RESEARCH ARTICLE

# Genetic Divergence Between Two Subspecies of Eld's Deer: Siamese Eld's Deer (*Cervus eldii siamensis*) and Burmese Eld's Deer (*Cervus eldii thamin*) Based on Y Chromosome Variation

Manakorn Sukmak[1,2], Suthatip Dejchaisri[1,2], Waradee Buddhakosai[3], Worawidh Wajjwalku[4*]

## Abstract

**Objective**—To verify the genetic diversity on Y chromosome and investigate the speciation in two subspecies of Eld's deer.

**Materials and Methods**—Three Y chromosome regions (DBY, ZFY, SRY) and one mtDNA specific region (CYTB) were amplified. Their sequences were analysis to verify the variation between two subspecies. The phylogenetic tree was constructed to compare their divergence pattern.

**Results**—We successfully amplified DBY, SRY and ZFY (508, 2,697, 1,945 bp, respectively) and CYTB (1,140 bp). For Y chromosome diversity, no variation site was found within subspecies but 10 variation sites were found when compared between Burmese Eld's deer and Siamese Eld's deer. Both phylogenetic trees from Y chromosome and CYTB revealed clearly divergence between these two subspecies.

**Conclusion**—Y chromosome was not appropriated to study the genetic diversity in intra-subspecies level but can used as a marker for study male introgression between two subspecies of Eld's deer. Our Y chromosome phylogenetic tree confirmed the speciation of Eld's deer.

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**Keywords:** Y chromosome; CYTB; Eld's deer

[1]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140.

[2]Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand.

[3]Interdisciplinary Graduate Program in Genetic Engineering, Kasetsart University, Bangkok, Thailand.

[4]Department of Pathology, Faculty of Veterinary Medicine, Kamphaeng Saen Campus, Kasetsart University, Nakhon Pathom, 73140.

[*]**Corresponding author** E-mail: fvetwww@yahoo.com

# ความแตกต่างทางพันธุกรรมบนโครโมโซมวาย (Y chromosome) ระหว่างละมั่งชนิดย่อยไทย (*Cervus eldii siamensis*) และละมั่งชนิดย่อยพม่า (*Cervus eldii thamin*)

มานะกร สุขมาก[1,2], สุธาทิพย์ เดชชัยศรี[1,2], วราดี พุทธโกสัย[3], วรวิทย์ วัชชวัลคุ[4*]

## บทคัดย่อ

**วัตถุประสงค์** เพื่อศึกษาความหลากหลายทางพันธุกรรมบนโครโมโซมวายภายในละมั่งชนิดย่อยเดียวกัน และความแตกต่างระหว่างละมั่งสองชนิดย่อย

**วัสดุ อุปกรณ์ และวิธีการ** ทำการเพิ่มจำนวนยีนที่จำเพาะต่อโครโมโซมวายสามยีน ได้แก่ DBY, ZFY และ SRY และยีน CYTB ที่จำเพาะต่อไมโตคอนเดรียดีเอ็นเอ ด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอร์เรส ชิ้นส่วนดีเอ็นเอที่เพิ่มจำนวนถูกส่งไปหาลำดับเบส และทำการเปรียบเทียบเพื่อศึกษาความหลากหลายทางพันธุกรรม รวมทั้งความแตกต่างระหว่างละมั่งสองชนิดย่อย

**ผลการศึกษา** ยีน DBY, SRY, ZFY ประสบความสำเร็จในการเพิ่มจำนวนแสดงขนาดชิ้นดีเอ็นเอ 508, 2,697, 1,945 คู่เบสตามลำดับ ยีน CYTB ปรากฏขนาด 1,140 คู่เบส เมื่อเปรียบเทียบความหลากหลายทางพันธุกรรมบนโครโมโซมวายพบว่าไม่มีความแตกต่างภายในละมั่งชนิดย่อยเดียวกัน แต่พบความแตกต่างจำนวน 10 ตำแหน่งเมื่อเปรียบเทียบระหว่างละมั่งสองชนิดย่อย แผนภูมิต้นไม้แสดงความสัมพันธ์ทางพันธุกรรม (phylogenetic tree) ที่สร้างขึ้นจากลำดับเบสของโครโมโซมวายและไมโตคอนเดรียดีเอ็นเอ แสดงความแตกต่างระหว่างละมั่งสองชนิดย่อยอย่างชัดเจน

**ข้อสรุป** โครโมโซมวายไม่มีความหลากหลายทางพันธุกรรมเมื่อพิจารณาในละมั่งชนิดย่อยเดียวกัน แต่มีความแตกต่างอย่างชัดเจนระหว่างละมั่งสองชนิดย่อย ดังนั้นโครโมโซมวายจึงสามารถใช้เป็นเครื่องมือในการติดตามการเกิดลูกผสมระหว่างละมั่งสองชนิดย่อยนี้ได้

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**คำสำคัญ:** โครโมโซมวาย ไมโตคอนเดรียดีเอ็นเอ ละมั่ง

[1]ศูนย์เทคโนโลยีชีวภาพเกษตร มหาวิทยาลัยเกษตรศาสตร์ วิทยาเขตกำแพงแสน จ.นครปฐม 73140

[2]ศูนย์ความเป็นเลิศด้านเทคโนโลยีชีวภาพเกษตร สำนักพัฒนาบัณฑิตศึกษาและวิจัยด้านวิทยาศาสตร์และเทคโนโลยี (สบว.) สำนักงานคณะกรรมการ อุดมศึกษา

[3]โครงการสหวิทยาการ สาขาพันธุวิศวกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยเกษตรศาสตร์ บางเขน

[4]ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

[*]**ผู้เขียนที่ให้การติดต่อ** E-mail: fvetwww@yahoo.com

## Introduction

Eld's deer (*Cervus eldii*) or brown-antlered deer is an endanger wildlife in Southeast Asia. Eld's deer has been categorized as endangered species by the International Union for Conservation of Nature (IUCN). In the past, two subspecies of Eld's deer including Siamese Eld's deer (*Cervus eldii siamensis*) and Burmese Eld's deer (*Cervus eldii thamin*) had existed in Thailand [1] but their population has been lost from the wild. Up to date, two subspecies of Eld's deer have been successfully bred in captivity by the Department of National Parks Wildlife and Plant Conservation (DNP), Zoological Park Organization (ZPO) and other private zoos.

Study on genetic diversity help scientist to know their genetic status e.g. risk of inbreeding, construct pedigree, species or subspecies identification. One of the most common genetic markers which have widely been used for genetic diversity study is mitochondrial DNA (mtDNA). Mitochondrial DNA is inherited from mother to their children without DNA recombination and have a lot of copies. For this reason, mtDNA have been used to investigate the maternal lineage and also evolutionary of animal. Previously, study on genetic diversity of Eld's deer in captivity has been established (Dejchaisri: personal communication) and have to date relied on control region (d-loop) of mtDNA. The study revealed 15 and 1 maternal haplotypes of Burmese Eld's deer and Siamese Eld's deer, respectively. Moreover, another mtDNA marker is cytochrome *b* gene (CYTB) which also located on mtDNA, can be used as a marker to describe evolution histories that also inherited from maternal linage such as species boundaries, appointed subspecies and find the geographical origin [2].

Contradictory, Y chromosome is a male specific chromosome in mammal species and inherited from father to son. Therefore, it can be used to elucidate paternal linage history. The Y chromosome has non-recombining regions called male-specific region of Y chromosome (MSY) where no material is exchanged through the recombination process and contain approximately 27 distinct proteins in human [3]. For this reason, the Y chromosome is an interesting subject that has been used as a molecular tool in population genetic such as genetic diversity study [4], phylogeography [5-7], hybridization identification [8-9] and sex identification. The application has been successfully used in many species for instance human, bovine, non-human primate and so forth. Recent study in swap buffalo reported 4 haplotypes of them in Thailand based on comparing sequences of the Y chromosome fragments [10].

Recently, the Eld's deer reintroduction project has been established in Thailand. Burmese Eld's deer is a delegate served for this scheme. The genetic data was employed to design the releasing strategies. Even though the genetic diversity has been observed in Eld's deer, there is no available Y chromosomal information, especially for Y specific polymorphic markers. To comprehensively elucidate the evolution of Eld's deer in Thailand based on both paternal and maternal lineage, cytochrome *b* region were added in this study. We compare a sequence of 5,150 bp of the Y chromosomal nucleotide sequence (DBY gene: 508 bp; ZFY gene: 1,945 bp; SRY gene: 2,697 bp) and also 1,140 bp of cytocrome *b* region of mitochondrial DNA between Siamese Eld's deer (*Cervus eldii siamensis*) and Burmese Eld's deer (*Cervus eldii thamin*).

## Materials and Methods

Blood samples of male Siamese Eld's deer from 2 different source (SATO from *Banglamung Wildlife Breeding Center*, Thailand; DUSIT from Dusit zoo, Bangkok, Thailand) and male Burmese Eld's deer were subjects for DNA extraction. The representatives were selected from 15 mtDNA haplotypes previously reported (Dejchaisri: personal communication), including 9 male Burmese Eld's deer from different maternal haplotypes (CET02, CET03, CET04, CET07, CET10, CET12, CET13, CET14 and CET15) and 2 Siamese Eld's deer (SATO and DUSIT). The target fragment was generated by Y chromosome specific primers. We amplified one fragment of DBY (Dead box polypeptide; Y-linked) [11], three fragments of ZFY (zinc finger protein; Y-linked) and three fragments of SRY (sex determining region Y) gene [12] by using 8 pairs of primer. We newly developed primers for CYTB fragment amplification. The detail of the sequences, position, expected length of each primer pair was shown in **Table 1**. Each Y chromosome specific primers were tested against male and female Eld's deer. Total volume of 10 µl reaction contained 1 µl DNA template (~10 pg – 1 µg), 1 µl of 10x $NH_3SO_4$ buffer, 0.1µl (0.1-1 µM) for each primers, 0.8 µl of 50mM $MgCl_2$, 0.2 µl of 10mM dNTP, 1.25 U Taq polymerase™Fermentas and add RNase-free water up to 10 µl. The condition composed of initial activation step at 95 °C for 5 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing step at 55 °C 30 s for each primers (see **Table 1**), extension step at 72 °C 30 s and final extension step at 72 °C 5 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. PCR products

**Table 1.** The Sequences, Annealing Temperature (Ta) and Expected Size of Primers Used in This Study

| Name | Forward primer | Reverse primer | Condition Annealing (Ta) | Size (bp) |
|---|---|---|---|---|
| DBY7 | GGTCCAGGAGARGCTTTGAA | CAGCCATTCTCTTGTTGGG | 55 ℃ | 508 |
| DBY8 | CCCCAACAAGAGAATGGCT | CAGCACCACCATAKACTACA | 55 ℃ | 508 |
| ZFY1 | CAGGTGAGGGCACATGAG | ATCACATTCGATGGCCTT | 55 ℃ | 1,033 |
| ZFY2 | ATATGCTTGAAGAGACGACAAC | AGTCAGAAGACAAATGTCACA | 55 ℃ | 671 |
| ZFY3 | TTCTAATTTGAAGACGCATGTG | CAACTTCTTTATGGTGTCGTG | 55 ℃ | 622 |
| SRY3 | AGCCTTTGAAGTTTCTACTGTC | CCCCAATACCTCCCCTCAATAC | 55 ℃ | 1,118 |
| SRY4 | GTCTGCTGCACCTTCATC | CTTATTGTGGCCCAGGCTTGTC | 55 ℃ | 970 |
| SRY5 | CCGGGCTATAAATATCGACCTC | GATGAAACCTTGGGTCTCACAG | 55 ℃ | 1,081 |
| CB0 | CATGACTAATGATATGAAAAACC | - | 55 ℃ | 1,140 |
| DloopRe | - | CTTTTCTGGVTTACAAGACCA | 55 ℃ | 1,140 |

were sent for sequencing by First Base Laboratory, Shah Alam Malaysia and analysis by using BioEdit software (Hall, 1999).

## Results

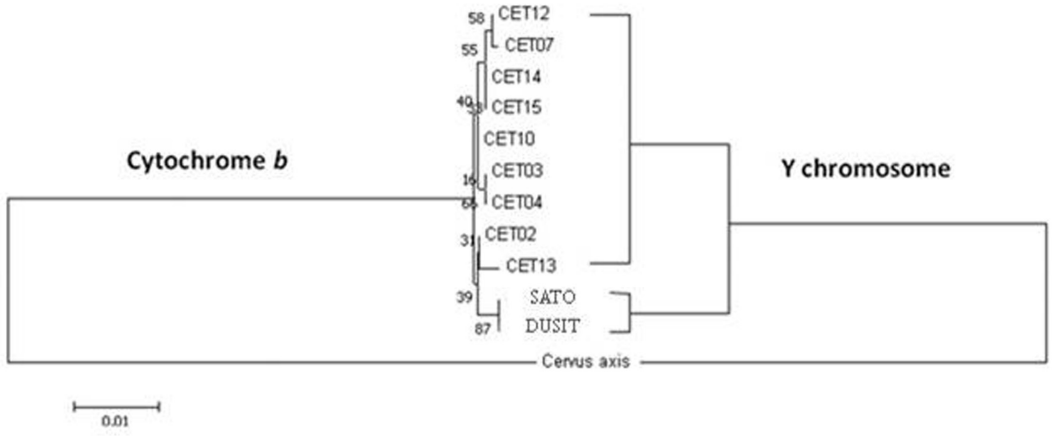
We analyzed the sequences of DBY (508 bp), SRY (2,697 bp) and ZFY (1,945 bp) gene of 9 of Burmese Eld's deer from different mtDNA haplotyps and 2 Siamese Eld's deer. No segregation site was found in these regions of both Siamese and Burmese Eld's deer. However, the sequence comparison between these two subspecies indicated one segregation site (C/T) on DBY fragment, two segregation sites (T/G and A/C) and two deletions on SRY fragment, three segregation sites (T/C, G/A and A/G) and one deletion on ZFY fragment. The segregation sites were noted in **Table 2** with the relative positions that refer to the alignment of sequence reported in [13]. The divergence comparison between phylogenetic tree from CYTB and Y chromosome was showed in **Figure 1**. The phylogenetic tree showed clearly separation between two subspecies of Eld's deer both based on both CYTB and Y chromosome. Furthermore, no evidence of hybridization between two subspecies was revealed in the captivity.

**Table 2.** Comparison of Single Nucleotide Positions Between Siamese Eld's Deer and Burmese Eld's Deer[a]

| | DBY | | SRY | | | ZFY | | | |
|---|---|---|---|---|---|---|---|---|---|
| Name/ | (508 bp) | | (2,697 bp) | | | (1,945 bp) | | | |
| Positions | 447 | 753 | 1,973 | 1,973 | 2,088 | 106 | 284 | 331 | 1,513 |
| CET02 | C | G | A | A | C | - | T | G | A |
| CET03 | . | . | . | . | . | - | . | . | . |
| CET04 | . | . | . | . | . | - | . | . | . |
| CET07 | . | . | . | . | . | - | . | . | . |
| CET10 | . | . | . | . | . | - | . | . | . |
| CET12 | . | . | . | . | . | - | . | . | . |
| CET13 | . | . | . | . | . | - | . | . | . |
| CET14 | . | . | . | . | . | - | . | . | . |
| CET15 | . | . | . | . | . | - | . | . | . |
| SATO | T | T | - | - | A | T | C | A | G |
| DUSIT | T | T | - | - | A | T | C | A | G |

[a]Dash(-) represents to Indel. For DBY sequences, data are accessible via Genbank entries GU902196, GU902197; SRY (KF038140, KF038141) and ZFY (KF038142, KF038143)

**Figure 1.** Neighbor-joining Tree (NJ) Using Cytochrome *b* Sequences (1,140 bp) and 5,150 bp of Y Chromosome Sequences (5,150 bp) with 10,000 Replicated Bootstrap[a]




[a]Both phylogenetic trees from mitochondrial DNA and Y chromosome show clearly divergence between two subspecies of Eld's deer (*Cervus eldii thamin* vs *Cervus eldii siamensis*). Sequences of *Cervus axis* were added in this phylogenetic tree as an outgruop.

## Discussion

Phylogenetic trees constructed by using 5,150 bp of the Y chromosome and 1,140 bp of cytochrome *b* region provided a clear divergence between Siamese Eld's deer and Burmese Eld's deer. The Y chromosome has low diversity within intraspecies level [4, 12, 14-15]. Theoretically, non-recombination region of Y chromosome is expected to be lesser variable than recombination region and negative or positive selection can severely affect on this region. Differences in migration pattern between male and female animals can induce variation of genetic that inherited in maternal and paternal lineage. In patrilocal species, like Eld's deer, females migrate more than males. This implies that there is less variation in the Y chromosome [11]. Microsatellite marker for the Y chromosome, which has been studied in bovidae species [16-17] is alternative for diversity assessment on this chromosome.

Even though the Y chromosome has low diversity in intraspecies level, this study shows a relatively high divergence of the interspecies level which supported divergence between these two subspecies based on cytochrome *b* region. High variation in interspecies level has also been reported in swamp and river buffalo [10]. This divergence is caused by hitch-hiking during positive selection of Y chromosome gene or by sexual selection and supports evolution history of these two subspecies based on mitochondrial DNA [18] and intron of Protein Kinase C Iota (PRKCI) gene [19].

Although interbreeding across these two subspecies can increase viability of Eld's deer population for long-term conservation, hybridization between genetically distinct lineages can lead the loss of adaptive diversity and outbreeding depression. However, this study reveals the example of successful captive breeding program without incident of hybridization in captive males. This study shows a clear divergence between two subspecies base on the Y chromosome sequence. Therefore, we suggest that Siamese Eld's deer and Burmese Eld's deer should be maintained as a distinct unit for conservation until genetic data or degree of inbreeding in wild population has observed for supporting interbreeding strategies.

In conclusion, although Y chromosome markers are not appropriated for paternal lineage haplotype assignment within the population, they can be used as a phylogenetic marker to detect hybridization between Burmese and Siamese Eld's deer population.

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