*., 2016; Trouwborst, 2019; Ripple *et al*., 2015). However, approximately 60% of large herbivores are facing substantial population declines resulting from hunting, habitat destruction and fragmentation, and resource competition with livestock (Ripple *et al*., 2015; Trouwborst, 2019). Southeast Asia hosts the highest number of threatened large herbivores (Ripple *et al*., 2015).

Banteng (*Bos javanicus*) is a wild ungulate distributed in Southeast Asia. Three subspecies of banteng are recognised according to their geographic distribution: *B. javanicus javanicus* (Java and Bali), *B. javanicus birmanicus* (Asian mainland) and *B. javanicus lowi* (Borneo). Currently banteng is listed as Endangered (EN) species according to the IUCN Red List of Threaten Species with the global population approximately 4,000-8,000 individuals and a decreasing trend over time (Gardner *et al*., 2016). In Thailand, the banteng has been listed as a protected animal under the Wildlife Preservation and Protection act BE 2535 (1992). More than 50 individuals have been recorded in Huai Kha Khaeng Wildlife Sanctuary (HKK), the core area of Western Forest Complex (WEFCOM), while smaller populations have been detected in the northeast and eastern regions (Gardner *et al*., 2016; Srikosamatara and Suteethorn, 1995). Over 20 years, there has been a reported 85% reduction in overall banteng numbers in Thailand (Gardner *et al*., 2016). Even within the largest stronghold at WEFCOM, suitable habitats of banteng are limited compared to other large herbivores including gaur, sambar deer and Asian elephant (Trisurat *et al*., 2010). Some remnant, isolated populations are facing the potential loss of genetic diversity and adaptive potential. The loss of mitochondrial (mtDNA) genetic diversity in small population was detected in the introduced Indonesian banteng founders at Lam Pao Wildlife Conservation Development and Promotion Station, Thailand (Saijuntha *et al*., 2013). Consequently, effective conservation and management programs should be considered to restore not only population sizes, but evolutionary and ecological processes sustaining adaptation and biodiversity. In 2014, the first banteng reintroduction program in Thailand was established within the Salakphra Wildlife Sanctuary situated on the south of HKK within WEFCOM. Pre-release health check, the soft-release strategies and habitat management scheme have resulted in successful establishment of the self-sustaining population. The reintroduction program has been continuous with yearly and ongoing monitoring of their demographic trend (Kongsurakan *et al*., 2020; Chaiyarat *et al*., 2019).

A genetic-based approach is one of the important components contributing to reintroduction success and conservation priority. Maternal and paternal diversity based on mtDNA and Y-chromosome variation, respectively, is uniparental inheritance, leading to the lack of nucleotide ambiguities resulting from heterozygotes. Compared to functional nuclear DNA, mtDNA also has advantages in having higher copy number in the cell and a faster mutation rate. These properties of mtDNA and Y-chromosome polymorphism allow a rapid assessment of human disturbance consequences toward population genetic diversity and adaptive potential. However, there has been limited genetic study of banteng in Thailand. Thus, the current study determined maternal and paternal genetic diversity of both reintroduced and wild banteng populations in WEFCOM, Thailand. The genetic profiles will provide an insight into evolutionary potential and evaluate effectiveness of ongoing conservation practices including reintroduction strategies, conservation and population management.

Materials and Methods

Blood or hair samples were collected from male bantengs during health examination or necropsy. Each representative sampling was obtained from the Salakphra Wildlife Sanctuary (SL; reintroduced population, N=3), Huai Kha Khaeng Wildlife Sanctuary (HKK; wild population, N=2), Khao Keow Open Zoo (KKOZ; captive population, N=1) and Choeng Doi Su Thep Wildlife and Nature Education Center (CM; captive population, N=2). Genomic DNA was extracted using the modified phenol chloroform method for blood samples and a Phire Animal Tissue Direct PCR Kit (Thermo Scientific, USA) for hair samples following the standard protocols. PCR amplification were performed using Phusion High-fidelity DNA polymerase (Finnzymes, USA) targeting mitochondrial DNA (mtDNA) and the Y-chromosome. The partial Cytochrome *b* (Cyt *b*) and Control Region (D-loop) of mtDNA and Y-linked introns/exons were amplified using the reported primer set (Sukmak *et al*., 2013). The Y-chromosome analysis focused on the partial zinc finger protein Y-linked gene (ZFY) and sex determination region (SRY). Purified PCR products were sent to the First BASE laboratory, Malaysia for Sanger Sequencing services. MtDNA and Y-chromosome variation was compared to the report GenBank sequences from previous genetic studies (Hassanin and Ropiquet, 2007; Nijman *et al*., 2008; Hassanin *et al*., 2012). Multiple sequence alignments were performed using BioEdit 7.0 software (Hall, 1999). Phylogenetic relationships among Bovidae family based on the partial Cyt *b*-Dloop, partial ZFY and partial SRY genes were analyzed using the MEGA X program (Kumar *et al*., 2018). Additional sequences from Wajjwalku (2013) spanning Cyt *b* (GenBank Accession number: MZ173460 - MZ173461 for HapE01 and HapE02 from Khao Ang Rue Nai Wildlife Sanctuary, and MZ173462 - MZ173467 for HapW01 and HapW06 from Huai Kha Khaeng Wildlife Sanctuary), Dloop (GenBank Accession number: MZ173468 - MZ173469 for HapE01 and HapE02 from Khao Ang Rue Nai Wildlife Sanctuary, and MZ173470

- MZ173475 for HapW01 and HapW06 from Huai Kha Khaeng Wildlife Sanctuary). Mitogenome from other species in the tribe Bovidae were included within the phylogenetic analyses. Since the D-loop region provided such high variation and may not have been suitable for genealogy analysis, evolutionary relationships among banteng haplotypes was estimated using the Cyt *b* region (419 bp) to reveal the origin (ancestral sequences) among banteng populations by the TCS network using the PopART software (Leigh *et al*., 2015).

Results

All the mtDNA sequences from the current study were clustered within the clade of *B. javanicus birmanicus*, in Asian mainland. Phylogenetic analysis of bantengs from this study revealed 4 mtDNA haplotypes and the total of 11 mtDNA haplotypes were detected in Thailand when combined with previous study (Wajjwalku, 2013). Of the 4 haplotypes, 3 haplotypes found in captive and reintroduced individuals were not detected from previous genetic study (Wajjwalku 2013) in wild banteng populations from Huai Kha Khaeng Wildlife Sanctuary, the core area in the Western Forest Complex, and Khao Ang Rue Nai Wildlife Sanctuary in the Eastern Forest Complex. These 3 haplotypes showed close evolutionary relationships by having 1-2 bp differences from the western population (Fig 1). Likewise, the TCS network based on Cyt *b* supported close evolutionary relationships among the Thailand population (Fig 2). Only a single haplotype was found in the reintroduced individuals from Salakphra Wildlife Sanctuary (SL). This haplotype had 1-bp difference from the wild haplotype (HapW06) detected in western population. Limited sampling from western populations might contribute to the non-detection of SL haplotype from extant populations in the west. Alternatively, random genetic drift in small and isolated population might lead to the loss of this haplotype from the wild. HapW05, which was previously detected (Wajjwalku *et al*., 2013), was found from the wild HKK, while there was no variation detected in captive banteng (KKOZ and CM). Since most of samples had originated from western Thailand, each haplotype was named according to previous study (Wajjwalku, 2013) and displayed in Table 1.

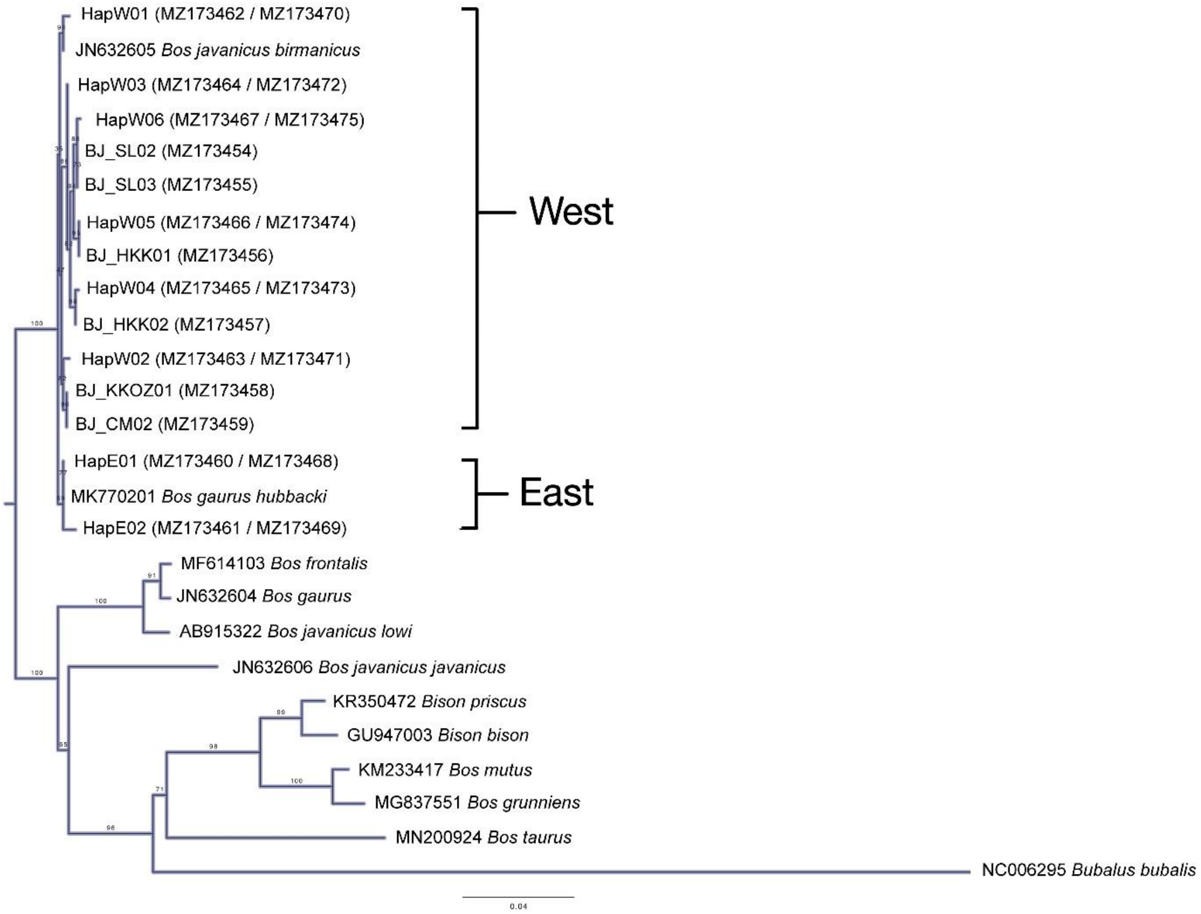

Figure 1 Phylogenetic relationship of banteng population in Thailand and of other Bovidae. Tree constructed based on mtDNA (partial Cyt B and D-loop) (1,660 bp) using a maximum likelihood approach, HKY+I+G nucleotide substitution model with bootstrap probability of 1,000 replicates. Bootstrap values greater than 50 are shown above branch length.

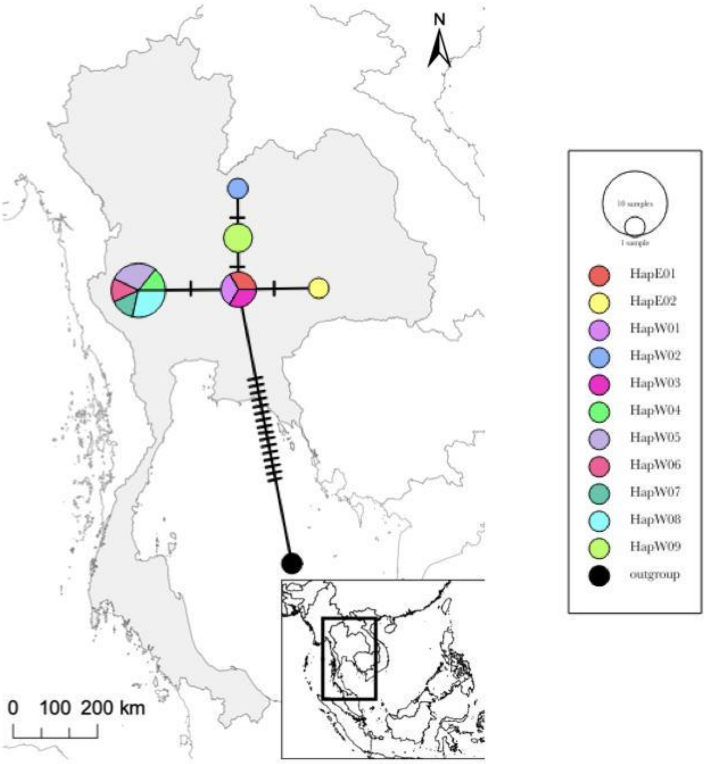

Figure 2 TCS network using Cty *b* (419 bp) showing different haplotypes of banteng from this study. Each circle represents a unique Cyt *b* haplotype, circle size represents number of sequences considered as the same genotype and different colored circles denote different D-loop haplotypes found in our samples. *B. javanicus lowi* (AB915322) used as an outgroup.

Table 1 Details of samples, origin, genes of interest, haplotype name and accession number in this study

Sample ID	Type	Genes	Origin	Haplotype (mtDNA/ZFY/SRY)	Accession No. (mtDNA/ZFY/SRY)
BJ SL01	Blood	ZFY, SRY	Salakphra Wildlife Sanctuary	NA/zfy_BJ_TH01/sry_BJ_TH01	NA/MZ173483/MZ173476
BJ SL02	Blood	mtDNA, ZFY, SRY	Salakphra Wildlife Sanctuary	HapW07/zfy_BJ_TH01/sry_BJ_TH01	MZ173454/MZ173484/MZ173477
BJ SL03	Blood	mtDNA	Salakphra Wildlife Sanctuary	HapW07/ NA / NA	MZ173455/NA/NA
BJ HKK01	Blood	mtDNA, ZFY, SRY	Huai Kha Khaeng Wildlife Sanctuary	HapW05/zfy_BJ_TH01/sry_BJ_TH01	MZ173456/MZ173485/MZ173478
BJ HKK02	Blood	mtDNA, ZFY, SRY	Huai Kha Khaeng Wildlife Sanctuary	HapW08/zfy_BJ_TH01/sry_BJ_TH01	MZ173457/MZ173486/MZ173479
BJ KKOZ01	Blood	mtDNA, ZFY, SRY	Khao Keow Open Zoo	HapW09/zfy_BJ_TH01/sry_BJ_TH02	MZ173458/MZ173487/MZ173480
BJ CM01	Hair	ZFY, SRY	Choeng Doi Su Thep Wildlife and Nature Education Center	NA/zfy_BJ_TH01/sry_BJ_TH02	NA/MZ173488/MZ173481
BJ CM02	Hair	mtDNA, ZFY, SRY	Choeng Doi Su Thep Wildlife and Nature Education Center	HapW09/zfy_BJ_TH01/sry_BJ_TH02	MZ173459/MZ173489/MZ173482

*NA = sequence not available

For Y-chromosome study, the current study characterized 2 segments of the banteng Y-chromosome: the ZFY (1,181 bp) and SRY (2,573 bp) genes. For the partial ZFY region, no genetic variation was detected among our samples (Fig 3). Compared with the single, available banteng ZFY sequence from previous study (Nijman *et al*., 2008), of the 1,181 bp partial ZFY gene, only single nucleotide polymorphism (A to G) was detected between the reference and our samples. Two SRY haplotypes were detected in Thai banteng populations. The first haplotype belonged to SL and HKK bantengs (western forest haplotype) and the second haplotype represented KKOZ and CM bantengs (captive haplotype) (Fig 4) (Table 1). The data from both MtDNA and Y-chromosome analysis in this present study revealed the different between domestic cattles (*Bos taurus* and *Bos indicus*) and banteng and no evidence of hybridization between the two different banteng subspecies.

Figure 3 Phylogenetic relationship of banteng in Thailand and of other Bovid species. Tree constructed based on partial ZFY gene (1,181 bp) using maximum likelihood (T92) model with bootstrap probability of 1,000 replicates. Bootstrap values greater than 50 are shown.

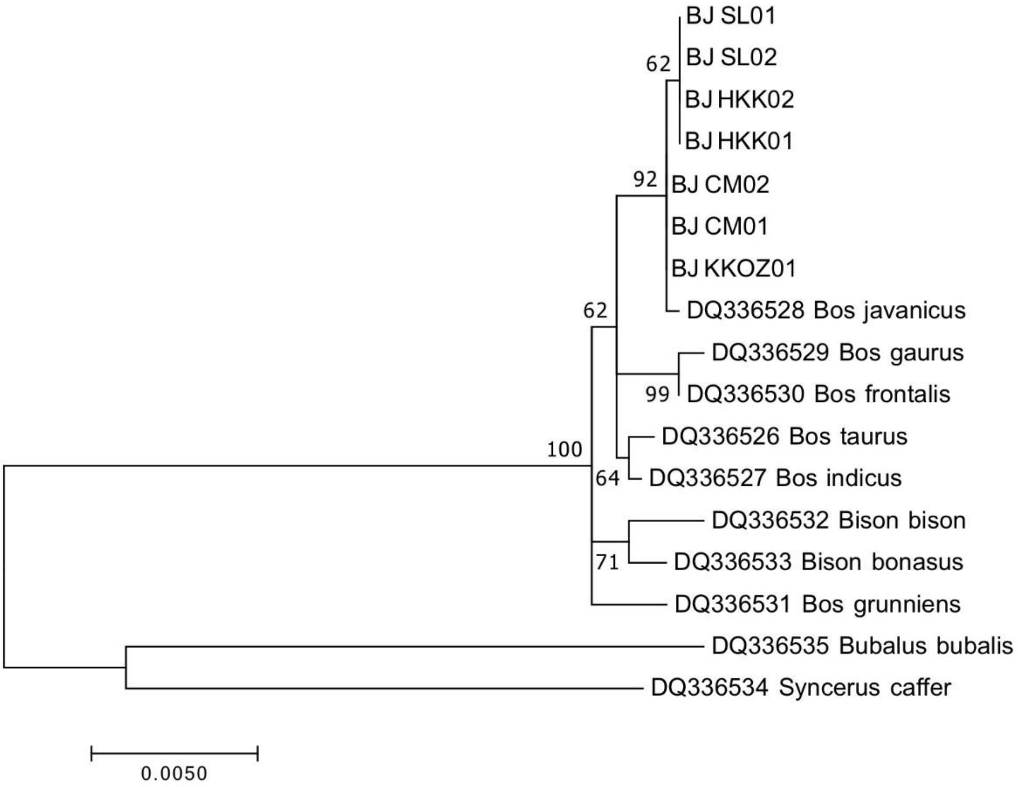

Figure 4 Phylogenetic relationship of banteng in Thailand and of other Bovid species. Tree constructed based on partial SRY gene (2,573 bp) using maximum likelihood (T92) model with bootstrap probability of 1,000 replicates. Bootstrap values greater than 50 are shown.

Discussion

MtDNA phylogenetic analyses are commonly used to examine intraspecific genetic diversity and phylogeographic partitioning within target species due to its high evolutionary rate, being maternally derived and having non-recombinant properties (Qiptiyah *et al*., 2019). The current study focused on Cyt *b* which is the most commonly used mtDNA protein coding genes and contains many informative SNPs for species identification or DNA barcoding (Muangkram *et al*., 2018). Compared to the protein coding gene, the D-loop region has higher mutation rates and therefore contains higher degree of variation (Arif and Khan, 2009) and is commonly used to evaluate population genetic diversity. According to Wajjwalku *et al*. (2013), 8 haplotypes of *B. javanicus birmanicus* (HapW01 to HapW06 and HapE01 to HapE02) have been reported in Thai banteng across western and eastern populations. The highest mtDNA diversity was detected in the HKK population (6 haplotypes), supporting that HKK is the stronghold of banteng population. Due to the contiguous habitats between the SL and HKK forests, the reintroduction of highly diverse founders to the western population might have a positive impact on adaptive potential, population fitness and resilience toward changing environments. However, more comprehensive genetic surveillance of banteng in the Western Forest Complex should be included in ongoing investigations.

With high bootstrap supports, the phylogenetic analysis revealed two different clades of *B. javanicus birmanicus*, corresponding to geographic partitioning into eastern and western populations according to Wajjwalku (2013) (Fig 1). All samples in this study were clustered with the western population clade. This grouping agreed with the geographic origin of each animal. However, the molecular clock or divergence time between these two populations was not established; further analysis is needed to prove this hypothesis. In addition, the eastern population (HapE01 and HapE02) was clustered with *B. gaurus hubbacki*, which is a Malayan guar populated Peninsular Malaysia. This tree topology might result from incomplete lineage sorting of mtDNA or ancient introgression between sister taxa. Recently, genetic study based on mitogenome data has provided strong support for close phylogenetic relationships between *B. gaurus hubbacki* and *B. javanicus birmanicus* (Rosli *et al*., 2011). Our results supported this phylogenetic analysis and close phylogenetic clustering between guar and Javan banteng was also described in Hassanin and Ropiquet (2007), and confirmed by nuclear DNA data (Hassanin *et al*., 2012). Thus, for further investigation of the hypothesis of incomplete lineage sorting of this species, analysis using autosomal markers or genome-wide SNPs should be added.

Y-chromosome variation is useful to define the evolutionary relationships along patrilines due to uniparental transmission and a non-recombining process (Di Lorenzo *et al*., 2016; Hellborg and Ellegren, 2003). In the Bovidae, Y-chromosome diversity is important to elucidate patterns and timing of speciation, phylogeography, and hybridization events (Nijman *et al*., 2008; Yindee *et al*., 2010; Kikkawa *et al*., 2003; Verkaar *et al*., 2004; Gou *et al*., 2010; Muhamad *et al*., 2007). The data of Y-chromosome analysis in this present study revealed that the banteng ZFY gene might be highly conserved within the subspecies level. Subspecies specification of Y-chromosome diversity has been reported in other ungulates such as swamp and river buffaloes (*B. bubalis carabensis* and *B. bubalis bubalis*) (Yindee *et al*., 2010) and Thamin and Siamese Eld's deer (*Rucervus eldii thamin* and *R. eldii siamensis*) (Sukmak *et al*., 2013). However, only a single sequence of Y-chromosome in banteng was reported in the database without subspecies information; thus we could not deduce the subspecies specific Y-region.

For SRY study, two SRY haplotypes were found in Thai banteng populations. The first haplotype belonged to SL and HKK bantengs (western forest haplotype) and the second haplotype represented KKOZ and CM bantengs (captive haplotype). The lack of diversity on the Y chromosome might have been due to a decreased number in the paternal lineage in limited number of male founders. For example, the male ungulate was selectively hunted for its antlers leading to a drastic decline in it numbers (Steinmetz *et al*., 2010). Although bantengs have also been hunted, a smaller level of selective pressure compared to other ungulate species might allow persistence of the large effective population size in the past, and therefore detection of two lineages of Y chromosome. Moreover, when our data were compared to the reported sequences, both Y haplotypes found in Thailand were not similar to the reported haplotype found in captivity (Blijdorp Zoo, Rotterdam; Verkaar *et al*., 2004; Nijman *et al*., 2008). This finding suggested that the SRY gene might be more diverse than ZFY and could possibly be useful for inferring diversification patterns of banteng populations from different geographical regions. To elucidate this hypothesis, complete study of other Y-chromosome regions with wider geographic coverage should be considered to gain more information on mechanisms underlying the observed phylogeographic patterns.

In conclusion, the current study demonstrated the genetic diversity of banteng in Thailand both from maternal and paternal lineages. There was no evidence of hybridization between the two different subspecies in this study. In addition, more study of Y-chromosome diversity in wild banteng populations in Thailand is urgently required for long-term population recovery, especially in the western forest population as well as reporting on the Y-chromosome in Javan populations (*B. javanicus javanicus*), to assist better understanding of evolutionary history based on Y-chromosome diversity and mtDNA population genetics in Bovidae.

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