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Use of mitochondrial cytochrome *b* sequences to determine the origin of captive Asian tapirs *Tapirus indicus*: implications for conservation

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ABSTRACT: Populations of the Endangered Asian tapir *Tapirus indicus* in Thailand have been severely fragmented and isolated and may be facing a high risk of extinction. Their genetic diversity and population viability remains unknown. The main aim of this study was to assess the genetic diversity of the Asian tapir using the complete mitochondrial cytochrome *b* gene (1140 bp). We collected 31 blood samples from captive individuals. Two polymorphic sites were found, contributing to 3 maternal lineages: Ti-1 (n = 15), Ti-2 (n = 11), and Ti-3 (n = 5). Comparative analysis with GenBank sequences of other Asian tapirs found another 17 variable sites. These results suggest that there may be up to 7 distinct haplotypes of *T. indicus*. Furthermore, the pattern of the haplotype distribution corresponded to natural geographic boundaries, including the Isthmus of Kra and the Malacca Strait. One unique haplotype found in Sumatra was genetically distinct from 3 haplotypes found in Thailand and 3 haplotypes from Malaysia. One haplotype shared between Thai and Malaysian populations indicated a possible origin in the tropical rainforest along the Thai-Malay border. In general, the diverged geographic distribution of these haplotypes illustrates the phylogeographic history of the family Tapiridae (*T. indicus*, *T. terrestris*, *T. pinchaque*, and *T. bairdii*) based on our 963 bp cytochrome *b* sequences. These baseline genetic data have the potential to enhance effective management of tapirs held in captive breeding programs. However, there is still an urgent need to identify and maintain the genetic diversity of these distinct populations in the wild.

KEY WORDS: Cytochrome *b* gene · Genetic diversity · Malayan tapir · Phylogenetic analysis · Thailand

INTRODUCTION

In Thailand, the Asian tapir *Tapirus indicus* (Desmarest 1819) has been categorized as a 'reserved' species by the Wildlife Preservation and Protection Act B.E. 2535, meaning that this species may not be illegally hunted, bred, possessed, traded, exported, or imported. It is also categorized as Endangered on the 2008 IUCN Red List of Threatened Species (www.iucnredlist.org) (Lynam et al. 2008) and on Appendix I of

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CITES. The geographic distribution of the Asian tapir is distinct from the other 3 extant species, which are found in Neotropical America, viz. lowland tapir *T. terrestris*, mountain tapir *T. pinchaque*, and Baird's tapir *T. bairdii* (Carter 1984). The pattern of genetic distribution of tapirs appears to correspond closely to their demographic history. Representatives of the family Tapiridae have been identified from Oligocene fossils found in North America and Europe (Schoch 1989). Neotropical tapirs diverged from the Asian tapir approximately 20 to 30 million years ago (Ashley et al. 1996, Norman & Ashley 2000, Pitra & Veits 2000) during the late Miocene (Padilla & Dowler 1994, Hulbert 1995). The earliest ancestor of the South American tapirs was recorded in the early Pleistocene in Argentina (Tonni 1992). The distribution of the newly derived clade of American tapirs is thought to be influenced by the presence of the Isthmus of Panama, and to have split from the ancestral clade approximately 3 million years ago (Ashley et al. 1996).

The current range of the Asian tapir is limited to the tropical rainforests of Southeast Asia, including Thailand, Myanmar, Malaysia, and the island of Sumatra in Indonesia (Woodruff 2003). Regionally, the species is sensitive to changes in forest cover, and the distribution of forest cover has been severely fragmented in parts of the tapir's range, leading to small populations (10 to 15 individuals) remaining in disjunct habitat (Lynam et al. 2008). In Thailand, tapir populations may be more stable, as rates of forest loss have decreased significantly in recent years (Lynam et al. 2012). Genetic monitoring has long been recognized as an important tool to protect endangered species from extinction (O'Brien 1994), and integrating genetic data with ecological data has been increasingly applied to conservation to assess the status of threatened and endangered species. The genetic management of *ex situ* populations is also critical to the persistence of small populations in captivity (Frankham 1995, Slate et al. 2000, Frankham et al. 2002, Keller & Waller 2002).

Previous genetic studies of the family Tapiridae have examined mitochondrial genes such as cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit II (Ashley et al. 1996), 12S rRNA (Springer 1997, Norman & Ashley 2000, Murphy et al. 2001), 16S rRNA (Murphy et al. 2001, O'Leary & Gatesy 2008), and cytochrome *b* (Pitra & Veits 2000, Arnason et al. 2008, Rovie-Ryan et al. 2008, Ogata et al. 2009, De Thoisy et al. 2010, Sanches et al. 2011, Steiner & Ryder 2011). Each coding gene has provided a different degree of phylogenetic resolution at the taxonomic level. Moreover, the mitochondrial cytochrome *b* gene has been reliably used for assessing genetic variation in mammals due to its moderate evolutionary rate. Previous studies of Asian tapirs have shown 6 haplotypes with 17 variable sites (n = 29) using available cytochrome *b* sequences compiled from GenBank (Pitra & Veits 2000, Rovie-Ryan et al. 2008, Ogata et al. 2009, Steiner & Ryder 2011). The distribution of each haplotype shows that the intra-species relationship is largely influenced by the Isthmus of Kra and the Malacca Strait. These 2 natural biogeographic disjunctions have been shown to act as barriers for other Southeast Asian species, including mammals, birds, frogs, and snakes (Hughes et al. 2003, Meijaard 2003, De Bruyn et al. 2005, Inger & Voris 2008, Liao et al. 2009).

In total, 243 polymorphic sites are recognized across all 4 extant species of tapir (Pitra & Veits 2000, Arnason et al. 2008, De Thoisy et al. 2010, Sanches et al. 2011, Steiner & Ryder 2011). De Thoisy et al. (2010) showed a phylogeographic relationship between lowland and mountain tapirs within South America, and a relationship between Baird's tapir and another extant American tapir was described by Steiner & Ryder (2011). However, little work has been done to describe the phylogenetic relationships of the Asian species.

The primary goal of this study was to investigate the genetic diversity of captive Asian tapirs in Thailand and to compare our sequences with available data from GenBank to provide baseline genetic information for this species, and a putative geographic origin for captive specimens. We also analyzed the comparative phylogenetic relationships within the family Tapiridae. The results from this study provide valuable data for the conservation management of both *ex situ* and *in situ* populations of the 4 extant species of this family.

MATERIALS AND METHODS

Blood samples were collected from 31 captive Asian tapirs held in 5 zoos registered under the Zoological Park Organization of Thailand (Dusit Zoo, Khao Kheow Open Zoo, Chiang Mai Zoo, Nakhon Ratchasima Zoo, and Song Khla Zoo) and 1 private zoo. For genetic analysis, DNA was extracted from each sample using the QIAamp® blood and tissue kit (Qiagen) according to the manufacturer's instructions. The complete mitochondrial cytochrome *b* gene was amplified using the forward primer on $tRNA^{Glu}$ (5′-CAT GAC TAA TGA TAT GAA AAA CC-3′) and the reverse primer on $tRNA^{Thr}$ (5′-CTT

TTC TGG TTT ACA AGA CCA-3′) with DreamTaq® DNA Polymerase (Fermentas International). DNA was amplified by PCR in a reaction volume of 100 µl which included 0.1 µM of each primer, 200 µM dNTP, 0.25 U *Taq* polymerase, 1 µl of 10× $(NH_4)_2SO_4$ buffer, and 1 mM of $MgCl_2$. Each PCR cycle consisted of 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 53°C, and extension for 45 s at 72°C. The correct PCR product was first confirmed using 2% agarose gel electrophoresis and purified using a QIAquick® PCR purification kit (Qiagen). The PCR products were then sent to First BASE Laboratories Sdn Bhd in Malaysia for sequencing. The cytochrome *b* fragments investigated in this study have been deposited in GenBank under accession numbers JX014321 to JX014351. The sequences were aligned using CLUSTAL W (Thompson et al. 1994) and combined with other *Tapirus indicus* sequences compiled from GenBank (Table 1). Cytochrome *b* sequences from sister taxa were also analyzed, including *T. terrestris*, *T. pinchaque*, and *T. bairdii* (Table 1), as well as a representative outgroup, *Rhinoceros sondaicus* (accession number AJ245725; Tougard et al. 2001).

Phylogenetic relationships among the compiled cytochrome *b* sequences were constructed using a 'find best-fit' substitution model with the best Bayesian information criterion (BIC) computed by the maximum likelihood statistical method (Saitou & Nei 1987, Nei & Kumar 2000) implemented in CLUSTAL W (Thompson et al. 1994) via MEGA5 (Tamura et al. 2011). The reliability of the nodes was assessed by 10 000 bootstraps (Felsenstein 1985). Median-joining networks between all 4 extant Tapiridae species were constructed via NETWORK 4.6.1.0 (Bandelt et al. 1999).

Table 1. *Tapirus* spp. Tapir sequences analyzed in this study. Length 1 (L1): 1140 bp; Length 2 (L2): 975 to <1140 bp; Length 3 (L3): 321 to 344 bp

GenBank accession no.	L1	L2	L3	Source
Tapirus indicus				
JX014321–JX014351	•			This study
AF145734	•			Pitra & Veits (2000)
AB469774–AB469776		•		Ogata et al. (2009)
EU224327–EU224339			•	Rovie-Ryan et al. (2008)
JF718881	•			Steiner & Ryder (2011)
Tapirus bairdii				
JF718880	•			Steiner & Ryder (2011)
Tapirus pinchaque				
JF718878	•			Steiner & Ryder (2011)
GQ259955–GQ259957		•		De Thoisy et al. 2010
Tapirus terrestris				
JF718879	•			Steiner & Ryder (2011)
AF056030	•			Pitra & Veits (2000)
AJ428947	•			Arnason et al. (2008)
GQ259910–GQ259954	•	•		De Thoisy et al. (2010)

RESULTS

Phylogenetic analysis of the 31 tapirs based on the complete mitochondrial cytochrome *b* gene (1140 bp) revealed 3 haplotypes: Ti-1 (n = 15), Ti-2 (n = 11), and Ti-3 (n = 5). Two singleton variable sites were found as 2 types of transitional substitution (C to T and T to C). Nucleotide diversity (Pi) was 0.00117. The most common nucleotide was adenine (31.05%) followed by cytosine, thymine, and 12.37% for guanine. For amino acid translation, we found amino shifts influenced by transitional substitutions at position 710 of the cytochrome *b* gene sequences that could be divided into 2 groups: (1) Ti-1 and (2) Ti-2 and Ti-3. Serine (237) of Ti-1 (TCA) shifted to leucine (237) of Ti-2 and Ti-3 (TTA).

Comparative analysis with previous studies of the Asian tapir (Pitra & Veits 2000, Rovie-Ryan et al. 2008, Ogata et al. 2009, Steiner & Ryder 2011) indicated 7 possible haplotypes with 17 polymorphic sites (Table 2). The substitutions included both transitions and transversions. Additional analysis of amino acid variation found 3 different sites: (1) serine 29 (TCC) ↔ alanine 29 (GCA) from the study of Rovie-Ryan et al. (2008), found only in Malaysia; (2) serine 237 (TCA) ↔ leucine 237 (TTA); and (3) isoleucine (ATC) ↔ leucine (CTC) from the study of Ogata et al. (2009), found only on the island of Sumatra in Indonesia.

The geographic distribution of haplotypes was divided into 5 possible geographic clusters — (1) Thailand: Ti-1 and Ti-2 (Ogata et al. 2009, present study); (2) Thailand and Malaysia: Ti-3 (Ogata et al. 2009, present study); (3) Malaysia: Ti-5 and Ti-6 (Rovie-Ryan et al. 2008); (4) the island of Sumatra, Indonesia: Ti-7 (Ogata et al. 2009); and (5) unknown origin: Ti-4 (Pitra & Veits 2000, Steiner & Ryder 2011; our Fig. 1).

Comparative phylogenetic relationships of the family Tapiridae based on the mitochondrial cytochrome *b* gene showed that each species was monophyletic (Fig. 2). The BIC selected the Kimura 2-parameter model with discrete gamma distribution as having the best fit.

The relationships of the 4 extant species in the family Tapiridae were determined using NETWORK (Bandelt et al. 1999) based on 963 bp cytochrome *b* sequences from the present study and reference sequences (Table 1) with 208 polymorphic sites. The

Table 2. *Tapirus indicus.* Seventeen polymorphic sites of *T. indicus* based on the mitochondrial cytochrome *b* gene (1140 bp) were analyzed using sequences obtained from this study and reference GenBank sequences from Table 1. The baseline for these sequences corresponds to position number 14176 of the complete mitochondrial genome of *T. terrestris*. Dots indicate base pairs identical to the Ti-1 haplotype of *T. indicus*. Dashes indicate no recorded nucleotide

		0 0 0 8 5	0 0 8 7	0 2 1 6	0 2 8 2	0 3 0 3	0 4 0 8	0 4 1 7	0 4 2 6	0 4 9 8	0 5 8 9	0 7 1 0	0 7 2 1	0 8 1 0	0 8 1 6	0 8 4 9	0 8 7 4	1 0 8 1	n
Ti-1	Group 1	T	C	T	G	C	C	T	C	G	T	C	A	A	G	C	T	T	15
Ti-2	Group 2	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	11
Ti-3	Group 3	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	5
Ti-4	AF145734, JF718881	.	.	.	.	.	.	.	.	A	.	.	.	C	A	.	C	.	2
Ti-2	AB469774	–	–	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	4
Ti-3	AB469775	–	–	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	5
Ti-7	AB469776	–	–	C	A	T	.	.	.	.	C	T	C	.	.	T	.	.	5
Ti-5	EU224327[a]	–	–	C	A	T	A	A	G	–	–	–	–	–	–	–	–	–	7
Ti-5	EU224338	G	A	C	A	T	A	A	G	–	–	–	–	–	–	–	–	–	1
Ti-6	EU224329[b]	–	–	.	.	.	A	A	G	–	–	–	–	–	–	–	–	–	3
Ti-6	EU224334[c]	G	A	.	.	.	A	A	G	–	–	–	–	–	–	–	–	–	2
																		Total	60

[a]Additional accession nos.: EU224328, EU224331, EU224332, EU224333, EU224335, EU224336 (n = 7); [b]additional accession nos.: EU224330, EU224337 (n = 3); [c]additional accession nos.: EU224339 (n = 2)

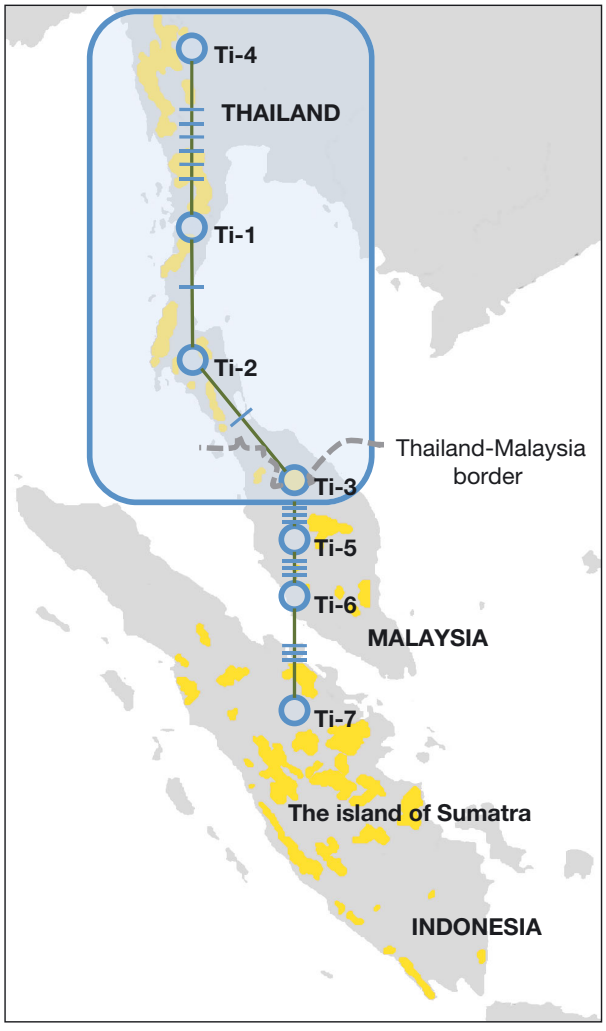

Fig. 1. *Tapirus indicus.* Comparative phylogenetic relationship between sequences from this study and reference sequences based on partial mitochondrial cytochrome *b* gene sequences (286 bp; position 142 to 427) of the Asian tapir. This information enabled us to distinguish haplotypes (blue circles) according to geographic region: 1 haplotype was found on the island of Sumatra, Indonesia and 2 haplotypes were found only on the Malay peninsula (Malaysia). One grouping of a large population (blue rectangle) based on longer mitochondrial cytochrome *b* gene sequences (975 bp) with 12 sites was grouped into Ti-1 and Ti-2 (Thailand), Ti-3 (Thailand/Malaysia), and Ti-4 (unknown origin). The blue horizontal lines refer to the number of nucleotide substitutions, and the yellow area indicates the current Asian tapir distribution modified from the IUCN Red List of Threatened Species (www.iucnredlist.org)

results do not include short length sequences (321 to 344 bp) of Ti-5 and Ti-6. The pattern of haplotype distribution is correlated with each geographic region (Fig. 3).

DISCUSSION

In total, the Asian tapir (n = 60) showed 7 possible haplotypes based on mitochondrial cytochrome *b* gene sequences. We identified 3 haplotypes in the captive tapir population in Thailand: Ti-1, Ti-2, and Ti-3. The majority of the captive individuals (48.4%) were haplotype Ti-1, which was a new haplotype found only in Thailand. Ti-2 and Ti-3 were similar to those described by Ogata et al. (2009). Ti-2 was found in Thailand and Ti-3 in Thailand and Malaysia. Ti-4

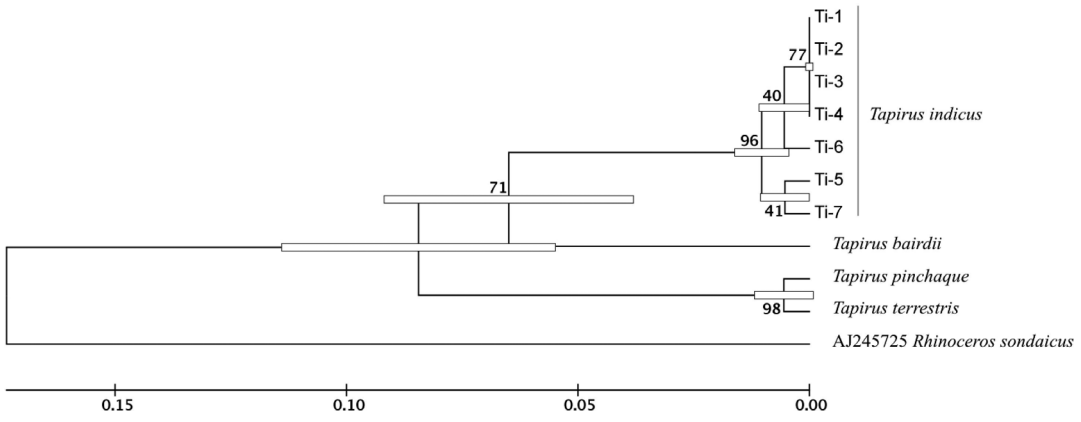

Fig. 2. *Tapirus* spp. Consensus maximum likelihood tree based on the mitochondrial cytochrome *b* gene, illustrating the phylogenetic relationship of the family Tapiridae with *Rhinoceros sondaicus* as the outgroup. The tree was computed using a discrete gamma distribution rate categories (rate = 5) Kimura 2-parameter model. Asian tapirs (Ti-1 to Ti-7) from Southeast Asia are isolated from 3 extant species of Neotropical tapirs (Table 1). Baird's tapir *T. bairdii* from Central America is also genetically isolated from South American tapirs. Mountain tapirs *T. pinchaque* have the closest relationship with lowland tapirs *T. terrestris*. All nodes were supported by the bootstrap values (10 000 replicates). Scale bar corresponds to nucleotide substitutions per site

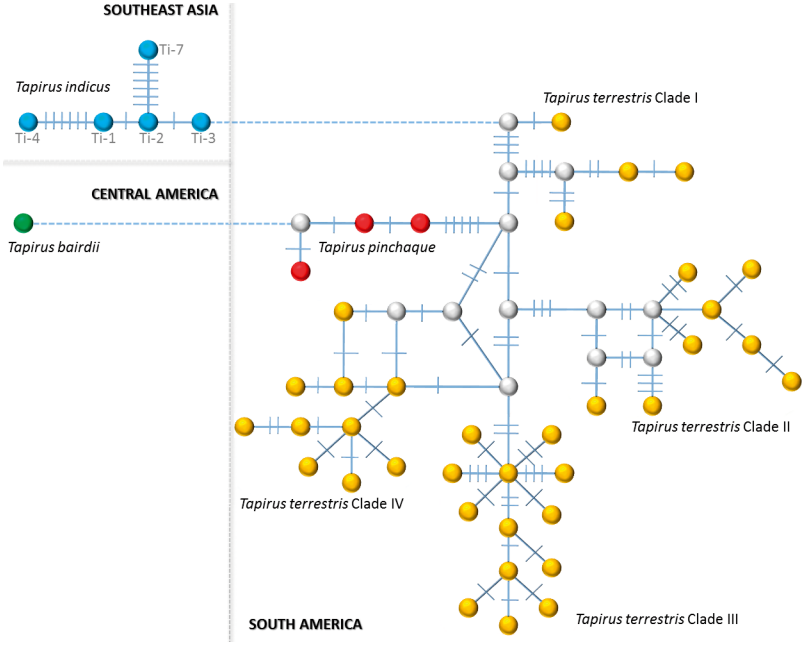

Fig. 3. *Tapirus* spp. Median-joining NETWORK diagram of the family Tapiridae. Colored circles refer to reference samples — blue: *T. indicus* from Southeast Asia; green: *T. bairdii* from Central America; red: *T. pinchaque* from South America; yellow: *T. terrestris* from South America. *T. terrestris* populations were divided into 4 clades as modified from De Thoisy et al. (2010), and the light grey circles refer to mutated positions. The short blue lines refer to the number of nucleotide substitutions. Dashed lines represent >10 nucleotide substitutions

had an unknown origin (Pitra & Veits 2000, Steiner & Ryder 2011). Ti-5 and Ti-6 were found on the Malay Peninsula (Rovie-Ryan et al. 2008). Ti-7 was located only on the island of Sumatra in Indonesia (Ogata et al. 2009). Ti-4 indicated conserved and variable sites, which were quite similar to tapirs originating from mainland Indochina. Despite 4 different nucleotides, the amino acid sequences of Ti-4 showed 100% match with Ti-1 from Thailand. Therefore, it is highly possible that Ti-4 was obtained from Thailand. For Ti-3, we suggest that this haplotype may have been derived from the extensive Thai-Malay transboundary rainforests. Future sampling of tapir populations from Peninsular Malaysia will further elucidate the demographic history of the Asian tapir.

In addition, landscape genetic sampling of wild tapirs will provide a better understanding of factors influencing phylogeographic patterns, for example, the Isthmus of Kra, which has previously been shown to act as a biogeographic barrier for birds and mammals endemic to Southeast Asia. The Isthmus of Kra might serve as a significant biogeographic transition between the Indochinese mainland and Sundaic regions, which include the Malay Peninsula and the Malay Archipelago islands west of the Wallace Line (Corbet & Hill 1992, Woodruff 2003).

Comparative phylogenetic analysis of the family Tapiridae with all available databases compiled from GenBank found 243 variable sites. The results showed that Southeast Asian and Central American

tapirs were completely isolated from South American tapirs. Lowland and mountain tapirs were closely related sister taxa. Our phylogenetic results based on cytochrome *b* revealed identical results as reported in studies of cytochrome *c* oxidase subunit II (Ashley et al. 1996) and 12S rRNA (Norman & Ashley 2000).

Recently, populations of wild Asian tapirs in Thailand have stabilized (Lynam et al. 2012) due to a decrease in the rate of deforestation following a ban on commercial logging in 1989, with protected areas now covering roughly 18% of land area across the country. In general, maintaining large intact areas of undisturbed rainforest is important for Asian tapir persistence (Novarino et al. 2005, Traeholt & Mohamed 2009, Mohamed & Traeholt 2011). In the remnant tapir populations in Thailand that are almost entirely restricted to protected areas, assessment of genetic diversity has become one of the top priorities and plays an important role in conservation management and captive breeding programs for small tapir populations. This study provides an important update on the genetic status of the Asian tapir based on mitochondrial cytochrome *b* gene sequences. Most significantly, our study provides a set of markers to which other captive specimens may be compared in order to determine their geographic origin. This is important given that Asian tapirs are listed in CITES Appendix I, so that any captive individuals with haplotypes of an origin outside of Thailand may have been derived from questionable sources. *Ex situ* conservation management needs integrated information from ecological, demographic, and genetic perspectives in order to make effective decisions for captive breeding management. Data from other faster-evolving polymorphic markers such as the D-loop, the Y chromosome, and microsatellites are also needed to provide a complete picture of genetic variability of the Asian tapir from both paternal and maternal lineages. Therefore, further sampling from a broader geographic area, as well as phylogenetic analyses based on genes with more variable sites will clarify the question of genetic variation and population structure in this species. The combined data can then be used to form recommendations to ensure effective management of the species.

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