International Journal of Agricultural Technology 2022Vol. 18(3):1247-1260
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Mitochondrial DNA analysis revealed genetic diversity of captive-bred hog deer (*Axis porcinus*) population in Thailand

Seritrakul, P.*, Kliangthip, W., Khamkha, S. and Laosutthipong, C.

Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Thailand 76120.

Seritrakul, P., Kliangthip, W., Khamkha, S. and Laosutthipong, C. (2022). Mitochondrial DNA analysis revealed genetic diversity of captive-bred hog deer (*Axis porcinus*) population in Thailand. International Journal of Agricultural Technology 18(3):1247-1260.

Abstract Hog deer (*Axis porcinus*) is an endangered member of the Cervidae family native to South and Southeast Asia. In Thailand, hog deer is nearly extinct in the wild due to unsustainable harvesting and deforestation over the past century. Recent captive breeding program was able to bring the population back to a sustainable level, and a number of hog deer was reintroduced back into the wild. However, due to the small starting number of individuals used at the beginning of the breeding program, there is a risk of genetic bottleneck effect, inbreeding, and loss of genetic diversity. Moreover, two possible subspecies of hog deer exist: *Axis porcinus porcinus* and *Axis porcinus annamiticus*. It was unclear which species is the one currently being used for captive breeding effort. The genetic diversity of captive-bred hog deer at nucleotide level was investigated here using the control region of mitochondrial DNA sequences. Overall, 11 haplotypes were identified from 30 individual hog deer at Huaisai Wildlife Breeding Station in Phetchaburi Province, which is one of the original sites of hog deer breeding programs in Thailand. The population still retained some nucleotide diversity and haplotype diversity. Tajima's D test indicated that the population was expanded in the past. Additionally, phylogenetic analysis revealed that all individuals were identified to be members of the *Axis porcinus annamiticus*, which is currently the prevalent subspecies in Southeast Asia. The results provided a starting point for further genetic analysis for hog deer conservation programs.

Keywords: Hog deer, Genetic diversity, Mitochondrial DNA, Subspecies

Introduction

Hog deer (*Axis porcinus*) is a species of small deer native to South and Southeast Asia. It is currently categorized as an endangered species by IUCN (www.iucnredlist.org/species/41784/22157664). Wild population of hog deer has been declining due to habitat loss, predation, and human activities (Brook *et al.,* 2015). A recent study shows that two subspecies of hog deer exist. *Axis porcinus porcinus* is found along the foothill of Himalayan mountain from

* **Corresponding Author**: Seritrakul, P.; **Email**: seritrakul_p@silpakorn.edu

Pakistan to the west of Myanmar. *Axis porcinus annamiticus* is found from Myanmar to Indo-China, although the exact eastern boundary of *Axis porcinus annamiticus* is not currently known (Gupta *et al.*, 2018). However, because the two subspecies of hog deer are similar in appearance, and no previous studies were able to clearly distinguish the two subspecies in Thailand, it is still unclear which subspecies of hog deer is the predominant one in Thailand (Figure 1). More importantly, because the ranges of *Axis porcinus porcinus* and *Axis porcinus annamiticus* are found adjacent to each other, it is possible that the two originated from a single population and may still undergo crossbreeding.

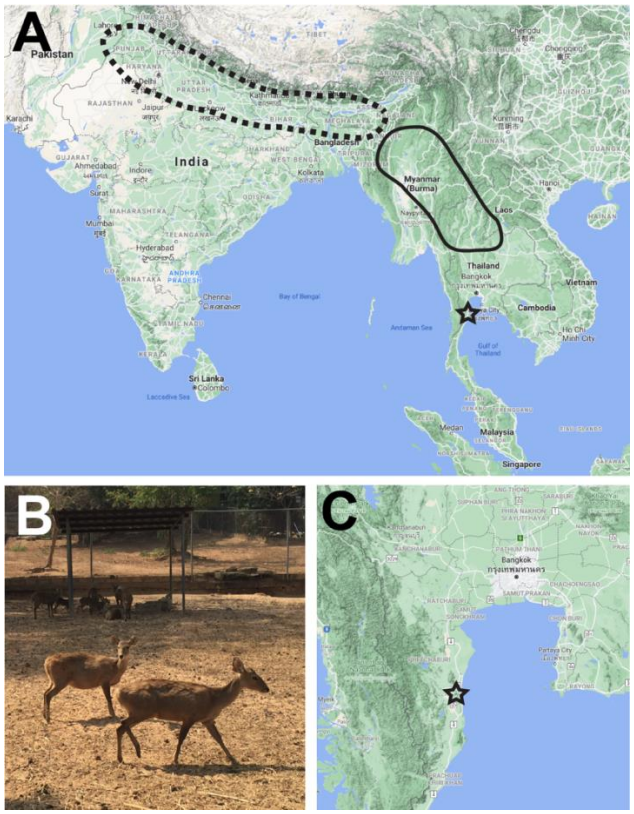

Figure 1. A: Geographic distribution of the current hog deer population, based on mitochondrial sequence data from Gupta *et al.*, 2018. *Axis porcinus porcinus* is found along the foothill of Himalayan mountain from Pakistan to the west of Myanmar (dotted line). *Axis porcinus annamiticus* is found from Myanmar and Indo-China, although the exact eastern boundary is not currently known (solid line). B: captive bred hog deer sampled in this study. C: site of Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand. Geographic map exported from Google Maps (accessed August 2021).

In Thailand, hog deer nearly went extinct during the 1950s until it was later put on the list of protected species by the Thai Government. Currently, hog deer is protected under the National Wildlife Protection and Preservation Act of 1992. Effort has been made to breed hog deer in captivity at zoos and wildlife breeding stations around the country and reintroduce them back into the wild. A survey in 2012 showed that reintroduced hog deer can survive in Thailand's protected forest areas with moderate success, but constant habitat management and population monitoring is required to ensure long term sustainability (Prasanai *et al.,* 2012). Hog deer in other parts of Southeast Asia are also under threat. For example, in Cambodia, wild populations of hog deer still exist, but they are facing extinction due to shrinking habitat and being hunted by human (Brook *et al.,* 2015).

Captive breeding of hog deer is underway at various locations throughout Asia, and due to hog deer's high rate of reproduction, it is possible to quickly expand the population from only a few individuals rescued from the wild. However, due to the small number of starting population used for breeding programs, there is a risk of inbreeding, bottleneck effect, and loss of genetic diversity. Inbreeding presents a serious threat to captive breeding of wildlife, especially for Thai hog deer population, which started with only a handful of breeding individuals. Due to the small starting gene pool and low genetic diversity, inbreeding may result in undesirable fitness-related traits such as low calf body weight, height, twin rate, disease resistance, and lower survival rate when released into the wild. This manifestation of inbreeding depression has been previously reported in a small isolated moose population in Norway (Haanes *et al.,* 2013), a confined population of Kashmir red deer (*Cervus elaphus hanglu*) (Park *et al.,* 2013), and an isolated population of Eld's deer (*Cervus eldi*) (Angom *et al.,* 2017).

Mitochondrial DNA has been extensively used for investigation of genetic diversity and phylogenetic relationship in numerous Cervid species such as in Black Muntjac (*Muntiacus crinifrons*) (Wu and Fang, 2005), Eld's deer (*Cervus eldi*) (Angom *et al.,* 2017; Balakrishnan *et al.,* 2003), red deer (*Cervus elaphus*) (Mahmut *et al.,* 2002) and populations of Indian hog deer (*Axis porcinus*) in the past (Angom *et al.,* 2015). More recently, mitochondrial DNA sequences was used to decipher red deer genetic and geographic distribution in Asia and Europe (Ludt *et al.,* 2004; Schnitzler *et al.,* 2018). The complete reference mitochondrial genome of hog deer has been previously sequenced and published (Hill *et al.,* 2017), thus facilitating the primer design and sequencing in this study.

The Huaisai Wildlife Breeding Station is located in Phetchaburi province, which is one of the original sites of hog deer breeding programs in

Thailand. In fact, the name Huaisai refers to the creek (*Huai*) of hog deer (*Nue-sai*) in Thai language, which indicates the abundance of hog deer that used to roam the area in the past (https://www.dnp.go.th/petchburi/royal/huaisaizoo.html). The starting population of hog deer were brought into the station in the early 1960s by locals (*personal communications*) and, therefore, the inherited genotypes are potential representative of hog deer genetic diversity of this area in the past. Over subsequent years, hog deer individuals were exchanged between other zoos and wildlife breeding stations around the country. Thus, investigating this population of hog deer genetic diversity offers a window into the overall genetics of hog deer in Thailand and southeast Asia.

The purposes were to identify the subspecies of hog deer currently being bred in captivity, which may represent the predominant hog deer species of Thailand, and to determine the existing genetic diversity among the current hog deer population in comparison to other populations and other related deer species.

Materials and methods

Sample collection

Hog deer blood samples were collected with permission from Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand (n=30). Each animal was individually tracked by identification on the ear tag previously established according to the standard protocol of Thailand Department of National Parks, Wildlife, and Plant Conservation (DNP). All animal procedure was done in accordance with the protocol reviewed and approved by Silpakorn University Animal Care and Use Committee (Project ID: 02/2562). Additionally, this research project was granted special permission from Thailand Department of National Parks, Wildlife and Plant Conservation (DNP) to collect data and samples from captive wildlife (letter number 0909.204/15026).

DNA extraction, PCR, and sequencing

DNA was extracted from blood samples using RBC Real Genomics extraction kit YGB50 (RBC Bioscience, Taiwan) according to the manufacturer's procedure. DNA samples were eluted into the elution buffer (100 µl) and stored at -20 ℃. PCR was performed using 1 µl of the extracted

DNA and 24µl of 1x OneTaq PCR master mix (NEB, USA), which contains a blend of Taq and high fidelity Deep Vent polymerases.

Primers used for hog deer mitochondrial DNA control region PCR were: ap15277 (forward primer): CAACCTCCTAAAATGAAGA**T**AAGT, and apH71 (reverse primer): GGCTGGGACCAAACCTAT. The primers were designed based on the position of mitochondrial DNA control region previously used in Muntjac deer, which encompasses the entire control region (Wu and Fang, 2005). However, in order to ensure that the primers will bind to hog deer's mitochondrial DNA properly, we modified a single base on the forward primer from C to T at position 20 (bold letter), in order to match with the published hog deer mitochondrial DNA sequence (Hill *et al.,* 2017).

Thermal cycling condition is as follows: pre-denaturation at 94℃ for 30 seconds, denaturation at 94℃ for 30 seconds, annealing for 30 seconds at decreasing temperature from 57℃ to 50℃ (decreasing 1℃ each cycle) for 6 cycles, extension at 68℃, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds, and extension at 68℃ for 30 seconds. The touchdown PCR protocol was used to ensure primer binding specificity during the annealing step. To ensure the correct product size of 956bp, 10µl of the PCR products were analyzed by running on a 1% agarose gel electrophoresis in 0.5% TBE buffer at 100 volt for 20 minutes and visualized under gel documentation equipment. The remaining PCR products were purified using GenepHlow gel/PCR purification kit (Geneaid, Taiwan) according to the manufacturer's protocol, eluted into ddH_20, quantified using a spectrophotometer and sent for sequencing by standard Sanger protocol (U2Bio, Thailand).

Mitochondrial DNA sequence analysis

DNA sequences from Sanger sequencing results were firstly checked visually for overall chromatogram peak quality, trimmed for high quality sequencing peaks using Geneious version R9 (Biomatters, USA). Multiple sequence alignment was performed and phylogenetic tree was constructed using neighbor-joining method, Jukes-Cantor genetic distance mode using Geneious version R9 (Biomatters, USA). Genetic diversity analyses (haplotype diversity, nucleotide diversity, Tajima's D and Fu's FS test) were performed using DnaSP version 6.12.03 (Rozas *et al.,* 2017) using the aligned sequences.

Results

Hog deer samples and genetic diversity

A total of 30 blood samples from hog deer (8 males and 22 females) were collected from Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand. Total genomic DNA samples were extracted, ran on 1% agarose gel electrophoresis to check for product size, purified, and sequenced using Sanger sequencing. The sequencing reactions typically yielded good quality sequences with clear chromatogram peaks at length of 850-900 bp. Alignment of the 30 hog deer sequences to the reference complete mitochondrial genome (MH443788) showed that the sequences aligned to the mitochondrial DNA control region at nucleotide position 15,340-16,147 of the complete genome.

Multiple sequence alignment of the 30 hog deer sequences identified 11 polymorphic sites out of the 807 bp region. Most of these polymorphic sites are single nucleotide polymorphisms, and one site (position 15495) is a single nucleotide insertion (Figure 2). Percent pairwise identities among the 30 aligned sequences showed overall high sequence similarity (98.23-100.00%), indicating that all of the hog deer were closely related with low genetic distances (Figure 3). Analysis of the aligned DNA sequences using DnaSP 6.12.03 revealed that the population contains a total of 11 haplotypes, with haplotype 1 being the most prevalent haplotype containing 9 members. Haplotype 2, 3, 4, and 6 contained 5, 4, 3 and 3 members, respectively, and the rest of the haplotypes were unique, containing only one member each. The haplotype diversity score was 0.867 and nucleotide diversity score was 0.003. Further evolutionary genetic analysis using Tajima's D test and Fu's FS test yielded results with negative values of -1.717 and -2.527, respectively, indicating that the population may have undergone a recent population expansion from a small number of breeding individuals (de Jong *et al.*, 2011; Ram ŕez-Soriano *et al.*, 2008).

Phylogenetic analysis

To further investigate the genetic relationship among the hog deer population at Huaisai Wildlife Breeding Station, in comparison to other hog deer populations in Asia, and other related *Cervidae* species, we performed phylogenetic analysis of all DNA sequences under investigation. Phylogenetic tree constructed using the neighbor-joining method from mitochondrial control region DNA sequences of hog deer and other related deer species showed an obvious clustering of the hog deer sequences together into a clade, while other

deer species including chital (*Axis axis*), red deer (*Cervus elaphus*), eld's deer (*Cervus eldi*), Sambar deer (*Cervus unicolor*), Javan rusa (*Cervus timorensis*), and elk (*Cervus canadensis*) formed a separated outgroup (Figure 4), confirming that hog deer is genetically distinct from other deer species. Within the hog deer clade, there are two obvious branches that split off into two clades: one containing *Axis porcinus porcinus* (Figure 4, green arrow) and a separate one containing *Axis porcinus annamiticus* (Figure 4, red arrows), indicating that these two subspecies are genetically different and can be unambiguously identified using the mitochondrial control region DNA. The sequences of hog deer from Huaisai Wildlife Breeding Station are clustered together among the sequences of other *Axis porcinus annamiticus* from northern India (sequences starting with KM88) (Gupta *et al.*, 2018) and from Thailand that were previously reported on Genbank (sequences starting with EF4911) (unpublished) with relatively short branch lengths, indicating that all *Axis porcinus annamiticus* are genetically closely related.

Sample	Nucleotide position within the mitochondrial genome										
	15385	15495	15552	15677	15680	15706	15774	15824	15852	15853	15918
	A	-	G	A	T	A	G	G	A	T	C
HS1	.	A	.	.	.	.	.	.	.	.	.
HS2	.	.	.	.	.	.	.	.	.	.	.
HS3	.	A	.	.	.	.	.	A	.	.	T
HS4	.	.	.	.	C	.	.	.	.	C	.
HS5	.	A	.	.	.	.	.	.	.	.	.
HS6	.	.	.	.	.	.	.	.	.	.	.
HS7	G	.	C	T	.	.	.	.	.	.	.
HS8	.	A	.	.	.	.	.	.	.	.	.
HS9	.	.	.	.	.	.	.	.	.	.	.
HS10	.	.	.	.	.	.	.	.	.	.	.
HS11	.	.	.	.	.	G	.	.	.	.	.
HS12	.	.	.	.	.	.	.	.	.	.	.
HS13	.	A	.	.	.	.	.	A	.	.	T
HS14	.	A	.	.	.	.	.	A	.	.	T
HS15	.	.	.	.	.	.	.	.	.	.	.
HS16	.	.	.	.	.	G	.	.	.	.	.
HS17	.	A	.	.	.	.	.	A	.	.	T
HS18	.	.	C	T	.	.	A	.	.	.	.
HS19	.	.	.	.	C	.	.	.	.	.	.
HS20	.	.	.	.	.	.	.	.	.	.	.
HS21	.	.	.	.	C	.	.	.	G	C	.
HS22	.	A	.	.	.	.	.	.	.	.	.
HS23	.	.	.	.	.	G	.	.	.	.	.
HS24	.	.	.	.	.	.	.	.	.	.	.
HS25	.	.	.	.	C	.	.	.	.	C	.
HS26	.	.	.	.	C	.	.	.	.	C	.
HS27	.	A	.	.	.	.	.	.	.	.	.
HS28	.	A	.	.	.	.	.	A	.	.	T
HS29	.	A	.	.	.	.	.	A	.	.	T
HS30	.	.	.	.	C	.	.	.	.	C	.

Figure 2. Nucleotide polymorphic sites among 30 mitochondrial control region DNA sequences (position 15385-15918) of hog deer from Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand. A Dot represents identical sequence compared to reference (top row)

To further verify this observation and investigate the nucleotide differences between *Axis porcinus porcinus* and *Axis porcinus annamiticus*, a pairwise sequence alignment between a representative hog deer mitochondrial control region DNA from Huaisai Wildlife Breeding Station (*Axis porcinus annamiticus*) was aligned with a hog deer mitochondrial control region DNA sequence from Corbett National Park, UK, India (*Axis porcinus porcinus*, MH392157) in order to pinpoint the exact positions of nucleotide differences (Figure 5). The pairwise alignment showed 96.9% pairwise identity, with 13 single nucleotide polymorphisms (SNPs) identified out of the 458 total nucleotide positions.

Sequence name	HS1	HS2	HS3	HS4	HS5	HS6	HS7	HS8	HS9	HS10	HS11	HS12	HS13	HS14	HS15	HS16	HS17	HS18	HS19	HS20	HS21	HS22	HS23	HS24	HS25	HS26	HS27	HS28	HS29	HS30
HS1																														
HS2	99.86																													
HS3	99.72	99.57																												
HS4	99.57	99.72	99.29																											
HS5	99.86	99.72	99.57	99.43																										
HS6	99.86	100.00	99.57	99.72	99.72																									
HS7	99.57	99.72	99.29	99.43	99.43	99.72																								
HS8	100.00	99.86	99.72	99.57	99.86	99.86	99.57																							
HS9	99.86	100.00	99.57	99.72	99.72	100.00	99.72	99.86																						
HS10	99.86	100.00	99.57	99.72	99.72	100.00	99.72	99.86	100.00																					
HS11	99.72	99.86	99.43	99.57	99.57	99.86	99.57	99.72	99.86	99.86																				
HS12	99.72	99.86	99.43	99.57	99.57	99.86	99.57	99.72	99.86	99.86	99.72																			
HS13	99.57	99.43	99.86	99.15	99.43	99.43	99.15	99.57	99.43	99.43	99.29	99.29																		
HS14	99.72	99.57	100.00	99.29	99.57	99.57	99.29	99.72	99.57	99.57	99.43	99.43	99.86																	
HS15	99.86	100.00	99.57	99.72	99.72	100.00	99.72	99.86	100.00	100.00	99.86	99.86	99.43	99.57																
HS16	99.72	99.86	99.43	99.57	99.57	99.86	99.57	99.72	99.86	99.86	100.00	99.72	99.29	99.43	99.86															
HS17	99.72	99.57	100.00	99.29	99.57	99.57	99.29	99.72	99.57	99.57	99.43	99.43	99.86	100.00	99.57	99.43														
HS18	99.15	99.29	98.87	99.01	99.01	99.29	99.57	99.15	99.29	99.29	99.15	99.15	98.72	98.87	99.29	99.15	98.87													
HS19	99.72	99.86	99.43	99.86	99.57	99.86	99.57	99.72	99.86	99.86	99.72	99.72	99.29	99.43	99.86	99.72	99.43	99.15												
HS20	99.86	100.00	99.57	99.72	99.72	100.00	99.72	99.86	100.00	100.00	99.86	99.86	99.43	99.57	100.00	99.86	99.57	99.29	99.86											
HS21	98.58	98.72	98.30	99.01	98.44	98.72	98.44	98.58	98.72	98.72	98.58	98.58	98.16	98.30	98.72	98.58	98.30	98.01	98.86	98.72										
HS22	99.86	99.72	99.57	99.43	100.00	99.72	99.43	99.86	99.72	99.72	99.57	99.57	99.43	99.57	99.72	99.57	99.57	99.01	99.57	99.72	98.44									
HS23	99.72	99.86	99.43	99.57	99.57	99.86	99.57	99.72	99.86	99.86	100.00	99.72	99.29	99.43	99.86	100.00	99.43	99.15	99.72	99.86	98.58	99.57								
HS24	99.86	100.00	99.57	99.72	99.72	100.00	99.72	99.86	100.00	100.00	99.86	99.86	99.43	99.57	100.00	99.86	99.57	99.29	99.86	100.00	98.72	99.72	99.86							
HS25	99.57	99.72	99.29	100.00	99.43	99.72	99.43	99.57	99.72	99.72	99.57	99.57	99.15	99.29	99.72	99.57	99.29	99.01	99.86	99.72	99.01	99.43	99.57	99.72						
HS26	99.57	99.72	99.29	100.00	99.43	99.72	99.43	99.57	99.72	99.72	99.57	99.57	99.15	99.29	99.72	99.57	99.29	99.01	99.86	99.72	99.01	99.43	99.57	99.72	100.00					
HS27	99.86	99.72	99.57	99.43	100.00	99.72	99.43	99.86	99.72	99.72	99.57	99.57	99.43	99.57	99.72	99.57	99.57	99.01	99.57	99.72	98.44	100.00	99.57	99.72	99.43	99.43				
HS28	99.72	99.57	100.00	99.29	99.57	99.57	99.29	99.72	99.57	99.57	99.43	99.43	99.86	100.00	99.57	99.43	100.00	98.87	99.43	99.57	98.30	99.57	99.43	99.57	99.29	99.29	99.57			
HS29	99.72	99.57	100.00	99.29	99.57	99.57	99.29	99.72	99.57	99.57	99.43	99.43	99.86	100.00	99.57	99.43	100.00	98.87	99.43	99.57	98.30	99.57	99.43	99.57	99.29	99.29	99.57	100.00		
HS30	99.57	99.72	99.29	100.00	99.43	99.72	99.43	99.57	99.72	99.72	99.57	99.57	99.15	99.29	99.72	99.57	99.29	99.01	99.86	99.72	99.01	99.43	99.57	99.72	100.00	100.00	99.43	99.29	99.29	

Figure 3. Percent pairwise identities among 30 mitochondrial control region DNA sequences of hog deer from Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand

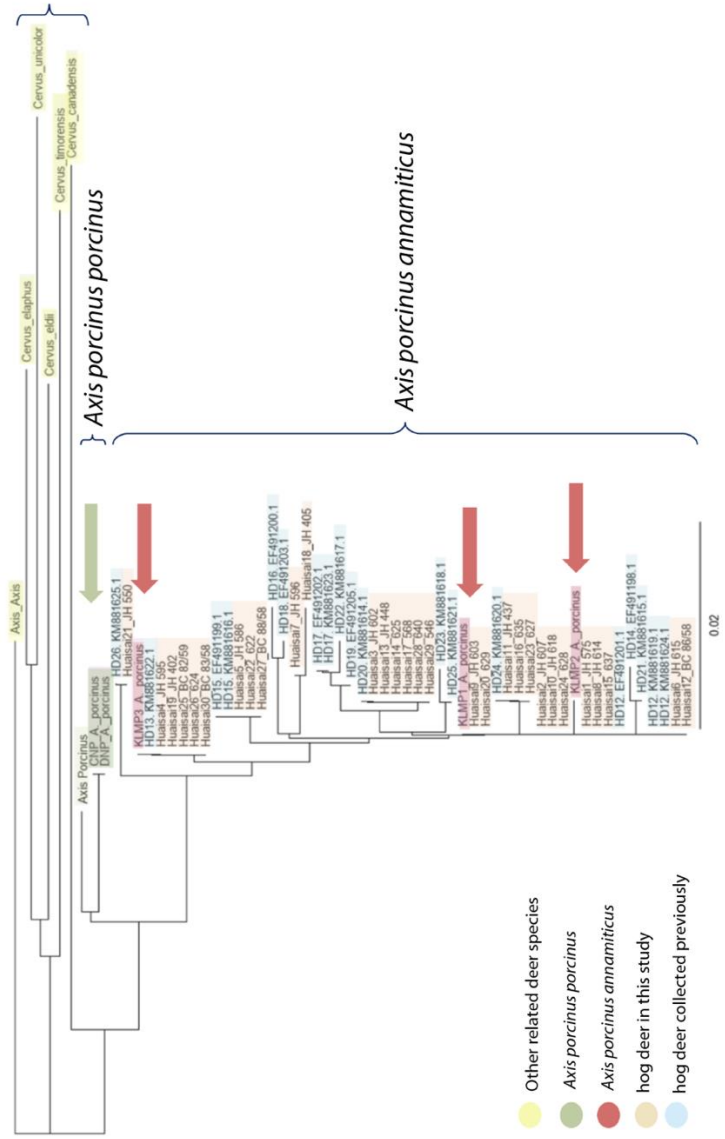

Figure 4. Phylogenetic tree constructed using neighbor-joining method from mitochondrial control region DNA sequences of hog deer from Huaisai Wildlife Breeding Station in Phetchaburi Province, Thailand (orange), compared with sequences from Gupta *et al.*, 2018 (sequences starting with KM88) and Thai hog deer sequences previously deposited on NCBI Genbank (sequences starting with EF4911) (blue). Reference sequences of other deer species (yellow) were used as an outgroup. Green and red arrows indicate known and published sequences of *Axis porcinus porcinus* and *Axis porcinus annamiticus*, respectively

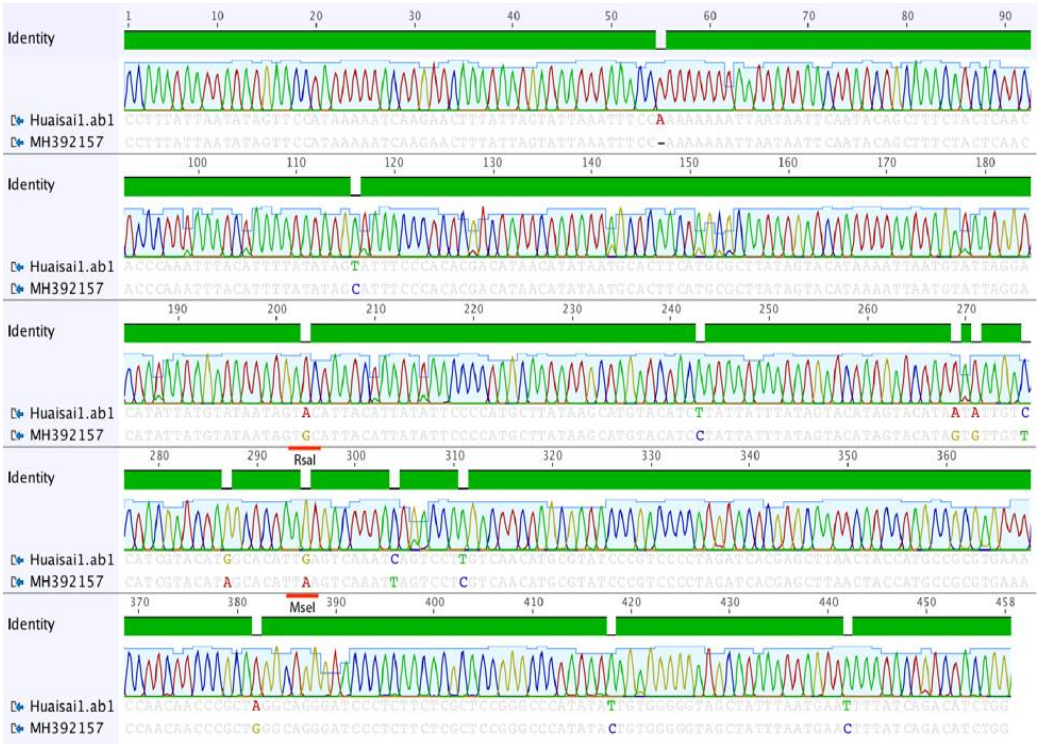

Figure 5. A representative sequence alignment between *Axis porcinus porcinus* and *Axis porcinus annamiticus* showed positions of nucleotide polymorphisms: A sequence of a hog deer mitochondrial control region DNA from Huaisai Wildlife Breeding Station (top sequence with chromatogram) was aligned with a hog deer mitochondrial DNA sequence from Corbett National Park, UK, India (MH392157, bottom sequence). The sequence alignment showed 96.9% pairwise identity. 13 single nucleotide polymorphisms (SNPs) were identified out of the 458 total nucleotide positions. Green bars represents identical sequences between *Axis porcinus porcinus* and *Axis porcinus annamiticus*. Red lines indicate potential restriction enzyme recognition sites for designing genotyping assays for further experiments

Discussion

Hog deer is a species of endangered deer native to Asia. The population has been declining in the wild, and conservation effort and captive breeding have been done over the past decades. In this study, genetic diversity and phylogenetic analysis of 30 hog deer mitochondrial DNA control region

sequences were performed using samples collected from captive-bred hog deer at Huaisai Wildlife Breeding Station in Phetchaburi province, Thailand. Of the 30 sequences investigated, 11 haplotypes were identified, with a haplotype diversity score of 0.867 and nucleotide diversity score of 0.003. Evolutionary genetic analysis showed Tajima's D test score of -1.717 and Fu's FS test score of -2.527.

The data in this work were overall similar to a previous study on the Black Muntjac (*Muntiacus crinifrons*), an endangered deer species native to China, where mitochondrial DNA sequences identified 9 haplotype from 44 samples, with nucleotide diversity of 0.0056, haplotype diversity of 0.862, and negative Tajima's D and Fu's FS scores (Wu and Fang, 2005). In this case, the data indicated that the population went through a bottleneck event historically, and improvement to the current breeding program is needed to increase genetic diversity, either by balancing the existing haplotypes or by reintroducing more founders.

However, the gathered data were in contrast to the mitochondrial DNA genetic diversity data of Eld's deer (*Cervus eldi*), another endangered deer species found in overlapping geographic region with hog deer. In a 2017 study of 30 captive and wild Eld's deer individuals, only one haplotype was identified and no genetic variation was detected (Angom *et al.*, 2017). This indicated that the Eld's deer population went through a very strong bottleneck effect and the population possess essentially no remaining genetic diversity, while the hog deer population in this study still retains an appreciable level of genetic diversity as indicated by the haplotypes and nucleotide diversity data. Therefore, Eld's deer is at an even greater risk of extinction compared to hog deer, and very careful conservation, breeding, and reintroduction are required urgently.

An earlier karyotype study was performed to examine an "Indian hog deer" population bred in captivity at a zoo in northeastern Thailand, although it was not known if this population was *Axis porcinus porcinus* or *Axis porcinus annamiticus* (Pinthong *et al.*, 2017) as the karyotyping assay did not reveal the detailed genetic information at a single nucleotide resolution. Therefore, a careful examination of the genetic signature of each subspecies is critical to maintain and propagate the proper genotype, and to prevent accidental introduction of a non-native subspecies to the fragile ecosystem such as protected forest or nature reserves.

A recent study indicated that two possible subspecies of hog deer exist, with native habitat ranging from western India to the Indo-China subcontinent (Gupta *et al.*, 2018). *Axis porcinus porcinus* occupies the western region, while *Axis porcinus annamiticus* occupies the eastern region. However, it was not

clearly established which subspecies the population of hog deer in Thailand belongs to. Our phylogenetic analysis using the 30 hog deer mitochondrial DNA, in conjunction with other available sequences on public databases, revealed that all 30 hog deer sequenced in this study, as well as those previously collected by others in Thailand (unpublished), belonged to *Axis porcinus annamiticus*, with sequences closely matching with previously verified individuals (Gupta *et al.*, 2018), and phylogenetic tree showed an obvious clustering into a clade with short branch lengths. These results indicated that the seemingly separated populations of Thailand's *Axis porcinus annamiticus* possesses low but appreciable level of genetic diversity and may in fact belong to the same population. Because hog deer from Huaisai Wildlife Breeding Station were originally collected from the local area, these data also further pinpoint the eastern range of *Axis porcinus annamiticus* to the central part of Thailand.

Molecular analysis using Mitochondrial DNA sequence has proven to be a reliable and useful tool for investigating the genetic diversity and phylogenetic relationship within and between species. In this work, mitochondrial DNA control region sequences confirmed that there were different subspecies of hog deer, *Axis porcinus porcinus* and *Axis porcinus annamiticus*, occupying adjacent geographic regions (Gupta *et al.*, 2018). Similarly, a recent study using mitochondrial DNA sequences and paleogenetic analysis tracked populations of French red deer (*Cervus elaphus*) revealed that two different subpopulations occupied two distinct habitats in the same area, which were similarly disturbed by human activities in the recent past (Schnitzler *et al.*, 2018). This highlights the importance of proper genetic identification of each subspecies, as accidental reintroduction of the wrong subspecies could result in catastrophic ecological consequences or even hybridization between subspecies. This has previously occurred in Victoria, Australia, where the non-native hog deer and chital (*Axis axis*) were introduced together in the 1860s. The two species promptly hybridized resulting in a persistent hybrid population that could not be reintroduced to their native habitats in Asia (Hill *et al.*, 2019).

To address this issue of rapid and accurate subspecies identification, a sequence alignment and comparison between *Axis porcinus porcinus* and *Axis porcinus annamiticus* were performed (Figure 5). The sequence alignment revealed several SNPs that are distinct and unique to each subspecies. Specifically, two SNPs at position 203 and 295 overlap with recognition sites of restriction endonuclease enzymes RsaI and MseI, respectively. This is useful for developing further molecular genotyping assay using restriction fragment length polymorphisms (RFLP) method (Williams, 1989). Alternatively, for

SNPs that did not overlap with restriction sites, one could employ the methods such as dCAP PCR (Neff *et al.*, 2002; Yang *et al.*, 2013), which would aid in rapid identification of the hog deer into proper subspecies for planning and selection in conservation and breeding program.

In conclusion, the hog deer genetic diversity was studied using mitochondrial DNA control region sequences, along with publicly available sequences of other related populations and species. The genetic analysis identified the subspecies of captive-bred hog deer in Thailand as *Axis porcinus annamiticus*, thus pinpointing the eastern range of this subspecies. The hog deer population in this study still retains some level of genetic diversity, although careful genotyping is required for planning and breeding of future generations. It is important to note that, although this study focused on a small population of hog deer that likely represented the local genetic diversity of hog deer in this region, future studies would need to involve a larger sample size from other geographic locations around the country, and this will be beneficial for the survey of the overall hog deer population in Thailand and the surrounding regions.

Acknowledgements

This study was funded by a research grant from the Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Thailand. All animal procedure was done in accordance with the protocol reviewed and approved by Silpakorn University Animal Care and Use Committee (SUACU, Project ID: 02/2562). Additionally, this research project was granted special permission from Thailand Department of National Parks, Wildlife and Plant Conservation (DNP) to collect data and samples from captive wildlife (letter number 0909.204/15026). We acknowledge the support from staff members of the Huaisai Wildlife Breeding Station, DNP, in Cha-am district, Phetchaburi province, Thailand, who helped with animal handling.

References

Angom, S., Gupta, S. K., Kumar, A. and Hussain, S. A. (2015). Identification of globally threatened cervids from problematic samples using cytochrome b and control region genes. Conservation Genetics Resources, 7: 647-650.

Angom, S., Kumar, A., Gupta, S. K. and Hussain, S. A. (2017). Analysis of mtDNA control region of an isolated population of Eld's deer (Rucervus eldii) reveals its vulnerability to inbreeding. Mitochondrial DNA Part B, 2:277-280.

Balakrishnan, C. N., Monfort, S. L., Gaur, A., Singh, L. and Sorenson, M. D. (2003). Phylogeography and conservation genetics of Eld's deer (*Cervus eldi*). Molecular Ecology, 12:1-10.

Brook, S. M., Nasak, C. and Channa, P. (2015). Indochinese hog deer *Axis porcinus annamiticus* on the brink of extinction. Deer Specialist Group News, 27:14-31.

de Jong, M. A., Wahlberg, N., van Eijk, M., Brakefield, P. M. and Zwaan, B. J. (2011). Mitochondrial DNA signature for range-wide populations of *Bicyclus anynana* suggests a rapid expansion from recent refugia. PLoS One, 6:1-5.

Gupta, S. K., Kumar, A., Angom, S., Singh, B., Ghazi, M. G. U., Tuboi, C. and Hussain, S. A. (2018). Genetic analysis of endangered hog deer (*Axis porcinus*) reveals two distinct lineages from the Indian subcontinent. Scientific Reports, 8:1-12.

Haanes, H., Markussen, S. S., Herfindal, I., RØed, K. H., Solberg, E. J., Heim, M., Midthjell, L. and Sæther, B. E. (2013). Effects of inbreeding on fitness-related traits in a small isolated moose population. Ecology and Evolution, 3:4230-4242.

Hill, E., Linacre, A. M. T., Toop, S., Murphy, N. P. and Strugnell, J. M. (2017). The complete mitochondrial genome of *Axis porcinus* (Mammalia: Cervidae) from Victoria, Australia, using MiSeq sequencing. Mitochondrial DNA Part B, 2:453-454.

Hill, E., Linacre, A., Toop, S., Murphy, N. and Strugnell, J. (2019). Widespread hybridization in the introduced hog deer population of Victoria, Australia, and its implications for conservation. Ecology and Evolution, 9:10828-10842.

Ludt, C. J., Schroeder, W., Rottmann, O. and Kuehn, R. (2004). Mitochondrial DNA phylogeography of red deer (*Cervus elaphus*). Ecology and Evolution, 31:1064-1083.

Mahmut, H., Masuda, R., Onuma, M., Takahashi, M., Nagata, J., Suzuki, M. and Ohtaishi, N. (2002). Molecular Phylogeography of the Red Deer (*Cervus elaphus*) Populations in Xinjiang of China: Comparison with other Asian, European, and North American Populations. Zoological science, 19:485-495.

Neff, M. M., Turk, E. and Kalishman, M. (2002). Web-based primer design for single nucleotide polymorphism analysis. Trends Genet, 18:613-5.

Park, D. N., Sharma, L. K., Kumar, V. P., Charoo, S. A., Mohan, N. and Goyal, S. P. (2013). Loss of genetic diversity and inbreeding in Kashmir red deer (*Cervus elaphus hanglu*) of Dachigam National Park, Jammu & Kashmir, India. BMC Research Notes, 6:326.

Pinthong, K., Tanomtong, A., Khongcharoensuk, H., Chaiphech, S., Rattanayuvakorn, S. and Supanuam, P. (2017). Karyotype and Idiogram of Indian Hog Deer (*Hyelaphus porcinus*) by Conventional Staining, GTG-, High-Resolution and Ag-NOR Banding Techniques. Cytologia (Tokyo), 82:227-233.

Prasanai, K., Sukmasuang, R., Bhumpakphan, N., Wajjwalku, W. and Nittaya, K. (2012). Population characteristics and viability of the introduced hog deer (*axis porcinus zimmermann*, 1780) in Phu Khieo wildlife sanctuary, Thailand. Songklanakarin Journal of Science and Technology, 34:263-271.

Ram ŕez-Soriano, A., Ramos-Onsins, S. E., Rozas, J., Calafell, F. and Navarro, A. (2008). Statistical power analysis of neutrality tests under demographic expansions, contractions and bottlenecks with recombination. Genetics, 179:555-567.

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E. and Sanchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. Molecular Biology & Evolution, 34:3299-3302.

Schnitzler, A., Granado, J., Putelat, O., Arbogast, R. M., Drucker, D., Eberhard, A., Schmutz, A., Klaefiger, Y., Lang, G., Salzburger, W. *et al.* (2018). Genetic diversity, genetic structure and diet of ancient and contemporary red deer (*Cervus elaphus* L.) from north-eastern France. PLoS One, 13:1-19.

Williams, R. C. (1989). Restriction Fragment Length Polymorphism. Yearbook of Physical Anthropology, 32:159-184.

Wu, H. L. and Fang, S. G. (2005). Mitochondrial DNA genetic diversity of black muntjac (Muntiacus crinifrons), an endangered species endemic to China. Biochemical Genetics, 43:407-416.

Yang, W., Kang, X., Yang, Q., Lin, Y. and Fang, M. (2013). Review on the development of genotyping methods for assessing farm animal diversity. Journal of Animal Science and Biotechnology, 4:2-7.

(Received: 20 August 2021, accepted: 16 April 2022)