

RESEARCH ARTICLE

# A New Haplotype Revealed by Using Mitochondrial DNA in Captive Dholes (*Cuon alpinus*) in Thailand

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## Abstract

**Objective**—To assess the genetic diversity of maternal lineage by using partial sequences of cytochrome b and control region of mitochondrial DNA in eight captive dholes in Thailand.

**Materials and Methods**—Faecal samples were preserved in preservative buffer. Mitochondrial DNA of eight faecal samples of dhole were extracted, purified, and amplified with specific primers for cytochrome b and control region of dhole using Polymerase Chain Reaction (PCR) technique. The PCR products were used for DNA sequencing and compared with data in GenBank.

**Results**—The 407-base pair (bp) fragments of cytochrome b and 246-bp fragments of control region were analyzed. We revealed no variable site on cytochrome b but seven variable sites on control region were detected. These variable sites were identified as two haplotypes (R and U) among eight samples. Haplotype U was a new control region haplotype.

**Conclusion**—There are only two haplotypes of captive dholes in Thailand. This result has implications toward conservation management and captive breeding of dholes in Thailand.

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**Keywords:** Dhole; Genetic diversity; Mitochondrial DNA

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# รูปแบบพันธุกรรมสายแม่กลุ่มใหม่ที่พบจากการศึกษาดีเอ็นเอบนไมโทคอนเดรียของหมาใน (*Cuon alpinus*) ในกรุงเลียง

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## บทคัดย่อ

**วัตถุประสงค์** เพื่อศึกษาความหลากหลายทางพันธุกรรมสายแม่ของหมาในในกรุงเลียง โดยศึกษาลำดับเบสของยีน cytochrome b และ control region บนไมโทคอนเดรียของหมาใน

**วัสดุอุปกรณ์และวิธีการ** นำตัวอย่างมูลของหมาในในกรุงเลียงจำนวน 8 ตัวมาสกัดดีเอ็นเอและเพิ่มจำนวนชิ้นดีเอ็นเอด้วยวิธี Polymerase Chain Reaction (PCR) โดยใช้ไพรเมอร์สองคู่ที่ออกแบบให้จำเพาะในส่วน of cytochrome b และ control region เพื่อใช้ในการวิเคราะห์ลำดับเบส

**ผลการศึกษา** ไม่พบความแตกต่างของลำดับเบสในส่วน cytochrome b ความยาว 407 คู่เบส ของหมาในทั้ง 8 ตัว แต่ส่วน control region ความยาว 246 คู่เบส พบว่ามีความแตกต่างของลำดับเบสทั้งหมด 7 ตำแหน่งและเมื่อเปรียบเทียบกับข้อมูลในธนาคารพันธุกรรม สามารถแบ่งรูปแบบพันธุกรรมสายแม่ตามลำดับเบสที่แตกต่างกันเป็น 2 กลุ่มคือ haplotype R และ U โดย U เป็นสายแม่กลุ่มใหม่ที่พบในการศึกษาครั้งนี้

**ข้อสรุป** หมาในในกรุงเลียงในประเทศไทยมีรูปแบบพันธุกรรมสายแม่เพียง 2 สาย โดย haplotype U เป็นพันธุกรรมสายแม่ที่พบใหม่ ข้อมูลเหล่านี้สามารถนำไปใช้เป็นฐานข้อมูลสำหรับการจัดการประชากรหมาในในกรุงเลียงต่อไป

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**คำสำคัญ:** หมาใน ความหลากหลายทางพันธุกรรม ไมโทคอนเดรียดีเอ็นเอ

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## Introduction

Molecular genetics have an important role for wildlife conservation. Information on genetic diversity can identify and category species, subspecies, as well as estimate population size [1] and genetically deteriorated populations [2]. Previous studies have indicated that losing of genetic diversity in wildlife populations might increase the risk of population-size reduction such as in cheetah [1]. Some studied reported free-ranging wild animals have higher levels of genetic diversity than captive animals such as in African wild dog (*Lycaon pictus*) [3]. However, it depended on the captive management and population size [4]. Despite well-designed breeding programs to maintain genetic diversity in Thamin Eld's deer (*Cervus eldi thamin*) at the Conservation and Research Center, Smithsonian Institution, Virginia, the populations have the same haplotype diversity level as the population in Chattin Wildlife Sanctuary, Myanmar. Both populations have a higher haplotype diversity than unmanaged population in Yangon Zoo in Myanmar [5]. The wild animals that were kept in captivity usually have a low effective population size and limited chance and choice for mating. As a result, the captive populations might suffer from inbreeding depression, lose genetic variation, and subsequently increase the risk of extinction. For long-term conservation strategies of endangered species, genetic diversity information have important role to reduce deleterious effects of inbreeding and genetic drift [2, 3].

In Thailand, little is known about molecular genetics in wild animals especially in endangered species. The dhole (*Cuon alpinus*), also called the Asiatic wild dog, Indian wild dog, red dog, or “maanay” in Thai [6] is an endangered species. It is listed as an endangered species by the International Union for the Conservation of Nature and Natural Resources (IUCN) Species Survival Commission [7]. Based on morphological data, it has been classified to eleven subspecies using differences in fur length, thickness and color [8]. Their distribution is found through most of South, East and Southeast Asia [9]. Dhole is one of two canine species in Thailand. Two-hundred and twenty-five dholes are keeping in captivity [10]. However, only eight animals are keeping in Thai zoos. There is no information about genetic diversity of captive dhole in Thailand.

The molecular genetics study such as evolutionary relationship and genetic diversity among various species of domestic and wild animals are usually evaluated by using mitochondrial DNA (mtDNA) [2, 11-14]. The mtDNA is maternally inherited without recombination and repairing system. Their substitution rate is higher than nuclear genome [11, 15]. Due to their high polymorphism, cytochrome b and control region of mtDNA have been used to evaluate the genetic diversity among various species of wild animals including canid species [16], Iberian lynx (*Lynx pardinus*) [17], dhole (*Cuon alpinus*) [8], Indian wolf species [18]. In this study, we evaluated partial sequences of cytochrome b and control region of mtDNA of eight captive dholes. The obtained data will improve our knowledge about their haplotype diversity for further use in conservation programs.

## Materials and methods

### *Sample collection*

Eight faecal samples were collected from two populations; Chiang Mai Zoo (CMZ) and Chiang Mai Night Safari (CNS). Faecal samples were collected from each dhole within 2-4 hours post-defecation. Samples were placed into 50 ml tubes containing DET buffer (20% dimethyl sulfoxide, 0.25 M ethylenediamine tetra-acetic acid, 100 mM Tris, pH 7.5, saturated with NaCl [19]). Samples were added into collecting buffer with 4 (DET buffer) : 1 (faeces) ratio [20] and were stored at 4°C until DNA extraction.

### *DNA extraction and polymerase chain reaction (PCR) amplification*

DNA from eight faecal samples was extracted and purified using silica and guanidinium thiocyanate protocol [21] and then eluted with 50 µl of 10 mM Tris-HCl, pH 8.0. The extracted DNA was used for PCR amplification. The partial sequence of mitochondrial cytochrome b region (440 base pairs) was amplified with primers CB0 (5'-CATGACTAATGATATGAAAAACC-3') and CB2 (5'-CTCAGAATGATATTTGTCCTCA-3') and partial sequences of the control region sequence (340 base pairs) was amplified with primers D-loopFw (5'-CACCATCAACACCCAAAGCT-3') and D-loopRe (5'-CCTGAAGAAAGAACCAGATGC-3'). The PCR reaction was carried out in a 30 µl volume, each reaction consisted of 3 µl of faecal DNA extracts as template, 1x (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> buffer, 4 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.3 µM each primer, 1.25 U *Taq* DNA polymerase (Fermentas, USA). PCR reactions were performed in a Peltier thermal cycler machine (USA), Model PTC-200 programmed for an initial denaturation cycle of 95°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 53°C (cytochrome b) or 54°C (control region) for 30 sec, extension at 72°C for 45 sec and followed by a final extension at 72°C for 10 min. The success of PCR reaction and the contamination were monitored by including positive (diluted blood DNA template from a captive dhole) and negative DNA controls (distilled water) were included with each set of PCR.

Amplification products were stained with ethidium bromide (Glocount™, D&F Control Systems, Inc.) and electrophoresed on a 1.5% agarose gels in TAE buffer (40 mM, Tris/acetate, 1 mM EDTA). The gels were visualized under UV Electronic UV Transilluminator and photographed with a digital image system (Alphadigidoc™, EEC).

### *DNA sequencing*

Positive PCR products were cut out from electrophoresis gel and were purified using QIAQuick gel purification columns (Qiagen Inc., Germany). All samples were sequenced using BigDye Terminator Cycle Sequencing Kit and run on an A-373 automated sequencer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's instructions, and with the same primers used for PCR amplification.

### *Phylogenetic analysis*

The sequences were confirmed by BLAST in GenBank and aligned with GenBank accession number NC013445 [22] using the program BIOEDIT VERSION 7.0.9 [23]. To determine the number of

unique haplotypes among eight faecal samples from CMZ and CNS, the 246-bp control region product were compared with the published homologous sequences in same species (GenBank accession numbers AY682699-682717).

Phylogenetic relationships among haplotypes were analyzed using maximum likelihood (ML) approaches in PAUP, version 4.0b10 [24] with a heuristic search with 1000 bootstrap replicates. The grey wolf sequence in GenBank accession number AF098117 was used as an outgroup following previously reported [8].

## Results

In this study, all faecal DNA samples of captive dholes were successfully amplified. The total of 440-bp fragment length of the cytochrome b region mitochondrial was obtained in this study. From whole sequences, the selected 407-bp of the cytochrome b was aligned and compared with the dhole complete mitochondrial DNA reported in GenBank accession number NC013445 [22]. The results revealed no

**Figure 1.** Alignment of Individual Eight Captive Dhole Samples in the 246 Base Pairs of Control Region with Haplotype A-S\*

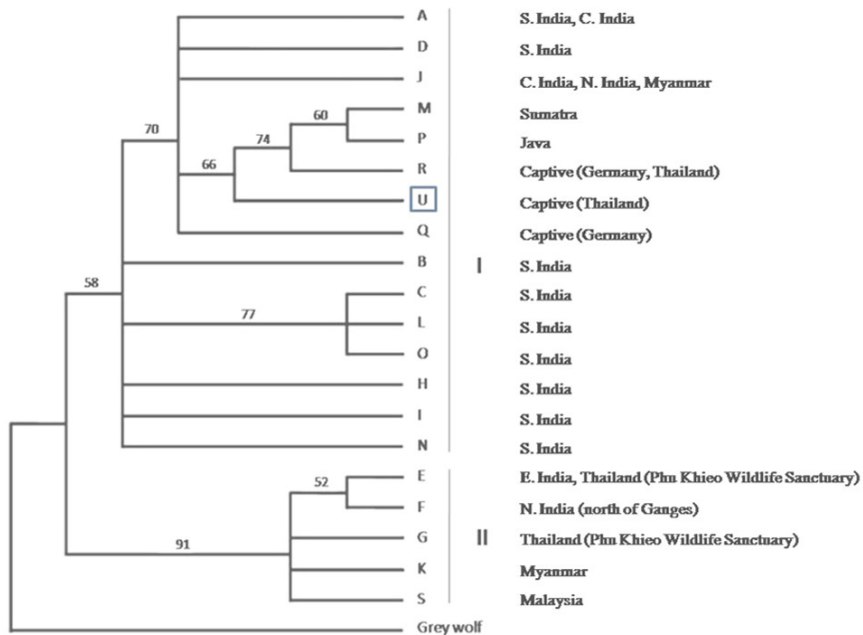
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\*Alignment of individual eight captive dhole samples in the 246 base pairs of control region with haplotype A-S in GenBank accession numbers AY682699-AY682717 [8]. Seven variable sites within among eight faecal samples in captive dholes were found and can identified two haplotypes (R and U). Dots indicate identity with haplotype A in GenBank accession number AY682699.

**Table 1.** Haplotypes of Eight Captive Dholes\*

Sample number	Age	Gender	Source	Haplotype	
				Cytochrome b 407 base pairs	Control region 246 base pairs
CMZ 1	8 years	Male	Chiang Mai Zoo	1	U
CMZ 2	8 years	Male	Chiang Mai Zoo	1	U
CNS 1	6 years	Female	Chiang Mai Night Safari	1	R
CNS 2	8 years	Male	Chiang Mai Night Safari	1	U
CNS 3	6 years	Female	Chiang Mai Night Safari	1	R
CNS 4	6 years	Male	Chiang Mai Night Safari	1	R
CNS 5	8 years	Female	Chiang Mai Night Safari	1	U
CNS 6	2 years	Female	Chiang Mai Night Safari	1	R

\*Eight captive dholes from Chiang Mai Zoo (CMZ) and Chiang Mai Night Safari (CNS) were revealed one cytochrome b haplotype (haplotype 1) and revealed two control region haplotypes (haplotype R and U).

**Figure 2.** Bootstrap Concensus Tree\*

\*Bootstrap concensus tree showed haplotype relationships within among nineteen haplotypes (haplotype A-S) [8] and a new haplotype (U), which was identified in captive dholes from Thai zoos based on maximum likelihood (HKY + G) analysis. Number above branch is bootstrap support from 1,000 replicates with ML bootstrap values.

substitution of nucleotide on cytochrome b sequence among eight faecal samples from both populations. The total of 340-bp fragment length of the control region was successfully PCR amplified from all samples. In the same way, the 246-bp control region was used to align and compare with haplotype A-S, which sequence reported in GenBank accession numbers AY682699-AY682717 [8]. Based on control region sequences from eight faecal samples, two haplotypes (R and new haplotype) were identified. Haplotype U is a new haplotype. Seven variable sites between two control region haplotypes (R and U) were found (**Figure 1**). The sample number 1, 3, 4, and 6 belonged to CNS are haplotype R. The sample number 1, 2 belonged to CMZ and sample number 2 and 5 belonged to CNS are haplotype U (**Table 1**).

Previous studies have identified two clades among nineteen haplotypes of dholes using one substitution on partial sequence of cytochrome b (C-T transition) [8]. In this study, the results of maximum-likelihood (ML) analysis with bootstrap consensus tree (1,000 replication) showed haplotype R and U placed within clade I. This relationship was based on cytochrome b sequence haplotype 1 with supported by medium ML bootstrap values and moderately supported grouping of haplotype R and U (**Figure 2**).

## Discussion

Non-invasive sampling method is usually used for genetic analysis of elusive species in the wild and [17] in captivity. Faecal samples are highly prefer for DNA sources, including mtDNA [25] that can be used to evaluate genetic diversity or phylogenetic relationships in threatened or endangered species. Although, several factors are affected the quality and quantity of genomic DNA extraction such as age of faeces, diet type, storage protocol and extraction method, in this study, all samples (n=8) were successfully PCR amplified. The reason may be come from the fresh samples, low influenced by non expected DNA and the highly specific of primer pairs.

In this study, the 407 bp of the mitochondrial cytochrome b region and 246 bp of control region were used to analyze. Concordant with previous studies [8, 18], the control region showed higher polymorphism than cytochrome b.

In conclusion, two haplotypes (R and U) were found by using control region analysis among eight captive dholes from both zoos. Furthermore, by using cytochrome b analysis, we identified captive dholes in both zoos into clade I, which differ from previously reported about our wild dholes in Thailand. They have been identified into clade II [8]. However, because of all captive dholes in both zoos are obtained from zoo in The Netherlands. Four captive dholes, which were revealed as haplotype R, were presumed to have China origin [22]. The other four captive dholes belonged to the new haplotype U, revealed uncertain subspecies and geographical wild origin. However, due to a closely related lineage clusters between haplotypes R and U, we presumed that they might be belonged to same subspecies of haplotype R or *Cuon alpinus lepturus*. The crossbreed between haplotype R and U to improve their genetic diversity and reduce inbreeding opportunity for long term conserving of captive dhole should be concerned.

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