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NAME: Miss Kanita Ouithavon

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR
(Assistant Professor Savitr Trakulnaleamsai, Ph.D.)

COMMITTEE MEMBER
(Associate Professor Naris Bhumpakphan, Ph.D.)

COMMITTEE MEMBER
(Assistant Professor Jessada Denduangboripant, Ph.D.)

COMMITTEE MEMBER
(Mr. Boripat Siriaroonrat, D.V.M., Ph.D.)

GRADUATE COMMITTEE CHAIRMAN
(Mr. Surapol Patharakorn, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON

DEAN
(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

MOLECULAR PHYLOGENETIC RELATIONSHIPS AMONG THAI DEER (SUBFAMILY CERVINAE)

KANITA OUITHAVON

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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The five extant Thai deer species and spotted deer (*Axis axis*) were analyzed by using molecular phylogenetic approach from genes that consist of two rapidly evolving mitochondrial markers; cytochrome *b* and control region and one slower nuclear marker; intron1 of the protein kinase C, iota gene (PRKCI). The aims of this study were to assess the molecular phylogenetic relationships among Thai Cervinae species, to clarify the taxonomic position of hog deer and to understand evolutionary history of Thai deer.

The results reveal that hog deer (traditionally named *Cervus porcinus*) and spotted deer (*Axis axis*) were grouped together as monophyly. Therefore, this study suggests the taxonomic position of hog deer to be placed into the genus *Axis* and confirms a scientific nomenclature of hog deer as "*Axis porcinus*". This study also confirms that Schomburgk's deer (traditionally named *Cervus schomburgki*) and swamp deer (*Cervus duvaucelii*) were grouped together as sister lineage close to the genus *Axis* clade, with clearly separated from the genus *Cervus* clade. Therefore, this study suggests the splitting Schomburgk's deer into different genus depart from the genus *Cervus* by changing the taxonomic position to be placed into the genus *Rucervus* and confirms a scientific nomenclature of Schomburgk's deer as "*Rucervus schomburgki*". Thus, these results were evidence for three distinct genera of Cervinae in Thailand; *Cervus*, *Rucervus* and *Axis*. This new classification should be considered by reclassifying these species into the new zoological names under the Thai Wildlife Protection Act.

Student's signature Thesis Advisor's signature

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Kanita Ouithavon
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LIST OF ABBREVIATIONS

A = adenine
A. = *Axis*
αLAlb = the alpha-lactalbumin
asl = above sea level
ATP = adenosine triphosphate
BLASTn = Basic Local Alignment Search Tool-nucleotide
bp = base pairs
C = cytosine
C. = *Cervus*
C. e. = *Cervus eldii*
C. p. = *Cervus porcinus*
°C = degree Celsius
CITES = the Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO2 = subunit II of the cytochrome oxidase
CR = control region
CSB = conserved sequence block
Cyt.*b* = cytochrome *b*
cyt. b = cytochrome *b* gene
DD = the Data Deficient
D-loop = a displacement loop
DNA = deoxyribonucleic acid
dNTP = deoxynucleotide triphosphate
EDTA = ethylenediamine tetraacetic acid
EtBr = ethidium bromide
G = guanine
H-strand = heavy strand
HSP = the promoters for H-strand transcription
IUCN = the World Conservation Union

LIST OF ABBREVIATIONS (Continued)

Kb = kilobase pairs

KCl = potassium chloride

Kg = kilogram

L = the likelihood

L-strand = light strand

LSP = the promoters for L-strand transcription

M = molar

M. = *Muntiacus*

M. m. = *Muntiacus muntjak*

ME = the minimum evolution

$MgCl_2$ = magnesium chloride

min = minute

ML = maximum likelihood

ml = milliliter

mM = millimolar

mm = millimeter

MP = maximum parsimony

mRNA = messenger ribonucleic acid

mtDNA = mitochondrial DNA

myr = million years

µg = microgram

µl = microliter

µm = micrometer

ND = NADH dehydrogenase

NJ = neighbor joining

nm = nanometer

No. = number

O_H = origin of H-strand replication

PCR = Polymerase Chain Reaction

LIST OF ABBREVIATIONS (Continued)

PKC = the protein kinase C

pmol = picomole

PRKCI = the protein kinase C, iota gene

RNA = ribonucleic acid

S = Svedberg unit

s = second

spp. = species (in the plural)

T = thymine

TAE = Tris-acetate-EDTA electrophoresis buffer solution

TAS = termination associated sequence

TBR = tree-bisection-reconnection

TE = buffer solution contains Tris and EDTA

Tm = melting temperature

tRNA = transfer ribonucleic acid

UPGMA = unweighted Pair group method with arithmetic means

UV = ultra violet

VNTRs = variable numbers of tandem repeats

MOLECULAR PHYLOGENETIC RELATIONSHIPS AMONG THAI DEER (SUBFAMILY CERVINAE)

INTRODUCTION

Cervinae is old world deer that is the one most specious subfamily among the family Cervidae, with extensive of morphological and ecological divergence. The diversity of their terrestrial habitats reflects to several adaptive radiations (Grubb, 1993; Geist, 1998). This group is characterized by antlers that normally have at least three tines, which includes four genera of (1) *Dama,* with two species of fallow deer in Eurasia, (2) *Axis,* with four species in Asia, (3) *Elaphurus*, with only one species of Père David's deer in China and (4) *Cervus,* the widely distributed genus with nine species in Asia and Red deer which is prevalent throughout the northern hemisphere (Groves and Grubb, 1987).

In the classification of Lekagul and McNeely (1977), the only one genus of Cervinae are recognized for Thai deer, which compose of four *Cervus* species that are the sambar (*Cervus unicolor*), Schomburgk's deer (*C. schomburgki*) the extinct species, Eld's deer (*C. eldii*) and Hog deer (*C. porcinus*), as a matter of fact that the two latter species are extinct from Thailand's natural habitats for a long time (Humphrey and Bain, 1990), but only still living in captivity. This classification is different from many modern authors' criteria that still placed Hog deer in genus *Axis*, based on the lack of upper canines variably present in others *Cervus* and in the presence of glands in the feet (Ellerman and Morrison-Scott, 1951). This contrast is one example of many inconsistencies remains concerning the intergeneric relationships among Cervinae morphological classifications.

The discrepancies between morphological phylogenies of Cervinae have clarified by the fact that previous studies which have used different methods of characters (Groves and Grubb, 1987; Meijaard and Groves, 2004). It is difficult to appraise the reliability of these inferences because of their high level of homoplasy affect to the usefulness of morphological characters; consequently, the result from deciphering in the phylogeny of Cervinae has been repeatedly questioned (Gentry, 1994; Hassanin and Douzery, 2003; Janis and Scott, 1987).

Molecular phylogenetic investigations have greatly helped in taxa organization also in more understanding the evolution of morphological characters. Several molecular studies on Cervidae and Cervinae have been contributed considerably to resolve evolutionary relationships among deer species based on amino acid, mitochondrial DNA and nuclear DNA sequences (Miyamoto *et al*., 1990; Cronin *et al*., 1996; Douzery and Randi, 1997; Randi *et al*., 2001; Pitra *et al*., 2004 and Gilbert *et al*., 2006), but these previous studies did not have sufficient gene and taxon of Thai deer sampling and did not clearly define to Thai deer which still have unclear genera within Cervinae.

In this study, Thai deer species and spotted deer (*Axis axis*) were being analyzed by molecular phylogenetic approach from genes that consist of two rapidly evolving mitochondrial markers; cytochrome *b* and control region and one nuclear marker; that is intron1 of the protein kinase C, iota gene (PRKCI) for clarifying the taxonomic position of hog deer and to understand the evolutionary history of Thai deer.

OBJECTIVES

1. To assess the molecular phylogenetic relationships among Thai Cervinae species.
2. To clarify the phylogenetic position of Hog deer (*Cervus* or *Axis porcinus*).
3. To understand the evolutionary history of Thai deer based on molecular phylogenetic approach.

LITERATURE REVIEW

1. General Introduction of Deer

A. Description of Cervidae and Cervinae

Cervinae is the one member of family Cervidae, which is the family of the deer, elk, caribou and moose, occur nearly throughout North America, South America, Northern Africa and Eurasia. They have been introduced widely elsewhere such as Cuba, New Guenea, Australia and New Zealand (Vaughan, 1986; Nowak, 1999). The recent living members among Cervidae include 23 genera and 47 species (Grubb, 2000).

Cervidae or in the general common name calls “deer” are a significant family among order Artiodactyla; the even-toed ungulates and also is dominant herbivores, suborder Ruminantia; the browsers or grazers that have highly specialized stomach composed of four chambers for digesting the cellulose-containing plant food which could be effectively digested by the involvement of symbiotic bacteria and infraorder Pecora; the advanced artiodactyls which the upper incisors are absent, the upper canines are usually absent, the lower canines are incisiform and the cheek teeth are selenodont and brachyodont, reflecting a browsing habit. Exception to Père David’s deer (*Elaphurus*) has relatively hypsodont cheek teeth (Lekagul and McNeely, 1977; Vaughan, 1986; Scott and Janis, 1987). Leinders and Heintz (1980) affirm that Cervidae characters of double lacrimal orifice and closed metatarsal gully are derived conditions with in the Pecora. Their plant food may include grass, bark, twigs and young shoots. The extremely varied habitats of Cervidae include forest, swamps, brushy country, deserts and arctic tundra (Nowak, 1999).

Antlers are the best recognized characteristic of Cervidae. Antlers are appendages of the skull, composed of a solid bony core and supported on permanent skin-covered pedicles. In most species, antlers are shed annually and grow again in the summer. The new antlers are covered with soft skin having soft brown hairs, antlers in this stage are called “velvet” or soft antlers which are tender and bleed easily if they get scratched. Antlers are presented in males, except in *Rangifer*, in which both sexes have antlers and the single genus *Hydropotes*, which are lacking antlers (Lekagul and McNeely, 1977; Nowak, 1999).

The external form of Cervidae is slim, long legs, short tail, and lack extreme elongation of the metapodials. Their head and body length is 720-2,700 mm, shoulder height is 320-1,900 mm and weight is 7-825 kg. Females are slightly smaller and more delicately shape than males, with more slender neck that differ from hairy neck of males during the rutting season. There are usually large lachrymal glands below the inner margin of their eyes and there are often glands between their hoofs which produce strong odor secretion for using in their communication. For the body size, *Alces* is the largest genus, while *Pudu* is the smallest genus among this family (Lekagul and McNeely, 1977; Scott and Janis, 1987; Nowak, 1999).

To recognize subfamily Cervinae, which includes four genera of *Dama*, *Axis*, *Cervus* and *Elaphurus*, this group is characterized by antlers that normally have at least three-tines which expose much feature variation (Groves and Grubb, 1987). Groves and Grubb (1987) and Grubb (1993) indicate the specific characteristics of each genus in Cervinae (Figure 1) as below:

1. *Dama*: Fallow deer; primitive and peculiar features with flattened and numerous points of palmate antlers, inducing the pedal glands on feet, spotted pelage and no mane. Only single species of *Dama dama,* that occurred in the Mediterranean region.
2. *Axis*: Spotted deer; antlers are carried on pedicles and are three-tined, upper canine teeth are absent, foot glands are presence and retain a full spotted adult pelage.
3. *Cervus*: *Cervus* deer; antlers shape like *Axis*, but are at least three-tined, upper canine teeth are presence, no foot glands, unspotted adult pattern and prominent rump patch.

4. *Elaphurus*: Père David's deer; no foot glands, unspotted adult pattern like *Cervus*, but having and abundance of long and somewhat wavy guard hairs most of the year The legs are heavy-boned and the feet are broadly splayed, with naked skin between the hooves. The tail is the longest of Cervinae. Only single species of *Elaphurus davidianus*, that occurred in the lowlands of northeastern and east-central China.

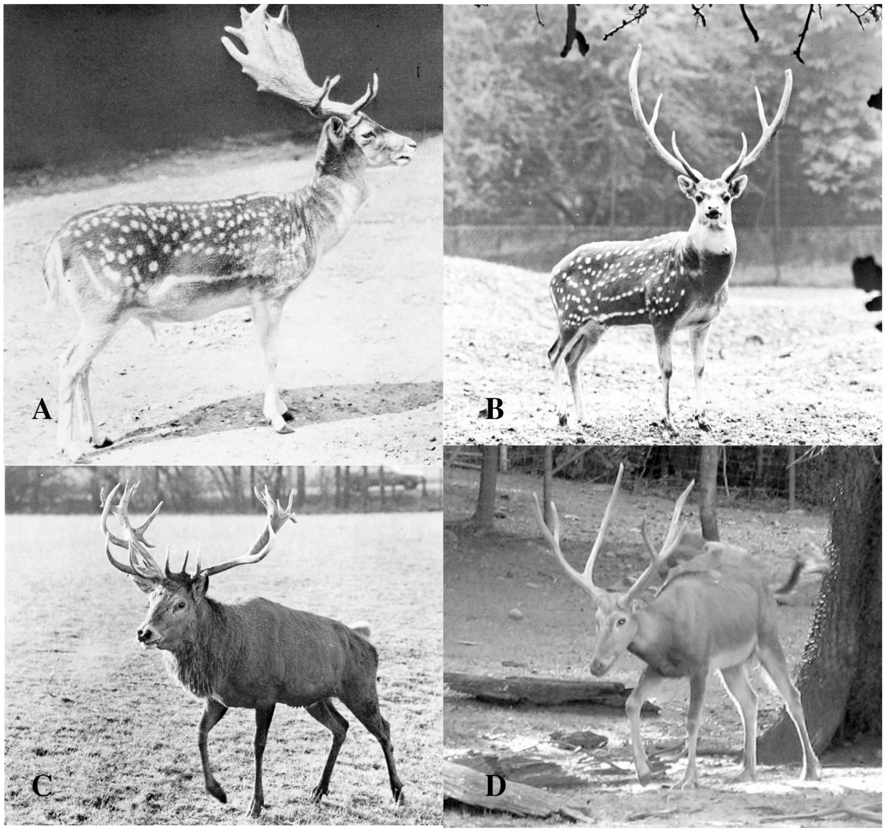


Figure 1 Cervinae in each genus

(A) *Dama*: Fallow deer (*Dama dama*)

(B) *Axis*: Chital or spotted deer (*Axis axis*)

(C) *Cervus*: Red deer (*Cervus elaphus*)

(D) *Elaphurus*: Père David's deer (*Elaphunus davidianus*)

Source: Nowak (1999)

B. Taxonomy History of Cervidae and Cervinae

Lekagul and McNeely (1977) proposed the classification of Cervidae into four subfamilies:

1. Moschinae: the Asian musk deer, which has only one genus of *Moschus*.
2. Muntiacinae: two genera of Asian barking deer; *Elaphodus* (Tufted deer) and *Muntiacus* (Muntjac).
3. Cervinae: three genera of Asian, European and North American Typical deer, which compose of *Cervus* (common Eurasian deer), *Elaphurus* (Père David's deer) and *Platyceros* (Fallow deer).
4. Odocoileinae: five genera of South American deer, two genera of Eurasian deer and three genera of North American, European, and Asian deer.

In the classification of Eisenberg (1981), Janis and Scott (1987) and Groves and Grubb (1987), there are six subfamily of Cervidae:

1. Hydropotinae: the antlerless deer, which has only one species of Chinese water deer (*Hydropotes inermis*).
2. Alcinae: the largest Cervidae, which has only one species of Moose (North American term) or Elk (European term) (*Alces alces*).
3. Rangiferinae: the single species of Caribou (North American term) or reindeer (European term) (*Rangifer tarandus*).
4. Muntiacinae: two genera of Asian barking deer, *Elaphodus* (Tufted deer) and *Muntiacus* (Muntjac).
5. Cervinae: the old world deer, which compose of four genera of *Dama*, *Axis*, *Cervus* and *Elaphurus.*
6. Odocoileinae: the new world odocoileines and the old world roe deer.

The formal classifications of Cervidae are mainly based on morphological traits, in which presence or absence of antlers is considered to be the first characteristic using in division. From this criterion, Hydropotinae (antlerless deer) is categorized to be the sister group to all other Cervidae (Scott and Janis, 1987; Groves and Grubb, 1987). The second characteristic using in subdivision is based on differences in the lateral metacarpals that the antlered cervids are separated into two distinctive groups such as:

1. The Telemetacarpalians: the taxon which includes Alcinae, Rangiferinae and Odocoileinae, as all possess the distal parts of the second and fifth lateral matacapals of hoof.
2. The Plesiometacarpolians: the taxon, which unites Cervinae and Muntiacinae, as both possess the proximal part of the second and fifth lateral matacarpals of hoof (Groves and Grubb, 1987).

Based on an analysis of morphological and karyotypic data, Groves and Grubb (1987) and Grubb (1993) proposed the subdivision of the plesiometacarpalia deer, which includes Cervinae and Muntiacinae as below:

a) Muntiacinae: consist of two genera with eleven species of Asian barking deer.

(1) *Elaphodus*: only one species of Tufted deer (*Elaphodus cephalophus*)
(2) *Muntiacus*: consist of ten species of muntjac
  - Indian muntjac or common barking deer (*Muntiacus muntjak*)
  - Reeves's muntjac (*M. reevesi*)
  - Hairy-fronted muntjac (*M. crinifrons*)
  - Fea's muntjac or Fea's barking deer (*M. feae*)
  - Roosevelt's muntjac (*M. rooseveltorum*)
  - Gongshan muntjac (*M. gongshanensis*)
  - Bornean yellow muntjac (*M. atherodes*)
  - Giant muntjac (*M. vuquangensis*)
  - Truong Son muntjac (*M. truongsonensis*)
  - Leaf muntjac (*M. putaoensis*)

b) Cervinae: consist of four genera with sixteen species

(1) *Dama*: only one species of Fallow deer (*Dama dama*)

(2) *Axis*: consist of four species
- Spotted deer or chital (*Axis axis*)
- Hog deer (*A. porcinus*)
- Kuhl's deer (*A. kuhlii*)
- Calamian deer (*A. calamianensis*)

(3) *Elaphurus*: one species of Père David's deer (*Elaphurus davidianus*)

(4) *Cervus*: ten species, which have been separated into four subgenera

(a) *Rusa*: consist of four species
- Rusa deer (*Cervus timorensis*)
- Sambar deer (*C. unicolor*)
- Philippine spotted deer (*C. alfredi*)
- Philippine brown deer (*C. mariannus*)

(b) *Rucervus*: consist of three species
- Swamp deer or barasingha (*C. duvaucelii*)
- Eld's deer or thamin (*C. eldii*)
- Schomburgk's deer (*C. schomburgki*)

(c) *Cervus*: consist of two species
- Red deer or wapiti or Elk (*C. elaphus*)
- Sika deer (*C. nippon*)

(d) *Przewalskium*: one species of Thorold's deer (*C. albirostris*)

C. Evolutionary History of Deer

The original occurrence of Cervidae was early Oligocene in Asia, late Oligocene in Europe and early Miocene in Eurasia, and have been a predominately Eurasian and northern group. They have never been found in Africa or south of the Sahara, and did not appear in North or South America until the Late Pliocene (Scott and Janis, 1987). Cervids probably arose from Tragulidae, an ancestor resembling during the Oligcene. The primitive deer were small and antlerless, though they had enlarged upper incisors, like the modern genus *Moschus* (the Musk deer). The development of antlers began about twenty-five million years ago. The early antlers were still small and short on elongated bony pedicles, as a pair of unbranched "spikes" in the modern genus *Muntiacus* (the barking deer). However, the earliest known antlered deer (*Dicrocerus* and allies) had forked antlers, so the spikes-form of *Muntiacus* probably represents a secondary condition. The later forms tended to have larger and more complex on shorter study pedicles, as culminating development in genus *Megaloceros* (the extinct Irish Elk), which had antlers more than three meters from tip to tip (Figure 2). *Megaloceros* have been extinct during the Pleistocene and early Recent, through the North African species *Megaloceros algericus* may have survived until 4,000-5,000 year ago (Lekagul and McNeely, 1977; Klein, 1984; Groves and Grubb, 1987; Schloeth, 1990).

Figure 2 The Irish Elk (*Megaloceros giganteus*) which have been extinct about 12,000 years ago. The shown figure was found in the peat bogs in Ireland.

Source: Schloeth (1990)

The early history of cervid evolution is known that in the Oligocene of Asia *Eumeryx* existed. It was very similar in morphology to the present-day musk deer of the family Moschidae. This fossil record can be speculated an early cervid might have been like certainly small in stature and did not bear antlers. It was browser, adapted to a mixed feeding strategy in tropical forests (Eisenberg, 1887). The first records of ruminants that have bonny outgrowths on the frontal bone, with some species have permanent horns but others clearly had deciduous antlers, are called the Dromomerycines that Frick (1937) placed them within the Cervidae.

From the fossil record as summarized by Gentry (1994), the earliest antlered deer are *Dicrocerus* and *Heteroprox*, which appear in the Late Orleanian in Mammalian Neogene biostratigraphic divisions (about seventeen million years ago). *Dicrocerus* was the first true deer with deciduous antlered born on an elongated projection from the frontal bone (Eisenberg, 1887). In the addition notes supposed by Azanza (1993), *Euprox*, which appears in Early Astaracian in Mammalian Neogene biostratigraphic division (about sixteen million years ago), was classified to be the Muntiacinae, which has been considered as the primitive stem group of all other Cervidae. The primitive Muntiacinae survived in Europe until the late Mammalian Neogene, and then they were replaced by *Eustylocerus pierensis*, which as the earliest known member of the Cervidae. This replacement corresponds to the change from a subtropical to a more temperate climatic region (Azanza and Menender, 1990).

The oldest fossil remains assignable to the node of Muntiacinae and Cervinae splitting appear in the Miocene deposit of Lufeng in China or seven million years ago (Han, 1985). From the report of Di Stefano and Petronio (2002), The most primitive member of Cervinae is *Cervocerus novorossiae*, which appears in the Late Miocene, the earliest fossil of *Axis* relation is *Axis shansius*, which appears in the Early Pliocene or five million years ago, while the oldest fossil of the *elaphus* like group is *Cervus magnus*, which appears in the Middle Pliocene. In addition, *Rusa elegans* form the Middle Pliocene deposit of Shaanxi has been noted that it is very close to the living *Cervus unicolor.*

D. Cervidae and Cervinae in Thailand

From the classification of Lekagul and McNeely (1977), there are six species of Cervidae in Thailand, divided into two subfamilies. The first subfamily is Muntiacinae, consisting of two species of common barking deer (*Muntiacus muntjak*) and Fea’s barking deer (*Muntiacus feae*). The second one is Cervinae, consisting of four species includes sambar deer (*Cervus unicolor*), the extinct Schomburgk’s deer (*C. schomburgki*), Eld’s deer (*C. eldii*) and hog deer (*C. porcinus*). For hog deer, it was classified into genus *Axis* and was scientifically named as *Axis porcinus* by many modern authors (Nowak, 1999). For Eld’s deer by nowadays, its zoological name is rewritten from “*C. eldi*” to *“C. eldii*’, in which this name is its correct original spelling (Timmins and Duckworth, 2008). The antlers shape of each Thai Cervidae as Figure 3 and the taxonomy of cervidae and Cervinae in Thailand organized by Lekagul and McNeely (1977) as below:

A key to the Thai Cervidae

1. - Short antler on long pedicle .... *Muntiacus* (2)
   - Long antler on short pedicle .... *Cervus* (3)
2. - Dorsal surface of tail is the same
     color as back .... *Muntiacus muntjak*
   - Dorsal surface of tail is black .... *M. feae*
3. - Brow-tine makes acute angle with beam .... (4)
   - Brow-tine makes obtuse angle with beam .... (5)
4. - Skull longer than 300 mm .... *Cervus unicolor*
   - Skull shorter than 300 mm .... *C. porcinus*
5. - Beam shorter than 1/3 length of
     the antlers, which branch into many tines .... *C. schomburgki*
   - Beam long and curved outward to form
     a large hook with many short snags on
     the upper edge of the tip .... *C. eldii*

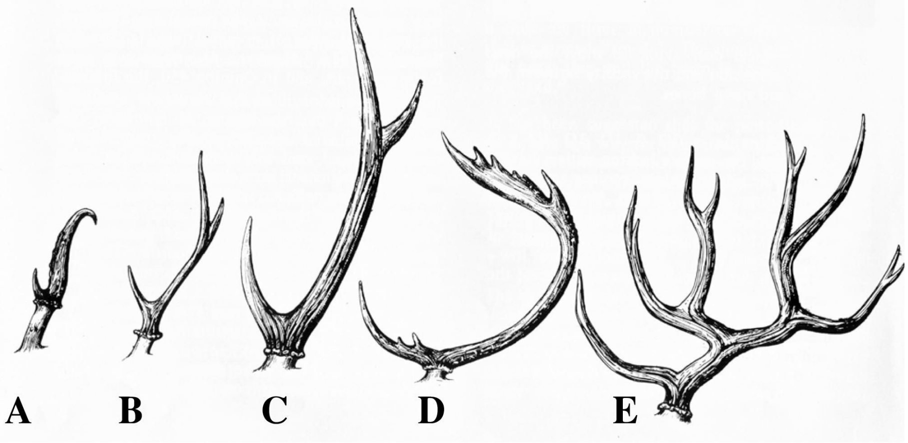


Figure 3 The antlers shape of Thai Cervidae

(A) *Muntiacus muntjak* and *M. feae*

(B) *Cervus porcinus*

(C) *C. unicolor*

(D) *C. eldii*

(E) *C. schomburgki*

Source: Lekagul and McNeely (1977)

The descriptions and some notes of each Thai deer species (Figure 4 and Figure 5) In the descriptions, the zoological names are adopted by the classification of Lekagul and McNeely (1977) as below:

1. Common barking deer

Zoological name: *Muntiacus muntjak* Zimmermann, 1780
Common names: Barking deer, Red Muntjac, Indian Muntjac
Synonyms: *Cervus muntjak* Zimmermann, 1780
Cervus moschatus Blainville, 1816
Cervus pleiharicus Kohlbrugge, 1896
Muntiacus bancanus Lyon, 1906
Muntiacus rubidus Lyon, 1911

There are 15 subspecies throughout India and Indo-Chinese range, but in Thailand, there are two subspecies includes *M. m. vaginalis*, which found throughout the country except the southern part and *M. m. peninsulae*, which found in the Southern part. *Vaginalis* subspecies is smaller size and duller color, while *peninsulae* subspecies is larger, brighter and bigger antlers. It has a wide altitudinal range and usually found from sea level to medium elevations in hilly country. Most of its range is dominated by evergreen vegetation, but it readily uses deciduous forests and mosaics of grassland, scrub, and forest. This species is browser more than grasser, in which mostly fallen fruits, buds, tender leaves, flowers, herbs and young grass are major food item. Both diurnal and nocturnal activity, but in Thailand, it is generally nocturnal, feeding at night or early in the morning. Common barking deer is a mostly solitary species that is capable of breeding through the year, and has been stated to be territorial (Lekagul and McNeely, 1977; Nowak, 1999).

2. Fea's barking deer

Zoological name: *Muntiacus feae* Thomas & Doria, 1889
Common names: Fea's Muntjac, Black Muntjac, Hairy-fronted Muntjac
Synonyms: *Cervulus feae* Thomas & Doria, 1889
Muntiacus feai Tortonese in Grubb, 1977

Its shape is similar to common barking deer, but slightly smaller, with recognized features including the dark brown upperparts, the legs darken to black above hoofs, the hind legs have a whitish line down the anterior portion of their thigh and the lachrymal glands are large and prominent. This species has usually been identified on the basis of dark pelage and especially the blackish tail. Little known about ecology and behavior, because very rare. The information available suggests that this species is tied to evergreen forests of the hills and mountains of western Thailand and adjacent Myanmar, and may be more common in montane evergreen forest above 1,000 m asl. It is categorized to be the Data Deficient (DD) in IUCN Red list and is the one species of Thai protected wildlife on Thai Wildlife Protection Act of 1992. On the notes in Thailand, some of them were found in tenasserim range until to the Phuket ridge in the Southern (Lekagul and McNeely, 1977; IUCN, 2004).

Figure 4 Muntiacinae (*Muntiacus* spp.) in Thailand

(A) Common barking deer (*Muntiacus muntjak*)

(B) Fea’s barking deer (*Muntiacus feae*)

3. Sambar deer

Zoological name: *Cervus unicolor* Kerr, 1792
Common names: Sambar, Sambar deer
Synonym: *Rusa unicolor* Kerr, 1792
Cervus equinus Cuvier, 1823
Cervulus cambojensis Gray, 1861
Rusa dejeani Pousargues, 1896

There are 16 subspecies throughout India and Indo-Chinese range, but the race in Thailand is *C. u. equinus*. Sambar is quite generalized deer in Thailand, found in a variety of habitats, but seems to prefer wooded areas. It occurs regularly on forested mountain slopes up to elevation of 3,825 m asl (on Siouguluan Mountain, Taiwan). The dominant features include the rump and underside of tail are white and as signal when the tail is raised, males may have a long and dense mane on the neck and forequarters, antlers have only three tines and are stouter and more rugose than those of most other *Cervus*. Most activity is crepuscular and nocturnal. Sambar deer is more of browser than grazer, generally feeding on leaves, buds, grass, berries and fallen fruit. It needs numerous amount of calcium when the antlers are growing, and visit salt licks especially oftentimes (Whitehead, 1972; Lekagul and McNeely, 1977; Nowak, 1999; Timmins and Duckworth, 2008).

4. Schomburgk's deer

Zoological name: *Cervus schomburgki* Blyth, 1863
Common name: Schomburgk's deer
Synonym: *Rucervus schomburgki* Blyth, 1863

Schomburgk's deer was endemic to Thailand. This extinct species was once still abundant in the Chao Phya River central plains, which is its swampy habitat, where there were long grasses, cane and shrubs. It avoided dense forest areas, which could trap it from moving freely. This species remarkable antlers is similar to those of Barasingha or Swamp deer (*Cervus duvaucelii*), but is more complex and has a very short beam. The large scale of commercial rice production began in the nineteenth century in Thailand caused of habitat lose and led this species to extinct. The last known wild animals were killed in 1932 and the last of living captive individual was killed in 1938 (Lekagul and McNeely, 1977; Nowak, 1999).

5. Eld's deer

5.1 Siamese Eld's deer

Zoological name: *Cervus eldii siamensis* Lydekker, 1915
Common names: Indo-Chinese Eld's deer, Brow-antlered deer
Synonyms: *Cervus eldii* M'Clelland, 1842
Cervus eldii siamensis Lydekker, 1915
Rucervus eldii siamensis Lydekker, 1915
Panolia platyceros Gray, 1843
Rucervus platyceros hainanus Thomas, 1918

5.2 Burmese Eld's deer

Zoological name: *Cervus eldii thamin* Thomas, 1918
Common names: Burmese Eld's deer, Thamin
Synonyms: *Cervus eldii* M'Clelland, 1842
Panolia acuticauda Gray, 1843
Cervus eldii thamin Thomas, 1918
Rucervus eldii thamin Thomas, 1918

There are two subspecies in Thailand including *C. e. siamensis*, which was once common throughout the northern country, but now no records were found in the natural habitats and *C. e. thamin*, which has been reported few from the Tenasserim. Its antlers is unique in that the brow-tine forms a continuous curve with beam, in which the *siamensis* differs from the typical *thamin* in having the beam palmated toward the crown, with several small snags on the posterior edge also lighter pelage. In Myanmar, most Burmese Eld's deer live in dry dipterocarp forest, which is usually dominated by *Dipterocarpus tuberculatus*, while the typical habitat of Eld's deer in Hainan Island (this animal is the same subspecies of Siamese Eld's deer in Thailand) is scrubland and dry grassland, with sparse trees in hills below 200 m asl. Eld's deer feeds on grass and some browse and also take fallen fruits and flowers, and reportedly can live without water for several days. The entire species of *C. eldii* are listed as appendix 1 of the CITES and are categorized as vulnerable in IUCN red list (Lekagul and McNeely, 1977; Nowak, 1999; IUCN 2004; Timmins and Duckworth, 2008).

6. Hog deer (*Cervus porcinus*)

Zoological name: *Cervus porcinus* Zimmermann, 1780
Common name: Hog deer
Synonyms: *Axis porcinus* Zimmermann, 1780
Axis oryzus Kalaart, 1852
Cervus minor Sclater, 1883

Hog deer is similar to sambar deer, but significantly smaller, more variable color and upper canines are lacking. There are two subspecies of *C. p porcinus* and *C. p. annamiticus*. Thai race is classified to be *C. p. annamiticus*. It was once plentifully found in low-lying grasslands throughout northern country, but now it was disappeared in the Thailand's natural habitats and become extinct by the mid-1960s (Humphrey and Bain 1990). Hence, it is listed as appendix 1 of the CITES and is categorized as the Data Deficient in IUCN red list. The special habit of it is running through the underbrush with its head held low rather than leaping over obstacles as other deer do, in which like the manner of hog, therefore, it has got the common name as "hog deer". It is essentially solitary, except on breeding season that is duration of large grouping for mating which probably as many as 40 individuals. (Schaller, 1967; Lekagul and McNeely, 1977; Nowak, 1999) Although no record from the Thailand's natural habitats for a long time, but hog deer is the most successful breeding in captivity. In Thailand, several herds have released to the wild for reintroduction pilot project, which was carried out in Phu Khieo Wildlife Sanctuary and Propagation Station in northern Thailand at a higher elevation than is typical for the natural range of the species in the early 1990s, and found that they could efficiently generate reintroduced population (Timmins and Duckworth, 2008).

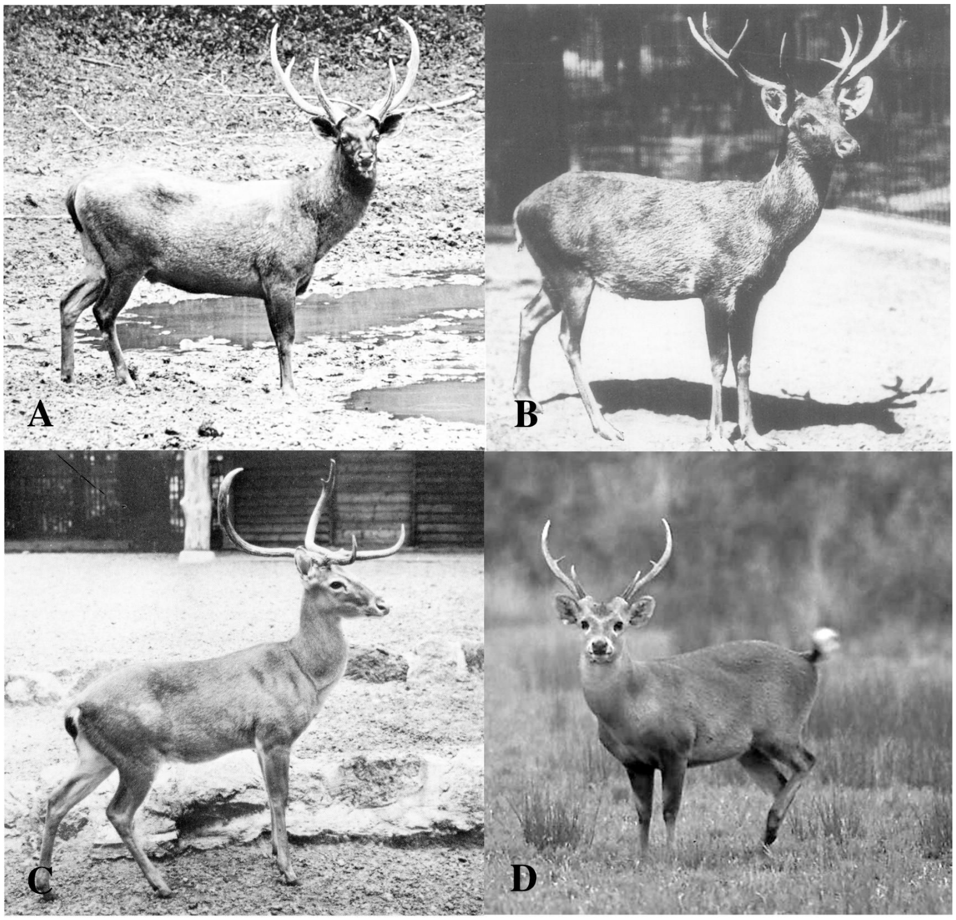


Figure 5 Cervinae in Thailand

(A) Sambar deer (*Cervus unicolor*)

(B) Schomburgk's deer (*C. schomburgki*)

(C) Eld's deer (*C. eldii*)

(D) Hog deer (*C. porcinus*)

Source: Nowak (1999)

Zoological names in the descriptions above, followed by the classification of Lekagul and McNeely (1977) in some species such as Sambar deer, Eld's deer, Schomburgk's deer and hog deer, are not accordant with those, which must be used today. Therefore, zoological names of Cervidae in Thailand were reviewed in this study as Table 1 (Pocock, 1943; Ellerman and Morrison-Scott, 1951; Lekagul and McNeely, 1977; Groves and grubb, 1987; Corbet and Hill, 1992; Grubb, 1993; Grubb, 2005). To understand some aspects of ecology of Thai deer easily, the comparison of their ecology and behavior is shown in Table 2 (Lekagul and McNeely, 1977; Grubb, 1993; Timmins and Duckworth, 2008).

E. Genus *Axis*: Spotted deer Versus Hog deer

According to Groves and Grubb (1987), the genus *Axis* contains four Asian species, in which two of them include spotted deer (*A. axis*) and hog deer (*A. porcinus*). In this classification, hog deer is categorized in Genus *Axis*, not in Genus *Cervus* as some author's classification, though its feature is similar to sambar deer (*Cervus unicolor*), but smaller.

For spotted deer (*A. axis*), which do not belong to Thailand's species, this species is slender, graceful and medium body build, whereas the other three species are stocky and have shorter legs. Like the others *Axis* spp., their coloration varies from season to season. The general color is a bright rufous fawn or yellowish brown to brownish. During part of the year, the upper parts of *A. axis* are beautifully marked with small white spots. Their distribution includes India, Nepal, Sikkim, Sri Lanka and Bangladesh (Nowak, 1999).

To compare spotted deer with hog deer (*C.* or *A. porcinus*), these two species are quite different in size and coat color, but both of them seem prefer living in grasslands and open forest more than penetrating into heavy jungles. (Nowak, 1999) Both species are found in Nepal, Sri Lanka and India, where their distributions sometimes overlap, in addition, the range of *A. axis* includes Bangladesh, while that of *C.* or *A. Porcinus* covers Indochina, Pakistan and China (Grubb and Gardner, 1998; Biswas, 1999) (Figure 6).

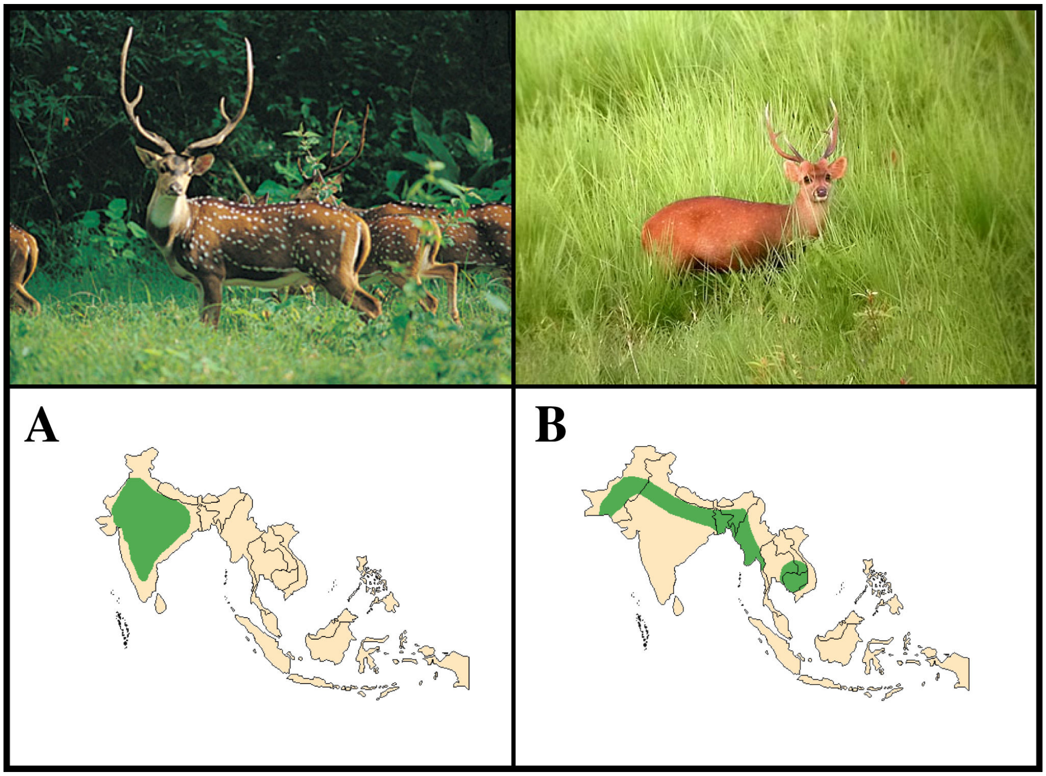


Figure 6 Spotted deer versus hog deer, with their distributions

(A) Spotted deer (*A. axis*)

(B) Hog deer (*A. porcinus*)

Source: Whitehead (1993)

Table 1 Zoological names of Cervidae in Thailand were reviewed from different authors.

Common name	Zoological name			
	Original	Pocock (1943)	Ellerman and Morrison-Scott (1951)	Lekagul and McNeely (1977)
Common barking deer	*Cervus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780
Fea's barking deer	*Cervulus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889
Sambar deer (Subspecies equinus)	*Cervus equinus* Cuvier, 1823	*Cervus unicolor* Kerr, 1792	*Cervus unicolor* Kerr, 1792	*Cervus unicolor* Kerr, 1792
Schomburgk's deer	*Cervus schomburgki* Blyth, 1863	Subgenus *Thaocervus* Pocock, 1943	*Cervus schomburgki* Blyth, 1863	*Cervus schomburgki* Blyth, 1863
Siamese Eld's deer	*Panolia platyceros* Gray, 1843	Subgenus *Panolia* Pocock, 1943	*Cervus eldii siamensis* Lydekker, 1915	*Cervus eldii siamensis* Lydekker, 1915
Burmese Eld's deer	*Cervus eldii thamin* Thomas, 1918	Subgenus *Panolia* Pocock, 1943	*Cervus eldii thamin* Thomas, 1918	*Cervus eldii thamin* Thomas, 1918
Hog deer (Subspecies *annamiticus*)	*Hyelaphus annamiticus* Heude, 1888	*Axis porcinus* Zimmermann, 1780	*Axis porcinus* Zimmermann, 1780	*Cervus porcinus* Zimmermann, 1780

Table 1 (Continued)

Common name	Zoological name			
	Groves and grubb (1987)	Corbet and Hill (1992)	Grubb (1993)	Grubb (2005)
Common barking deer	*Muntiacus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780	*Muntiacus muntjak* Zimmermann, 1780
Fea's barking deer	*Muntiacus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889	*Muntiacus feae* Thomas and Doria, 1889
Sambar deer	*Rusa unicolor* Kerr, 1792	*Cervus unicolor* Kerr, 1792	*Rusa unicolor* Kerr, 1792	*Rusa unicolor* Kerr, 1792
Schomburgk's deer	*Rucervus schomburgki* Blyth, 1863	*Cervus schomburgki* Blyth, 1863	*Rucervus schomburgki* Blyth, 1863	*Rucervus schomburgki* Blyth, 1863
Siamese Eld's deer	*Rucervus eldii siamensis* Lydekker, 1915	*Cervus eldii siamensis* Lydekker, 1915	*Rucervus eldii siamensis* Lydekker, 1915	*Rucervus eldii siamensis* Lydekker, 1915
Burmese Eld's deer	*Rucervus eldii thamin* Thomas, 1918	*Cervus eldii thamin* Thomas, 1918	*Rucervus eldii thamin* Thomas, 1918	*Rucervus eldii thamin* Thomas, 1918
Hog deer	*Axis porcinus* Zimmermann, 1780	*Axis porcinus* Zimmermann, 1780	*Axis porcinus* Zimmermann, 1780	*Axis porcinus* Zimmermann, 1780

Table 2 Some aspects of ecology comparison among Cervidae in Thailand.

Common name	Ecology				
	History range	Habitat	Altitude	Food	Life style
Common barking deer	Throughout the country	All type of forest, even in heavily degraded forest, areas adjacent to forest and in plantations	In the lowlands up to at least 1,000 m asl	Mostly fruits, buds, tender leaves, flowers, herbs and young grass	Mostly solitary and nocturnal, small groups probably refer to mating pairs
Fea's barking deer	The Tenasserim of western Thailand, the Isthmus of Kra north and the Thai-Myanmar borderlands	Evergreen forests and montane forest of the hills and mountains	Above 1000 m asl	Mostly fruits, buds, tender leaves, flowers and herbs	Usually solitary, more diurnal and less nocturnal than most other deer, pairs may form during the breeding season, live near water source
Sambar deer (Subspecies equinus)	Throughout the country	Wide variety of forest types and environmental conditions, even in heavily degraded forest, but seem to prefer dense forests	In the lowlands up to at least 3,825 m asl	Mostly fruits, buds, tender leaves, flowers, herbs and young grass	Mostly crepuscular and nocturnal, often in groups and transitory large herd were found occasionally

Table 2 (Continued)

Common name	Ecology				
	History range	Habitat	Altitude	Food	Life style
Schomburgk's deer	Lie within 13°30′–18°N, 98°30′–102°E, of the central plain of Thailand	Swampy plains with long grass, cane, and shrubs; it apparently avoided forest	In the lowlands	Young shoots of grass	Mostly crepuscular and nocturnal, typically stay in a family
Siamese Eld's deer	Throughout the country north of Kra	Deciduous Dipterocarp Forest, with an open understory and most burn annually during dry season	Lower than 1,000 m asl	Young shoots of grass and leaves, but also prefer wild fruits and cultivated crops	Often form rather large herds of up to 50 or more, typically found in open plains and generally avoid dense forests
Burmese Eld's deer	The Tenasserim and the Thai-Myanmar borderlands	Deciduous Dipterocarp Forest, with an open understory and most burn annually during dry season	Lower than 1,000 m asl	Young shoots of grass and leaves, but also prefer wild fruits and cultivated crops	Often form rather large herds, typically found in open plains and generally avoid dense forests
Hog deer (Subspecies *annamiticus*)	Throughout the country north of Kra	Floodplain and moist or wet tall grasslands	In the lowlands	Primarily a grazer of young grasses, also herbs, fruits and young leaves	Mostly crepuscular, nocturnal and solitary, transitory large herd were found occasionally

2. Molecular Evolution and Molecular Phylogenetic Approaches

A. Molecular Evolution and Molecular Phylogenetic

Due to the hereditary information of all living organisms in the world, with exception of some viruses, is consist of the same four types of nucleotides; adenine (A), thymine (T), cytosine (C) and guanine (G) and a basic process in the evolution of life is the substitution of one nucleotide for another during a long evolutionary time. Hence, these molecular characters of each individual could be compared together for knowing how their evolutionary relationship is (Graur and Li, 2000).

A new era in molecular science had begun after the entire genome of *Haemophilus influenzae*, the first of a free-living organism was published in July 1995 until the recent of entire human (*Homo sapiens*) genome was discovered. Therefore, DNA sequences are now recognized as the invaluable document of the history of life on the earth. The knowledge of what evolutionary information is written into gene sequences and how this information might be recovered is the science of molecular evolution (Page and Holmes, 1998).

Because of illusive direct observation from morphological data and also many extant groups do not have adequate fossil records; it has still not clear answers what are the general causes of speciation, and how do rates of speciation vary over the time which could lead to actual understanding the general patterns and processes of speciation and diversity of life. Hence, the expansion of molecular phylogenetics over the recently past decade has opened up a forceful new approach to these questions. Phylogenetic trees derived from DNA sequences provide an indirect record of the speciation events that have led to present day species. Together with data on geographical and ecological attributes of species, they can provide information on the causes of speciation within the group. The success of phylogenetics approach as a tool for solving speciation problems rests on two fundamental issues. First, it relies on reconstructing evolutionary species relationship within a clade and second, processes in addition to speciation influence the phylogeny and attributes of present-day species (Barraclough and Nee, 2001).

B. Appraisal of the species

The definitions of "Species" are evolved by the change with the additional knowledge which we have gained since the Linnaean Period until nowadays biochemical and molecular blossom (King, 1983). There are some important historical views of "Species concept" which at least have been proffered as usable models as following:

1. The morphological species concept

"Species are a group of individuals or populations with the same or similar morphological characters" (Cain, 1954). The concept was expanded by Linnaeus (1707-1778), the father of modern systematic who introduced the facility of binomial nomenclature and has the view that every organism must belong to the lowest taxonomic entity, the species.

2. The biological species concept

"Species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr, 1942). This concept was proposed, in which would apply to sexually reproducing species, but no application to asexual organisms. Some critics as Simpson (1961) disagreed to this concept on its failure to cope with temporally sequential species and also pointed to the difficulty in assessing the degree of reproductive isolation between species.

3. The evolutionary species concept

"A species is a single lineage of ancestral descendant population of organisms which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley, 1978). In the Wiley's opinion, he views that all species past and present belong to the same evolutionary species and no lineage may be subdivided into ancestral and descendant species. This view was attacked by Mayr (1982), who deemed that the weakness of evolutionary species definitions is that they minimize the crucial species problem, the causation and maintenance of discontinuities between species and concentrate instead on the problem of how to delimit multidimensional species taxa.

4. The phylogenetic species concept

"A species is the smallest diagnosable cluster of individual organism within which there is a parental pattern of ancestry and descent" (Cracraft, 1983). Cracraft (1983) reasoned that if reproductive isolation was not regarded as the central issue in taxonomic differentiation, then differentiation was the primary act and interbreeding was followed. This concept could eliminate any reference of reproductive isolation from other taxa. Species are recognized strictly in terms of their status as diagnosable evolutionary taxa, thus, two sister taxa could hybridize and still be considered as species if each was diagnosable as a discrete taxon.

C. Phylogenetic Concepts

Today those characters that have been long inherited as DNA sequences are used as supplement of morphological data for better insights of "Phylogeny" which refers to history of a species and to its relationships to other species. Phylogeny or evolutionary tree is a mathematical structure which is used to model the actual evolutionary history of group of sequences or organisms. A tree consists of "nodes" connected by "branches". Its structure includes "Terminal nodes" represent sequences or taxon which are data; may be both of extant or extinct, "internal nodes" which represent hypothesis ancestors and "the root", which represent the ancestor of all the sequences that comprise the tree (Stearns and Hoekstra, 2005).

Phylogenetic studies of speciation have focused on reconstructing species level phylogenies, namely the relationships among species within higher group such as genera. The internal nodes of the tree reflect speciation and the timing of those events (Barraclough and Nee, 2001). Page and Holmes (1998) and Stearns and Hoekstra (2005) explain that Phylogenies form the three basis of classification which is the formal naming of groups (Figure 7) in the following:

1. Monophyletic group: all species in this group are descended from a common ancestor that is not the ancestor of any other groups, whereas a non monophylelic group omits some of those descendants.

2. Polyphyletic group: its species are descended from several ancestors that are also the ancestors of species classified into the other groups.

3. Paraphyletic group: this group is an unnatural group that does not contain all species descended from the most recent common ancestor of its members.

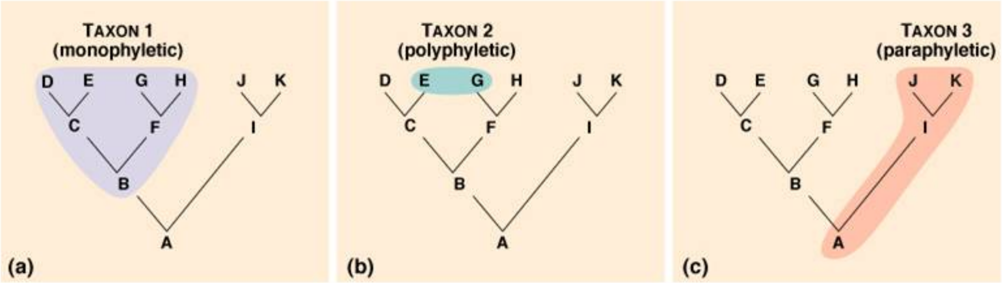


Figure 7 The difference of monophyly, polyphyly and paraphyly

Source: Page and Holmes (1998)

D. Terminology of Tree

The tree is related with a set of sequences which can imply a kind of evolutionary change. There are some of different ways of representing evolutionary change on tree which could be described as some basic terms (Figure 8) as below:

1. Plesiomorphy: a trait that sequence of taxon has the same base as the common ancestor of all the sequences being studied.

2. Apomorphy: opposite to plesiomorphy, in which has the different base from the common ancestor.

3. Autapomorphy: similar to apomorphy, but only unique derived character states.

4. Synapomorphy: similar to apomorphy, but shared derived states together and is not found in their closet relatives.

5. Homoplasy: a trait of sequences similarity which occur independent inheritance from their ancestor. This trait is poor indicator for evolutionary relationship because the similarity does not reflect shared ancestry (Page and Holmes, 1998).

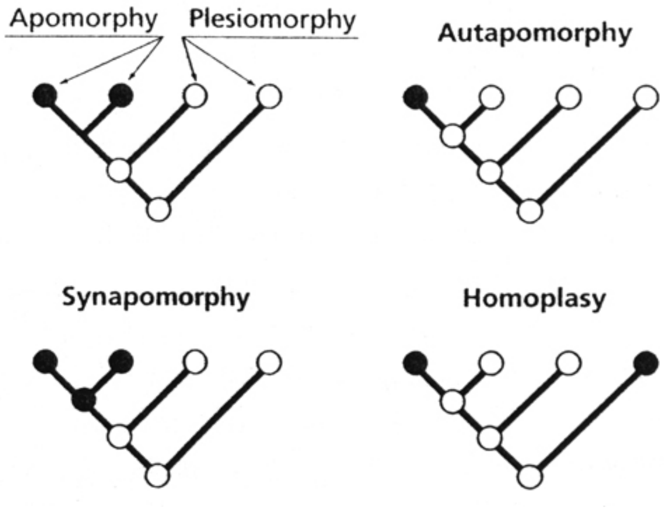


Figure 8 Different patterns of ancestral (○) and derived (●) charater states on tree

Source: Page and Holmes (1998)

E. Divergence, Convergence and Parallel Evolution

There are two major reasons that traits or molecular sequences can look similar character. The first one, because they are descended from their common ancestors and the second one, because of natural selection in the environment or genetic drift could shape them in similar ways and they now look the same, although they are descended from unrelated and looked deferent ancestors. The traits descended from the same common ancestors can now look different because evolution has adapted them to different ecological condition are considered as "evolutionary divergence", oppositely, the traits descended from different common ancestors can now look similar because evolution has adapted them to similar ecological conditions are through as "evolutionary convergence". Thus, both of these kind could delude us depart from the real genetic relationships (Stearns and Hoekstra, 2005).

The commonest cause of homoplasy in morphological traits is convergence in DNA sequences mutation. There are three kinds of homoplasy include (1) Parallel evolution: the independent evolution of the same feature from the same common ancestry, (2) Convergent evolution: the independent evolution of the same feature from different common ancestry and (3) Secondary loss the loss of a derived feature, which results in the apparent reversion to the ancestral condition (Figure 9) (Page and Holmes, 1998).

Due to these different kinds of evolution, there are some specific characters which could signify the kind of evolution "Homologous characters" are those that are derived directly from the same previous character states, are comparable parts of the same characters transformation series, or are identical with that of the last common ancestor, whereas "Analogous characters" appear to be similar because they serve the same function, but they are derived from different character states (Freeman and Herron, 2001) (Figure 10).

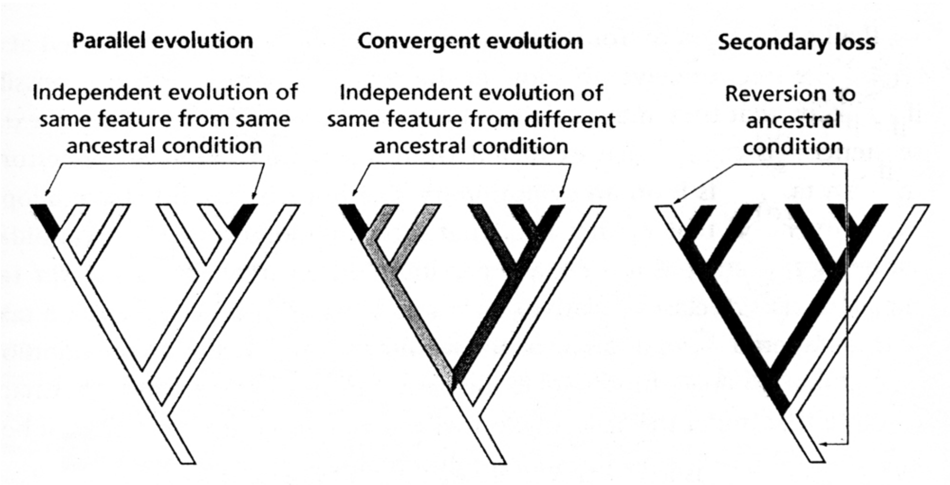


Figure 9 Three different kinds of homoplasy

Source: Page and Holmes (1998)

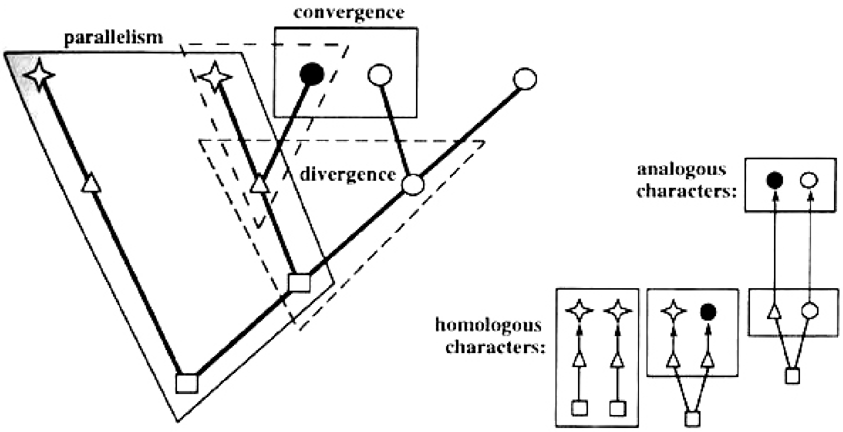


Figure 10 The three kinds of evolution, with two kinds of characters include homology and analogy (Symbols denote different states of a single phenotypic trait and arrows connecting symbols show character transformation among the states of the trait).

Source: Freeman and Herron (2001)

F. Reconstructing of Phylogenetic tree

Phylogenetic analysis of molecular data is important tool for studying the evolutionary history of life. The general task of molecular phylogenetic is to convert information such as DNA sequences into an evolutionary tree. There are two ways that seem useful for dividing the methods which is based on how the data are treated includes (1) Distance methods: beginning of convert aligned sequences into a pair-wise distance matrix and then input that matrix into a tree building method and (2) Discrete methods: considering each nucleotide site directly (Page and Holmes, 1998).

There are many statistical methods that can be used for reconstructing phylogenetic tree from molecular data that penny *et al*. (1992) have suggested these methods should have five criteria for desirable properties of tree building:

1. Efficiency: is how fast time, in which a computer program could find a tree using a given method effectively.

2. Power: is a measure of how much data the method need to produce a reasonable and correct result does.

3. Consistency: is whether the method will converge on the true tree if adding it more data.

4. Robustness: the sensitivity of method to violations of its underlying model.

5. Falsifiability: is whether violations are sufficient to rule out a particular model. If its assumptions are violated, should not be using the method at all.

Page and Holmes (1998) have evaluated each method based on these five criteria which are described below:

1. Distance methods

In the distance methods, evolutionary distances of nucleotide substitutions between two taxonomic units are computed for all pairs of taxa and a phylogenetic tree is constructed by using an algorithm based on some functional relationships among the distance values.

a) Goodness of fit measures: this method seeks the metric tree that best account for the observed distances as the first, the second step seeks the tree whose sum of branch lengths is the minimum.

b) Minimum evolution method: the minimum evolution (ME) tree is the tree which minimizes the sum of branch lengths; however, the length in this case is computed from the pair-wise distances between the sequences rather than from the fit of individual nucleotide sites to a tree. The neighbor joining (NJ) tree is first computed, then see if any local rearrangement of the NJ tree produces a shorter tree.

c) Neighbor joining method: neighbor joining (NJ) is a widely used method for tree building which combines computational speed with uniqueness of result. This method is a clustering method rather than an optimality method, however, it is a good heuristic method for estimating the minimum evolution (ME) tree and is regarded as a simplified version of the ME method. The principal point of NJ method is defined as two taxa that are connected by a single internal node is an unrooted bifurcating tree. So these two taxa are said to be neighbors if they are connected through a single internal node.

d) Unweighted Pair group method with arithmetic means (UPGMA): this is the simplest method for tree reconstruction. It's originally developed for constructing phonograms, but it can also used to construct phylogenetic tree if the rates of evolution are approximately constant. Unless the rate of gene substitution is constant, UPGMA often gives an incorrect topology. Actually, UPGMA is the best viewed as a heuristic for finding the least squares ultrametric tree for a distance matrix.

2. Discrete methods

In contrariety to distance method, this method operates directly on sequences or character data rather than on pair-wise distances. So, they attempt to avoid the loss of information that occurs when sequences are changed into distances.

a) Maximum parsimony (MP): the principle of MP concerns the identification of a topology that requires the smallest number of evolutionary change to explain the observed differences on the tree. In MP method, discrete character states are used, and the shortest pathway can lead to these character states is chosen as the best tree which is the one with the fewest changes in mutation and the least convergence.

b) Maximum likelihood (ML): in ML method, the likelihood (L) is the probability of observing the data given a specific model of character state changes. The aim of ML method is to find the tree with the highest L Value as formula, L=Pr (D/H); D is the set of sequences data and H is a phylogenetic tree. So, the tree that is the most probable evolutionary outcome is the maximum likelihood estimate of the phylogeny. The chosen tree is the basic model assumes the same type of random change down all branches of the tree.

3. Molecular Markers: Which Is Chosen for This Study

A. Mitochondria

Mitochondria are organelles within cytoplasm of the eukaryotic cells that have small amount of their own DNA, ribosome and can make many of their own protein. Their function are changing the energy from food into a form that cell can use or they are the sites of the oxidative phosphorylation process of the cells. These chemical reactions generate large amounts of adenosine triphosphate (ATP), an energy-rich molecule, with more than thirty molecules per one glucose molecule. Each mitochondrion size is approximately 0.5-1 µm, consists of two layers membrane. An outer covering consists of porin, the protein membrane that are actively controls the movement of molecules into and out of organelle. An inner membrane is a series of folds as the tubular or lamella structure call “cristae” which their inner space is the “matrix” substance. In matrix, is the location of genetic material calls “Mitochondrial DNA; mtDNA” in the form of small circular DNA (Klug and Cummings, 2002).

Mitochondria are thought to be derived from bacteria that were taken into cells in the distant past. Their origin is more than a billion years ago when a free-living, the closest relatives of which are the α-proteobacteria entered a eukaryotic cell. This endosymbiosis event was beneficial to both organisms are now an essential feature of modern eukaryotic cell. When cell divide, their mitochondria divide as well and pass on their DNA to their daughter mitochondria, so mtDNA are normally inherited exclusively from the maternal line (Brown *et al*, 1979).

1. Mitochondrial DNA; mtDNA

Each mitochondrion is estimated to contain 2-10 mtDNA copies. In mammals, each circular mtDNA molecule contains 15,000-17,000 base pairs, which encode the same 37 genes; 13 for polypeptides (mRNAs) which provide instructions for making enzymes involved in oxidative phosphorylation, 22 for transfer RNAs, (tRNA) and one each for the small and large subunits of ribosomal RNAs (16S and 12S rRNA). These types of RNA help assemble protein building blocks into function proteins. The protein products are components of respiratory chain complexes I (ND 1-6 and ND4L), complexes III (cytochrome *b*), complexes IV (COX 1-3) and ATP synthase (ATP8 and ATP6). The mitochondria genome is arranged very efficiently, no introns and has small intergenic spacers where the reading frames even sometimes overlap. On the circular genome, the control region is the primary non-coding region which is responsible for the regulation of heavy (H) and light (L) strand transcription and of H-strand replication (Figure 11).

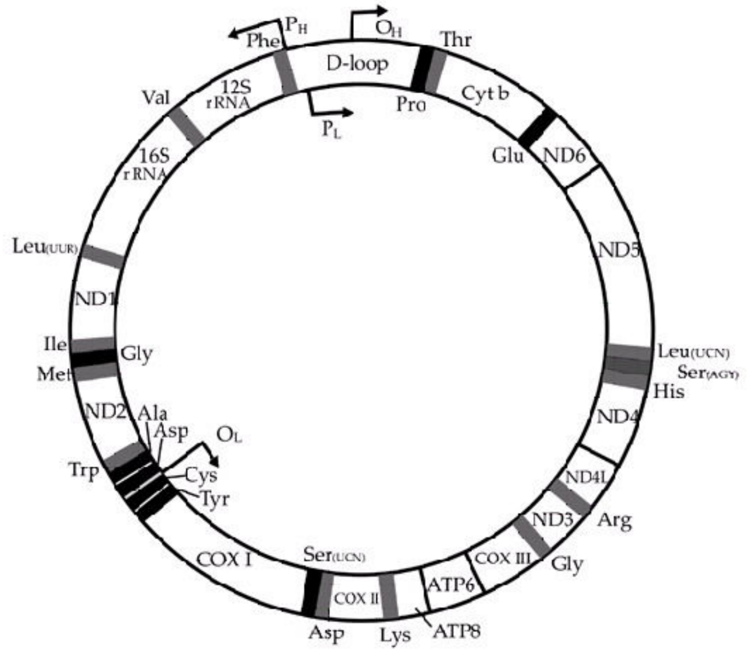


Figure 11 The mammalian mitochondrial genome ; the genome encodes 22 tRNA, 2 rRNAs and 13 proteins which their products are composed of respiratory chain complexes I (ND 1-6, 4L) , III (Cyt. *b*), IV (COX 1-3) and ATP synthase (ATP8 and ATP6). D-loop denotes the one substantial non-coding region.

Source: Johns and Avise (1998)

As a molecular marker, mitochondrial DNA has many advantages (Brown *et al*, 1982 and Thorne *et al.*, 1998 Arnason *et al.*, 2002), they are described as following:

a) The mutation rate of animal mtDNA is about tenfold value of nuclear DNA. It is faster base substitution, probably due to its inefficient replication repair. It is useful for assessing genetic relationships of individuals or groups within a species and also for identifying and quantifying the phylogeny among different species.

b) Different regions of mtDNA genome evolve at different rates, allowing suitable regions to be chosen for the question under study. This approach has limited that are imposed by the rate of each portion of mtDNA sequence change. In animal, the rapid rate of change makes mtDNA most useful for comparisons of individuals within species and for comparisons of species that are closely or moderately-closely related, whereas the species become more distantly related, the number of sequence differences become very large.

c) Because of no recombination process on mtDNA or there is usually no change in mtDNA from parent to offspring and because mtDNA is maternally inherited in most species, So mtDNA is a powerful tool for tracking back family tree down the maternal line and has been used in the role to track the ancestry of many species back hundreds of generation.

d) The mtDNA can be considered as neutral marker, because their mutations do not involve with the survival of their life, so there are no loss of new genotype due to the evolutionary natural selection.

2. Cytochrome *b* gene; *cyt. b*

Cytochrome *b* is one of the cytochromes (includes Cyt. *a*, *b* and *c*). Each group of them has different kinds of spectrum absorption ability. Their function involved in the electron transport in the respiratory chain of mitochondrion from co-enzyme Q to the oxygen molecules. It contains eight transmembrane helices connected by intramembrane or extramembrane domains (Figure 12) (Esposti *et al.*, 1993).

The cytochrome *b* is the most widely used gene for phylogenetic work for several reasons. Because some parts of gene are conserved due to their functional restrictions, they evolve slowly in terms of non-synonymous substitution Despite the most portion of gene are mutated slowly, but the rate of mutation in silent positions is relatively fast. So, they are thought to be variable enough for population level questions, and conserved enough for clarifying deeper phylogenetic relationships. Most of the variable positions seem to be located within the coding region for transmembrane domains or for the amino-terminal and the carboxy-teminal ends (Irwin *et al.*, 1991).

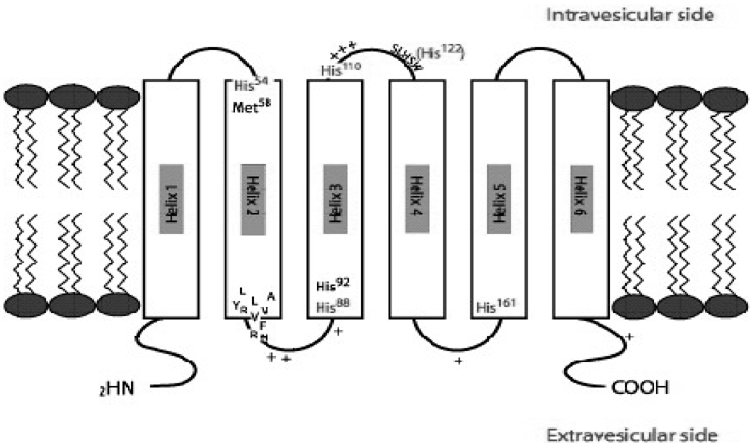


Figure 12 The cytochrome *b* protein structure, consist of 8 transmembrane helices connected by intramembrane or extramembrane domains.

Source: Esposti *et al.* (1993)

3. Control region; CR

The mtDNA control region is the non-coding region which contains the heavy-strand replication origin and the promoters for both the light (L) and the heavy (H) strand transcription. In mammals, CR is located between tRNA^{Pro} of 5$^{'}$ end light (L)-strand and tRNA^{Phe} of 3$^{'}$ end heavy (H)-strand, with 880-1,400 bp estimate long depending on insertion, deletion or variable numbers of tandem repeats (VNTRs) which are usually appeared on the end of 5$^{'}$-left and 3$^{'}$-right peripheral domain. CR in the same meaning calls a displacement loop or “D-loop” that is depiction for mtDNA structure where the two strands of double-stranded mtDNA molecule are separated and held apart by a third strand or displaced strand which formed the loop of the “ D ” on the replication duration (Saccone *et al.*, 1991; Arnason *et al.*, 2002).

CR is divided into three domain, with two feature portions, (1) The portion with slowly rate of mutation that is a conserved central region, CCR or domain II and (2) the portion with highly rate of mutation consist of 5'-left and 3'-right peripheral domains or domain I and domain III. In mammals, the first domain at the 5'end contains the putative termination associated sequence (TAS). The central domain is the most conserved contains several structural elements that can be readily aligned even between different families. These elements are conserved sequence block; CSB (B-F boxes) which is the recognition specific site for replication and transcription. The termination-associated sequence (TAS) site on domain I is the final point of new H-strand elongation, with CSB (B-F boxes) on domain II is the recognition site. Usually the most variable part is the third domain at the 3'end. This domain consists of conserved sequence block; CSB (1, 2+3) and is the location of the origin of H-strand replication (O_H) and also is the promoters for L-strand and H-strand transcription (LSP and HSP) (Figure 13) (Saccone *et al*., 1991; Clayton, 1992).

The control region in many species often evolves faster than the rest of the mitochondrial genome and also to be highly variable. This variability has lead to the expanding usage of CR to examine questions ranging in population structure or among closely related species level for resolving their phylogenetic relationships quite reliably (Ghivizzani *et al*., 1993; Douzery and Randi, 1997).

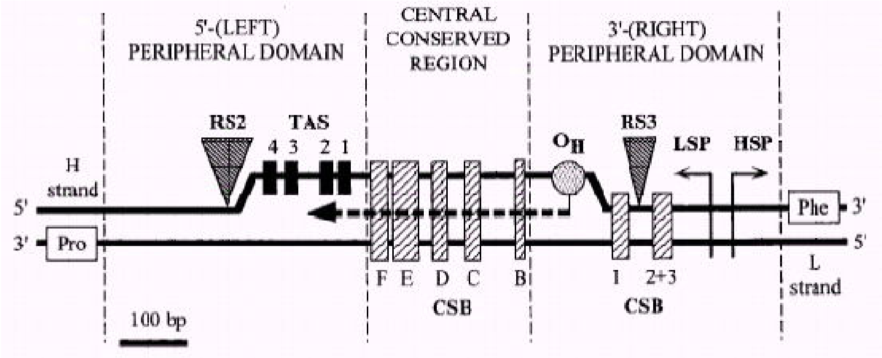


Figure 13 The mammalian control region structure

Source: Douzery and Randi (1997)

B. Introns of the Protein Kinase C, iota gene (PRKCI)

1. Nuclear introns

Introns are non-coding sequences portion of DNA between coding regions in a gene which is transcribed, but which does not appear in the mRNA product, whereas those of coding region retained and expressed or translated into protein are call exons (Klug and Cummings, 2002). Introns occur often within eukaryotic genes and make up most of the length of genes, with vary greatly in number, size and organization from gene to gene. Although introns are considered to be non-functional and are removed from genes, but there is a strong conservation of sequence around the intron-exon boundaries. The eukaryotes introns almost usually begin and end with the nucleotides GT and AG, respectively, probably this reason allows them to be accurately spliced themselves. Intron splicing process is itself an extremely important, in which if it is spliced at different places, a single gene can make different proteins (Graur and Li, 2000).

Most introns in the eukaryotes are “spliceosomal introns” or “nuclear introns” because they are spliced by a spliceosome which consists of proteins and RNA. An absence of introns in Bacteria, and the most divergent protists, as well as mitochondria suggests that they have only recently entered eukaryotic cell or the endosymbiotic process gave rise to the mitochondria, which has been known as the intron-late hypothesis (Page and Holmes, 1998).

2. The Protein kinase C, iota; PRKCI

The protein kinase C (PKC) family consists of 12 phospholipids-dependent serine/threonine kinases, which are differentially expressed and are involved in a wide variety of cellular processes. They are divided into three classes of (1) conventional, which require calcium and diacylglycerol for activation, (2) novel, which is calcium independent and (3) atypical, which require phosphatidylserine, but not calcium or diacylglycerol (Urban *et al*., 2004).

The Protein kinas C, iota, PRKCI is gene encodes a member of the protein kinase (PIC) and is a kind of atypical PKC enzyme. This protein kinase plays a requisite role in BCL-ABL-mediated resistance to chemotherapy-induced apoptosis, and is critical for epithelial cell polarity and also cell survival. As a human gene, their kinase products can protect leukemia cells against drug-induced apoptosis. This protein kinase can be recruited to vesicle clusters by direct interaction with the small GTPase RAB_2, where this kinase phosphorylates glyceraldehyde-3-phosphate dehydrogenase and also play a role in microtubules dynamics in the early secretary pathway (Suzuki *et al*., 2002; Murray *et al*., 2004).

4. The Previous Study on Molecular Phylogenetic Relationships among Cervidae and Cervinae

The antlered deer are considered as the most specious family or subfamily. Because of the high level of homoplasy and the high species richness of antlered deer is partly the result of their diverse ecological characteristics. Therefore, there are many studies concerning the evolutionary relationships within them were investigated based on several criteria such as palaeontological, morphological, behavioral, karyological data and also molecular data from various markers include mitochondrial DNA and nuclear DNA which those have been a matter of much speculation and debate, as is reflected in uncertainties about evolutionary relationships at different taxonomic levels. Examples of these previous studies on phylogeny of Cervidae and Cervinae were described in the following:

Miyamoto *et al*. (1990), Phylogeny and evolution of antlered deer includes subfamilies Cervinae, Muntiacinae and Odocoileinae were determined based on mtDNA sequences of both rRNA genes and three adjacent tRNA genes. They found that *Cervus* and *Muntiacus* are most similar, with lesser different mutation point than the others comparing. Parsimony solution indicates that rates of nucleotides change among antlered deer are similar.

Emerson and Tate (1993), 22 proteins electrophoretic patterns were analyzed and found that *Cervus* split into two distinct groups, with *C. elaphus* and *C. nippon* clade and *C. unicolor* and *C. timorensis* clade which this latter clade is more closely with genera *Dama* and *Axis*. Therefore, *Cervus* seemed to be paraphyletic.

Cronin *et al.* (1996), nuclear κ-casein sequences were analyzed. This study supported Emerson and Tate (1993), but addition was found that *C. unicolor* formed a monophyletic clade with *C. duvaucelii*.

Douzery and Randi (1997), the mtDNA control region (CR) sequence or D-loop were determined for Cervinae, Odocoileinae and Hydropotinae. Phylogenetic analyzed of CR supported the paraphyly of antlered deer, with the first cluster the *Cervus* and *Dama* were grouped within Cervinae as the old world deer and the second, the sole *Hydropotes* was group within Odocoileinae as the new world cervids.

Randi *et al.* (1998), the mtDNA cytochrome *b* (*cyt. b*) gene were compared among Cervidae include Cervinae, Muntiacinae, Odocoileinae and Hydropotinae. This study supported the monophyly of Plesiometacarpalians, with Cervinae and Muntiacinae are joined in a moderately to strongly supported clade of Eurasian species. They found contrasting with formal taxonomy that *Hydropotes* is not the sister group of all the antlered deer. Because of nesting within the Odocolienae, therefore, *Hydropotes* lost the antlers secondarily. Therefore, the mtDNA *cyt. b* phylogeny forks Cervidae into two groups of plesiometacarpal (Cervinae and Muntiacinae) versus telemetacarpal (Odocoileinae and Hydropotinae) conditions, which suggested paraphyly of antlered deer.

Randi *et al.* (2001), the evolutionary relationships within Cervinae using the mtDNA control region marker was elucidated paraphyly of the genus *Cervus*. This data suggested that *Elaphurus* should be merged with *Rucervus*, hog deer (*Axis porcinus*) did not closely with sambar deer (*Cervus unicolor*) and also found that subgenus *Rusa* did not monophyly, because *C. unicolor* and *C. timorensis* did not group with *C. alfredi.*

Liu *et al*. (2003), the mtDNA cytochrome *b* gene was analyzed. This data is similar to Randi *et al*. (2001) which found closely related between *Elaphurus* and *Rucervus*. But with the contrast, they found sambar deer (*C. unicolor*) was grouped with hog deer (*A. porcinus*).

Meijaard and Groves (2004), the craniometric data of Asian deer species skulls were analysed using multivariate statistics. Their results suggest that spotted deer (*Axis axis*) was grouped within *Cervus* group and was spitted from the others *Axis* or spotted deer (*A. axis*) was different group with hog deer (*A. porcinus*). These craniometrics data were combined with molecular data of Miyamoto *et al*. (1990) and Randi *et al*. (2001) and found that the hog deer position was more closely related to Spotted deer than sambar deer, in which the *Axis* lineage was diverged in the Early Pliocene, following by hog deer (*A. porcinus*) which was diverged in the Late Pliocene to the Early Pleistocene (Figure 14).

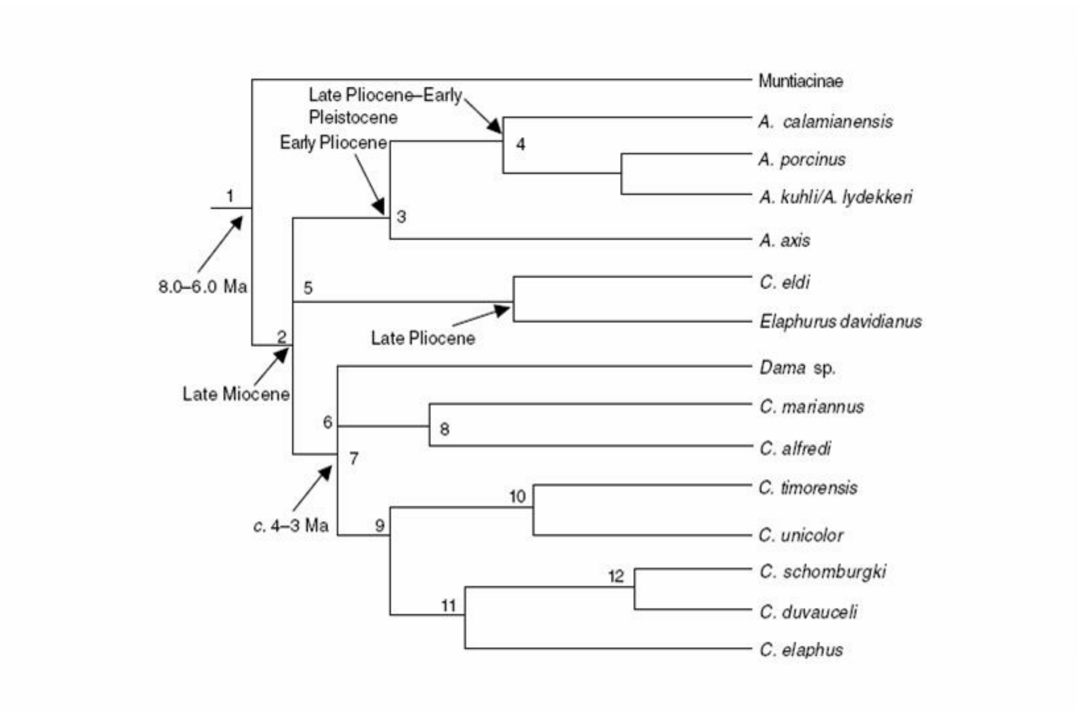


Figure 14 Phylogenetic tree of the Cevinae based on craniometrics data by Meijaard and Groves (2004) combined with molecular data by Randi *et al*. (2001).

Source: Meijaard and Groves (2004)

Pitra *et al.* (2004), the phylogenetic pattern and timing of the radiation of Cervinae was investigated based on the mtDNA cytochrome *b* gene. This study achieved strong support for monophyly of Cervinae, with muntiacinae are sister group, but did not support monophyly of *Axis*. From the base of Cervinae phylogeny, they found a split between spotted deer (*A. axis*), Schomburgk's deer (*C. schomburgki*) and swamp deer (*C. duvaucelii*), with Schomburgk's deer and swamp deer are clearly sister species. These results did not support a sister relationship between Spotted deer (*A. axis*) and hog deer (*A. porcinus*), which indicated that *Axis* is paraphyletic. Hog deer (*A. porcinus*) was unexpected finding that it was combined in the same clade of Rusa, with its sister species is sambar deer (*C. unicolor*) and also finding close relationship between Père David's deer (*Elaphurus davidianus*) and Eld's deer (*C. eldii*) (Figure 15).

Gilbert *et al.* (2006), the phylogeny of Cervidae was examined based on multigene of two mitochondrial protein-coding genes (Cytochrome *b*; *cyt. b* and subunit II of the cytochrome oxidase; CO2) and two nuclear introns (intron2 of the α-lactalbumin; αLAlb and intron1 of the protein kinase C, iota gene; PRKCI). The results within Cervinae, they suggested the monophyly of *Axis*, moreover, both species of *Axis* (Spotted deer; Axis axis and hot deer; (*A. porcinus*) are synapomorphy. By contrast, the polyphyletic of Cervus was found. Swamp deer (*Cervus duvaucelii*) was allied with *Axis*, whereas Eld's deer (*C. eldii*) was related to *Elaphurus*.

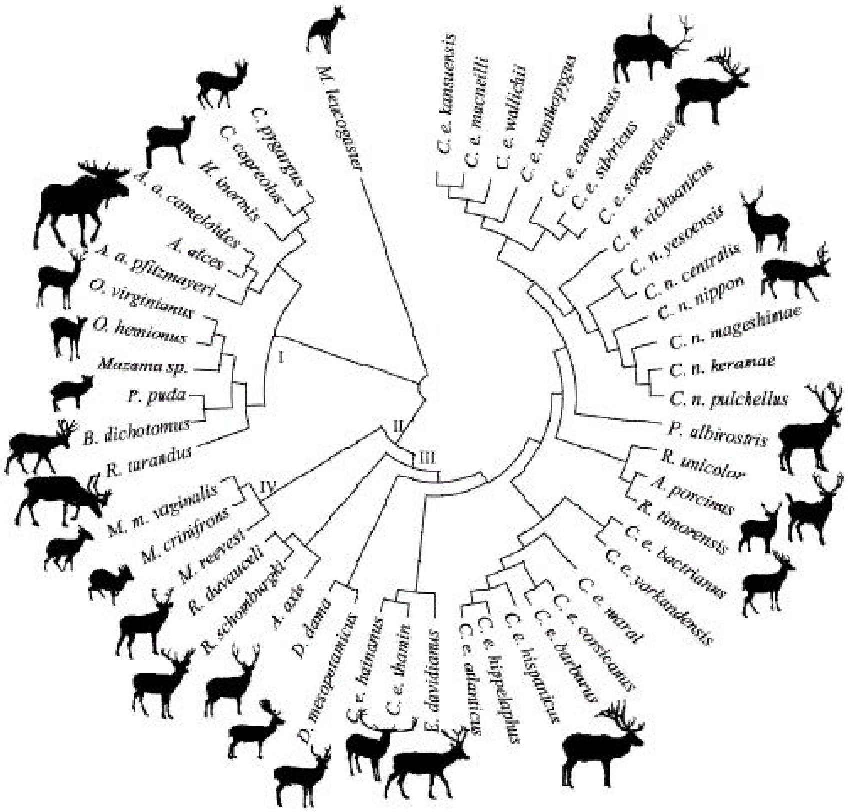


Figure 15 Topology of the intrafamilial relationships among Cervidae based on complete cytochrome *b* data set. They were splinted into two distinct lineages, (I) the telemetacarpalian and (II) the plesiometacarpalian from Bayesian analysis, identified (III) the monophyly of Cervinae (Old world deer), with (IV) muntjacs as the sister group.

Source: Pitra *et al*. (2004)

MATERIALS AND METHODS

Materials

1. Equipments for Samples Collection

a) Blood samples or hair samples of Thai deer and Spotted deer
b) Blood vacutainer 2.0 ml plus EDTA
c) Nipro disposable syringe 5 and 10 ml
d) Disposable needle No.18, 21 and 24
e) Zoletil and equipments for anaesthesia
f) Others medical equipments such as: alcohol 95%, cotton ball, cutting, forceps and disposable glove sterile, plastic bags etc.
g) Ice box and ice
h) Autoclave
i) Incubator
j) Freezer

2. Laboratory Chemical Substances

a) Aquapure genomic blood kit (Bio-Rad)
b) QIAamp DNA Micro Kit (Qiagen)
c) QIAquick PCR Purification Kit (Qiagen)
e) dNTP set 100 mM Solution (4 x 0.25 ml)
f) Taq DNA polymerase (recombinant) 500 U set, with 10X KCl Taq buffer and 2.5 mM $MgCl_2$ (Fermentas)
g) Gene Ruler 1 kb DNA Ladder 250 µg (Fermentas)
h) 50 nmole primer for mtDNA cytochrome *b* (cyt. *b*), mtDNA control region (CR) and intron1 of protein kinase C, iota gene (PRKCI) amplification
i) Distilled water
j) Agarose gel

k) 50X TAE buffer, which compose of Trisbase, glacial acetic acid, pH 8.0 EDTA and distilled water
l) Ethidium bromide (EtBr)

3. Laboratory Equipments

a) Micropipette 2, 20, 100, 200 and 1,000 µl
b) Micropipette tip
c) Microcentrifuge tube 1.5 ml
d) PCR tube
e) Agarose gel electrophoresis set
f) Refrigerated centrifuge
g) Ultracentrifuge
h) Spin down centrifuge
i) Thermal cycler for Polymerase Chain Reaction (PCR)
j) UV Gel documentation
k) Variable volume glassware such as: glass beaker, flask and measuring cylinder
l) Automated ABI 377 Genetic Analyzer
m) Spectrophotometer
n) Heating and Cooling equipments such as: incubator, hot water bath, microwave, fridge, freezer and scale ice maker
o) Basic laboratory equipments such as: vortex mixers, balances, stirrer, pH tester, hot plate, calculator and laboratory timer
p) Lab notebook

4. Computer and Programs for Phylogenetic Analysis

a) Computer Intel Pentium Core 2 Duo with at least 3.0 GHz CPU and at least 2.0 GB processing speed, which can operate internet connecting
b) Basic Local Alignment Search Tool-nucleotide; **BLASTn** from http://blast.ncbi.nlm.nih.gov/Blast.cgi

c) CAP3 Sequence Assembly Program; **CAP3** from http://pbil.univ- lyon1.fr/cap3.php
d) Multiple Sequence Alignment; **ClustalW** from http://align.genome.jp/ http://www.ebi.ac.uk/Tools/clustalw2/index.html
e) Multiple Sequence Alignment; **ClustalX** Version 1.82 (Thompson *et al.* 1997)
f) Primer Design with Oligo Primer Analysis Software Version 7.0; **Oligo** from http://oligo.net
g) Chromatogram viewer and base call editor Version 2.33 ; **Chromas** from http://technelysium.com.au/chromas.html
h) Phylogenetic Analysis Using Parsimony and other methods version 4.010b; **PAUP*** (Swofford, 2002)
i) **TREEVIEW** version 1.6.6 (Page, 1996)

Methods

1. Samples Collections

Deer species for this research include 6 species consisting of 5 species of Thai deer and 1 species of spotted deer as below:

a) Hog deer (*Cervus porcinus* or *Axis porcinus*)
b) Sambar deer (*C. unicolor equinus*)
c) Spotted deer (*A. axis*)
d) Eld's deer, with 2 subspecies
    (1) Siamese Eld's deer (*C. eldii siamensis*)
    (2) Thamin Eld's deer (*C. eldii thamin*)
e) Common barking deer (*Muntiacus muntjak*)
f) Fea's barking deer (*M. feae*)

From those deer species, the ingroups include four species of Cervinae (Hog deer, sambar deer, spotted deer and two subspecies of Eld's deer). Muntiacinae (common barking deer and Fea's barking deer) are determined as outgroups.

Blood and hair samples of those deer species were collected from seven sampling captivity areas of Thailand which are under operated by the Zoological Park Organization under the Royal Patronage of His Majesty the King and Wildlife Propagation Division, Department of National Park, Wildlife and Plants Conservation (DNP) (Appendix Figure A1). The locations are shown in Figure 16 and the samples from each source are described in Table 3. Blood samples were contained in vacutainers plus EDTA (Appendix Figure A2) and hair samples were filled in plastic bags, which those were kept in ice during transportation to laboratory room in Kasetsart University. Then, those blood samples were kept cool in 4°C refrigarator.

Figure 16 Location of seven sampling areas

(1) Khaokho wildlife propagation station, Phetchabun.

(2) Korat Zoo, Nakhon Ratchasima.

(3) Dusit Zoo, Bangkok.

(4) Hauysai wildlife propagation station, Phetchaburi.

(5) Pattalung wildlife propagation station, Phatthalung.

(6) Ton Nga-Chang wildlife propagation station, Songkhla.

(7) Songkhla Zoo, Songkhla.

Table 3 Deer samples from each source.

Species	Sample	Source	n
C. (or *A.*) *porcinus*	P1, P5, P6	Hauysai Wildlife Station[a]	6
	P9	Dusit Zoo[b]	
	P11	Korat Zoo[b]	
	P12	Songkhla Zoo[b]	
C. unicolor	U2, U3, U4	Hauysai Wildlife Station[a]	5
	U5	Songkhla Zoo[b]	
	U8	Khaokho Wildlife Station[a]	
C. eldii siamensis	S1	Dusit Zoo[b]	1
C. eldii thamin	T1, T2, T7	Hauysai Wildlife Station[a]	5
	T9, T11	Dusit Zoo[b]	
A. axis	A1, A2	Songkhla Zoo[b]	4
	A3, A4	Korat Zoo[b]	
M. mumtjak	M2, M3	Pattalung Wildlife Station[a]	2
M. feae	F1, F2	Ton Nga-Chang Wildlife Station[a]	2
Total			25

[a] Wildlife Propagation Division (DNP)

[b] the Zoological Park Organization under the Royal Patronage of His Majesty the King

2. DNA Extraction

Whole genomic DNA from blood was extracted using Aquapure genomic blood kit (Bio-RAD) and those from hairs were extracted using QIAamp DNA micro kit (Qiagen) according to the manufacturer's protocol. At the last step of protocol, the extracted DNA in total volume of 100 µl of the TE buffer was stored at –20°C in a refrigerator.

3. Determination of Yield and Quality of Extracted DNA Solution

DNA yield was determined by measuring the concentration from its absorbance at 260 nm (A_{260}). Fifty µl of 1/50 extracted DNA dilution was measured using spectrophotometer. DNA quality was determined by measuring the concentration from the absorbance at 280 nm for protein which indicates the quantity of contaminants. The A_{260} of 1 detection path corresponded to 50 µg of DNA per milliliter. Distilled water is used as diluents when measuring DNA concentration could be estimated by the relationship between absorbance and concentration which is biased on extinction coefficients calculated for nucleic acids in water. The ratio of A_{260}/A_{280} of 1.8 to 1.9 exposed the high purity of DNA (Kalia *et al*., 1999). For the same standard of DNA band yielded after DNA amplification, the extracted DNA was diluted into the same concentration of 100 ng/ µl in total volume of 100 µl.

4. Primers Design

One of the most important factors in successful DNA amplification and DNA sequencing is proper primer design (Kamel, 2003). Primers are short nucleotide sequences designed to bind to a desired segment of DNA target region. They must be able to anneal to the target DNA in a predictable location and on a predictable strand. They furthermore must be capable of extension by Taq DNA Polymerase (Rose *et al*., 2003). There are several properties that are to be carefully considered while designing primers as Rouchka *et al*. (2006) description as below:

1. Primer length: The optimal primer length of 18-24 bases long is long enough for adequate specificity and short enough for annealing temperature.

2. Melting temperature (Tm): Tm between 55-65°C works best in the most applications. Primers with low Tm and too high Tm are not desirable, because DNA polymerase will be less active at such temperature. The Tm of primers pair should be within 5°C of each other. The simplest formula of all for calculating Tm which is valid for oligos up to 18-24 bases long is: $Tm = 2 * (A + T) + 4 * (G + C)$.

3. GC content: The percentage of number of G and C of the total bases in primer should be 40-60 %. An optimal GC content enables specific binding and efficient melting temperature of the primer.

4. Self-Complementarity of primer sequences: A large number of complementary bases within the primer sequences allows them to self anneal to form stem loops and hairpin loops. The interaction of primers with each other at 3'-end produces primer dimmers. Also the runs of three or more C and G at the 3'-end of primers may promote mispriming at G or C-rich sequences. These mentioned cases should be avoided.

5. GC clamp: Presence of any GC nucleotide pair such as CC, GG, GC or CG at the 3' end of primer creates stable hybridization due to the stronger bonding of G and C bases. It also helps the efficiency of the reaction by minimizing any breathing that might occur.

The newly primer of cytochrome *b* gene and control region portion were designed in this study. In the first step, conserved regions both of forward and reverse directions were observed from the interest homologous nucleotide sequences alignment that using multiple sequences alignment tools such as ClustalW (http://align.genome.jp/). The sequences in FASTA format file was changed into *.seq and was launched into Oligo Primer Analysis Software. The pair of primers was picked up and presented in Table 4.

5. DNA Amplification

A PCR process follows in three steps, denaturation, annealing and extension. In The first step, double helix of DNA is separated into single strands by heating it at 94°C. In the second step, the temperature is lowered to proper Tm temperature (in commonly, should not be lesser than 50°C) to anneal with the complementary regions of the single stranded DNA molecules. And in the last step, Taq DNA polymerase attaches to the primers at 72°C and starts copying the strands. The reaction of DNA amplification in this study as below:

1. Cytochrome *b* gene (*cyt. b*)

The entire *cyt. b* gene (approximately 1,140 bp) was amplified using newly primers which were designed by Oligo Primer Analysis Software as Table 4. The total volume 50 µl of reaction mixture contain:

Taq DNA polymerase (Fermentas)	0.25	µl
10X KCl Taq buffer (Fermentas)	5	µl
2.5 mM $MgCl_2$ (Fermentas)	4	µl
2.0 mM dNTP mixture (Fermentas)	4	µl
Each of 5 pmol/µl CCyt1 and CCyt2 primers	2.5	µl
Sterile deionized water	27.75	µl
100 ng/µl DNA template solution	4	µl

Reaction mixtures were subjected to the thermal cycling consists of initial denaturation step at 94°C for 4 min, 35 cycles of denaturation step at (94°C, 20s), annealing step at (50°C, 30s) and extension step at (72°C, 1 min) and a final extension step of 5 min at 72°C.

2. Control Region (CR)

The entire portion of Control region (approximately 1,000 bp) was amplified using newly primer which was designed by Oligo Primer Analysis software as Table 4. The total volume 50 µl of reaction mixture contain:

Taq DNA polymerare (Fermentas)	0.25	µl
10X KCl Taq buffer (Fermentas)	5	µl
2.5 mM $MgCl_2$ (Fermentas)	4	µl
2.0 mM dNTP mixture (Fermentas)	4	µl

Each of 5 pmol/µl CerCR and CerCF primers	2.5	µl
Sterile deionized water	27.75	µl
100 ng/µl DNA template solution	4	µl

Reaction mixtures were subjected to the thermal cycling consists of initial denaturation step at 94°C for 4 min, 35 cycles of denaturation step at (94°C, 20s), annealing step at (52°C, 30s) and extension step at (72°C, 1 min) and a final extension step of 5 min at 72°C.

3. Intron1 of protein kinase C, iota gene (PRKCI)

Intron1 of PRKCI gene (approximately 600 bp) was amplified using a suite of known primer according to Ropiquet and Hassanin (2005) as Table 4. The total volume 15 µl of reaction mixture contain:

Taq DNA polymerare (Fermentas)	0.075	µl
10X KCl Taq buffer (Fermentas)	1.5	µl
2.5 mn $MgCl_2$ (Fermentas)	1.5	µl
2.0 mn dNTP mixture (Fermentas)	1.2	µl
Each of 5 pmol/µl U26 and L748 primers	0.75	µl
Sterile deionized water	7.225	µl
100 ng/µl DNA template solution	2	µl

Amplification were done on the thermal cycling consists of initial denaturation step at 94°C for 4 min, 35 cycles of denaturation step at (94°C, 20s), annealing step at (53°C, 30s) and extension step at (72°C, 1 min) and a final extension step of 5 min at 72°C.

Table 4 Primer pairs used for amplification and sequencing.

Marker	Primer name	Direction	Sequence (5′-3′)	Tm (°C)	Source
cyt. b	CCyt1	F	CAACCAAC (CT) CCACCACTCACAA	53.5	This study
	CCyt2	R	ATATTAATAAGCGTCAGGGAATAG	55.2	This study
CR	CerCF	F	ATACCAATCACCAGCACAATCGA	55.6	This study
	CerCR	R	GTGCTTGATACCAGCTCCTCT	51.3	This study
PRKCI (Intron1)	U26	F	TATGCTAAAGTACTGTTGGT	54.25	Ropiquet and Hassanin (2005)
	L748	R	CTGTACCCAGTCAATATCCT	58.35	Ropiquet and Hassanin (2005)

6. Agarose Gel Electrophoresis

Amplification products were tested and checked the size fragment by electrophoresis on 1% agarose gel, comparing with GeneRuler 1 kb DNA Ladder (Appendix Figure 3). Agarose gel in 1X TAE (Tris-Acetate EDTA) buffer was prepared by adding 1 g of agarose powder to 100 ml of 1X TAE. The mixture was heated in microwave until the agarose completely dissolves 1X TAE was added to evaporated mixture until to 100 ml. The warm agarose solution was cooled to 50-60°C and then poured into the mold which had a comb placed above it. The gel should be about 5 mm thick and air bubbles were not allowed to be under all of gel sheet. The gel was allowed to be set at room temperature for 30 min. Then, the comp was carefully removed form the solidified gel and ready for samples loading.

The gel was placed into the electrophoresis tank, with 1X TAE buffer adding to cover the gel until to tank level scale. The 2 µl of amplification products were mixed with 4 µl of 1X loading buffer. Then, these mixture solution and GeneRuler 1 kb DNA Ladder were pipetted into the slots of the submerged gel before closing the lid of the gel chamber. After that, the electrical leads were attached to the electrophoresis tank, so that, the DNA will move toward the anode, with the voltage of 100 volts for 30 min. The gel was run until the gel loading buffer has migrated the appropriate distance through the gel. After switched off the voltage, the gel was stained with 0.5 µg/ml ethidium bromide for 15 min and then examined under ultraviolet light of UV gel documentation to visualize DNA bands and to print the photograph. The resultful DNA band size was estimated by correct band of DNA marker.

7. DNA Purification and Sequencing

After checking of result DNA band by agarose gel electrophoresis, resultful amplification products were purified. 50 µl of *cyt. b* gene and control region products were purified by QIAquick PCR Purification kit (Qiagen) according to the supplier's protocol. 18 µl of purified DNA were sent to MACROGEN Inc., Korea and also KU-Vector, Kasetsart University for DNA sequencing.

An alternative means for DNA purification could be done by USB purification kit. 10-14 µl of intron1 of PRKCI products were purified by this means 1 µl of USB was added to 15 µl of intron1 of PRKCI products. The mixtures should be left at room temperature for 30 min before placing them into the thermal cycler for PCR. The standard condition used for DNA purification consists of first step at (37°C, 20 min) and second step at (80°C, 15 min). Whole purified DNA was sent to KU-Vector, Kasetsart University for DNA sequencing.

8. DNA Sequences Assembly

All sequences outputs were compared with GenBank database by BLASTn program (http://blast.ncbi.nlm.nih.gov/blast.cgi) for making sure those are correct sequences. Using Blastn, closely related to *cyt. b*, control region or intron1 of PRKCI sequences from public database were retrieved and added to the alignment. After checking for correct sequences, each base character of all sequences were edited by eye on Chromas program Version 2.33 and then edited sequences from each primers were assembled together for each one complete sequence by using CAP3 program (http://pbil.univ-lyon1.fr/cap3.php). Each complete sequence were aligned using the software (ClustalX Version 1.82 (Thompson *et al.*, 1997), followed by visual inspection. The excess from each target sequences must be removed and gaps were treated as a fifth character state in the phylogenetic analysis.

9. Phylogenetic Analysis

Phylogenetic analysis were carried out for four types of them to investigate evolutionary relationships as following: (1) neighbor-joining (NJ) and (2) maximum parsimony (MP) using PAUP* Version 4.010b (Swofford, 2002).

For the NJ tree, distances were calculated applying Tamura and Nei's (1993) method using an α-parameter of gamma distribution. Non-parametric bootstrap analyses with 1,000 replicates were performed to obtain estimates of support for each node of the NJ trees.

For MP analyses, the informative sites were analyzed using equally weighted characters and were searched by heuristic option with a stepwise starting tree, a random stepwise addition of 1,000 replicates, tree-bisection-reconnection (TBR), and steepest descent branch-swapping algorithm. Gaps were treated as missing data. If this heuristic search option yielded more than one of most parsimonious trees, they were joined in one tree of the semi-strict consensus tree, which was the tree show percent congruent proportion among all MP trees. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates. This algorithm was used for the data set that has more than twenty samples taxa.

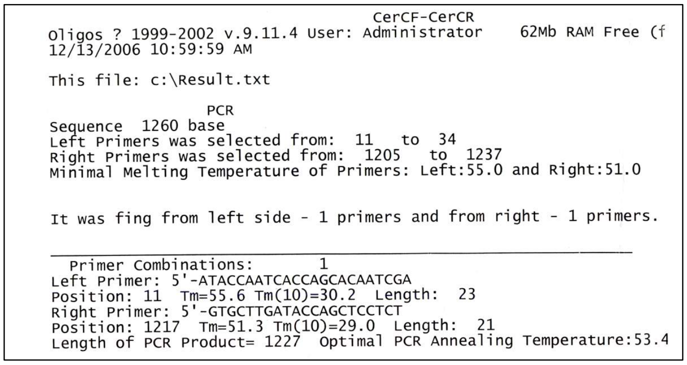
For the data set which was lesser than twenty samples taxa, the MP trees were searched by branch-and-bound search option and the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

RESULTS AND DISCUSSION

Results

1. A Newly Constructed Primer Pairs for the Mitochondrial DNA Amplification

New primers for mitochondrial DNA amplification included cytochrome *b* gene and control region (D-loop) portion were designed in this study. Each pairs of primer design intend to cover more than 1,000 base sites from forward and reverse directions of the DNA target. In the first step, the conserve sequences from the multiple sequences alignment using tool as ClustalW 1.81 (http://align.genome.jp/) were observed. The six mitochondrion complete genome sequences in the accession number NC_007704.1 (*Cervus elaphus*), EF058308.1 (*C. nippon taiouanus*), EF035448.1 (*C. unicolor swinhoei*), NC_006973.1 (*C. nippon yesoensis*), NC_004563.1 (*Muntiacus muntjak*) and NC_008491.1 (*M. reevesi*) were chosen from GenBank and were conducted for this alignment. In the second step, cytochrome *b* gene and control region portion as the DNA target were positioned in this alignment and the primers region in the range of 200 base sites from forward and reverse directions were inspected. Then, all sequences were cut, remaining only all interest nucleotides which covered the complete sequence of DNA target. Sequences were recognized by Oligo program if data file was saved as *.seq. In the last step, *. seq file was launched into Oligo Primer Analysis Software Version 7.0 for primer design. The example of outputs of this program as shown in Figure 17.



```
                                          CerCF-CerCR
Oligos ? 1999-2002 v.9.11.4 User: Administrator    62Mb RAM Free (f
12/13/2006 10:59:59 AM

This file: c:\Result.txt

                  PCR
Sequence  1260 base
Left Primers was selected from:  11   to  34
Right Primers was selected from:  1205   to  1237
Minimal Melting Temperature of Primers: Left:55.0 and Right:51.0


It was fing from left side - 1 primers and from right - 1 primers.

_______________________________________________________________
  Primer Combinations:       1
Left Primer: 5'-ATACCAATCACCAGCACAATCGA
Position: 11  Tm=55.6 Tm(10)=30.2  Length:  23
Right Primer: 5'-GTGCTTGATACCAGCTCCTCT
Position: 1217  Tm=51.3 Tm(10)=29.0  Length:  21
Length of PCR Product= 1227  Optimal PCR Annealing Temperature:53.4
```

Figure 17 Output from Oligo Primer Analysis Software

From Oligo primer designing software, the program produced the new primer for cytochrome *b* gene and control region amplification in the description as below:

1. Cytochrome *b* gene

In mammals, the mitochondrial cytochrome *b* gene generally spans about 1,140 bp. This gene is located between tRNA^{Glu} of $5'$ end light (L)-strand and tRNA^{Thr} of $3'$ end heavy (H)-strand. The pair of new primers consists of:

Left primer; CCyt1 (5’ – CAACCAAC (CT) CCACCACTACAA – 3’)
Right primer; CCyt2 (5’ – ATATTAATAAGCGTCAGGGAATAG – 3’)

The target DNA sequences from these primers pair is approximately 1,440 bp long. It includes partial sequence of NADH dehydrogenase subunit 6 (ND6), complete sequence of tRNA^{Glu} gene, complete sequence of cytochrome *b* gene, complete sequence of tRNA^{Thr} gene and partial sequence of tRNA^{Pro} gene.

2. Control region (D-loop)

The non-coding control region of mitochondrial DNA is highly variable in mammals. The control region is divided into three domains of the central conserved region which flanked by the left and the right domains. Both of the left and the right domains tend to be variable of tandem repeats (VNTRs) (Clayton, 1992). In the Cervidae, Cervus nippon was found a repeat in the left domain with more than 1,200 bp long (Douzery and Randi, 1997). Variability of base amount on this portion was found in deer samples sequences using in this study from approximately 900 bp up to more than 1,000 bp long depending on the insertion of tandem repeats. This non-coding portion is located between tRNA^{Pro} of $5'$ end light (L)-strand and tRNA^{Phe} of $3'$ end heavy (H)-strand. The pair of new primers consists of:

Left primer; CerCF (5’ – ATACCAATCACCAGCACAATCGA – 3’)
Right primer; CerCR (5’ – GTGCTTGATACCAGCTCCTCT – 3’)

The target DNA sequences from these primers pair is approximately 1,260 bp long. It includes partial sequence of cytochrome *b*, complete sequence of tRNA^{Thr} gene, complete sequence of tRNA^{Pro} gene, complete sequence of control region, complete sequence of tRNA^{Phe} gene and partial sequence of 12S ribosomal RNA gene.

After DNA sequences assembly in each DNA target portion (cytochrome *b* gene and control region portion) were finished. Each sequences portion from the same sample was assembled together using CAP3 program (http://pbil.univ-lyon1.fr/cap3.php). Each one of assemblage sequence is approximately 2,600 bp long which mainly includes complete sequences of cytochrome *b* and control region, additionally with several of tRNA genes. The position of each primer and DNA target are presented as Figure 18.

```
CLUSTAL W (1.81) Multiple Sequence Alignments

Sequence 1: NC_004563.1_Muntiacus_muntjak      2733 bp
Sequence 2: NC_008491.1_Muntiacus_reevesi      2730 bp
Sequence 3: NC_006973.1_Cervus_nippon_yeso     2920 bp
Sequence 4: EF058308.1_Cervus_nippon_taiou     2806 bp
Sequence 5: EF035448.1_Cervus_unicolor_swi     2854 bp
Sequence 6: NC_007704.1_Cervus_elaphus         2729 bp
Start of Pairwise alignments aligning...

NC_006973.1_Cervus_nippon_yeso      CCACCATCATTCCTCCCAAATAAATTAAGAATACCATTAAACCCAGAAAG
EF058308.1_Cervus_nippon_taiou      CCACCATCATTCCTCCCAAATAAATTAAGAATACCATTAAACCCAGAAAG
NC_007704.1_Cervus_elaphus          CCACCATCATCCCTCCTAAATAAATTAAGAACACTATTAAACCCAGAAAG
EF035448.1_Cervus_unicolor_swi      CCACTATCATCCCTCCTAAATAAATTAAGAATACCATTAAACCCAGAAAG
NC_004563.1_Muntiacus_muntjak       CCACCATCATTCCCCCCAAGTAAATCAAAAACACCATCAAACCTAAAAAA
NC_008491.1_Muntiacus_reevesi       CCACTATCATTCCCCCCAAATAAATTAAAAACACTATTAAACCTAAAAAA
                                    **** ***** ** ** ** ***** ** ** ** ** ***** * ***
                                                                     CCyt 1
NC_006973.1_Cervus_nippon_yeso      GACCCACCAAAATTTAACACAATACCACAACCAACTCCACCACTCACAAT
EF058308.1_Cervus_nippon_taiou      GACCCGCCAAAATTTAACACAATACCACAACCAACTCCACCACTCACAAT
NC_007704.1_Cervus_elaphus          GACCCACCAAAATTTAACACAATACCACAACCAACTCCACCACTCACAAT
EF035448.1_Cervus_unicolor_swi      GATCCACCAAAATTTAACACAATACCACAACCAACTCCACCACTCACAAT
NC_004563.1_Muntiacus_muntjak       GACCCACCAAAATTTAATACAATACCACAACCAACCCCACCACTCACAAT
NC_008491.1_Muntiacus_reevesi       GACCCACCAAAATTTAATACAATACCACAACCAACTCCACCACTCACAAT
                                    ** ** *********** ***************** **************

NC_006973.1_Cervus_nippon_yeso      TAATCCCAACCCCCCATAAATAGGCGAAGGTTTCGAAGAAAACCCTACAA
EF058308.1_Cervus_nippon_taiou      TAATCCTAACCCCCCATAAATAGGCGAAGGTTTCGAAGAAAATCCTACAA
NC_007704.1_Cervus_elaphus          TAATCCCAACCCCCCGTAAATAGGCGAAGGTTTCGAAGAAAATCCCACAA
EF035448.1_Cervus_unicolor_swi      TAATCCCAACCCCCCATAAATAGGCGAAGGTTTCGAAGAAAATCCTACAA
NC_004563.1_Muntiacus_muntjak       TAAACCCAACCCCCCATAAATAGGCGAAGGTTTTGAAGAAAATCCCACAA
NC_008491.1_Muntiacus_reevesi       TAAACCCAACCCCCCATAAATAGGCGAAGGTTTTGAAGAAAATCCTACAA
                                    *** ** ******** ***************** ******** ** ****
```

Figure 18 The position of newly constructed primers of CCyt1, CCyt2, CerCF and CerCR and location of DNA target

```
                                                        3’ ND6 <          > 5’tRNA-Glu
NC_006973.1_Cervus_nippon_yeso      AACCAAGCACAAAAATAATACTTAAGATAAATACAATGTATGTTATCATT
EF058308.1_Cervus_nippon_taiou      AACCAAGCACAAAAATAATACTTAAGATAAATACAATGTATGTTATCATT
NC_007704.1_Cervus_elaphus          ACCCAAGCACAAAAATGATACTTAAGATAAATACAATGTATGTTATCATT
EF035448.1_Cervus_unicolor_swi      AACCAAGCACAAATATGATACTTAAGATAAATACAATGTATGTTATCATT
NC_004563.1_Muntiacus_muntjak       AACCAAGCACAAAAATGATACTTAAAATAAATACAATGTATGTTATCATT
NC_008491.1_Muntiacus_reevesi       AACCAAGCACAAAAATGATACTTAAAATAAATACAATGTATGTTATCATT
                                    * *********** ** ******** ************************

NC_006973.1_Cervus_nippon_yeso      ATTCTCACATGGAATCTAACCATGACTAATGATATGAAAAATCATCGTTG
EF058308.1_Cervus_nippon_taiou      ATTCTCACATGGAATCTAACCATGACTAATGATATGAAAAACCATCGTTG
NC_007704.1_Cervus_elaphus          ATTCTCACATGGAATCTAACCATGACTAATGATATGAAAAACCATCGTTG
EF035448.1_Cervus_unicolor_swi      ATTCTCACATGGAATCTAACCATGACTAATGATATGAAAAACCATCGTTG
NC_004563.1_Muntiacus_muntjak       ATTCTCGCATGGAATCTAACCACGACTAATGATATGAAAAACCATCGTTG
NC_008491.1_Muntiacus_reevesi       ATTCTCACATGGAATCTAACCACGACTAATGATATGAAAAACCATCGTTG
                                    ****** *************** ****************** ********
                                             3’tRNA-Glu <> 5’  Cyt.b
NC_006973.1_Cervus_nippon_yeso      TCATTCAACTACAAGAACACTAATGACTAATATCCGAAAAACCCACCCAT
EF058308.1_Cervus_nippon_taiou      TCATTCAACTACAAGAACACTAATGACCAATATCCGAAAAACCCACCCGC
NC_007704.1_Cervus_elaphus          TCATTCAACTACAAGAACACTAATGACCAATATCCGAAAAACCCACCCAC
EF035448.1_Cervus_unicolor_swi      TCATTCAACTACAAGAACACTAATGACCAATATCCGAAAAACCCACCCAT
NC_004563.1_Muntiacus_muntjak       TCATTCAACTACAAGAACACCAATGACAAATATTCGAAAAACCCACCCAC
NC_008491.1_Muntiacus_reevesi       TCATTCAACTACAAGAACACCAATGACAAATATCCGAAAAACCCACCCAC
                                    ******************** ****** ***** **************


NC_006973.1_Cervus_nippon_yeso      TAATAAAAATTGTAAACAACGCATTCATTGACCTCCCCGCCCCATCAAAT
EF058308.1_Cervus_nippon_taiou      TAATAAAAATTGTAAACAACGCATTTATTGACCTCCCCACCCCATCAAAT
NC_007704.1_Cervus_elaphus          TAATAAAAATTGTAAACAACGCATTTATTGACCTCCCAGCCCCATCAAAT
EF035448.1_Cervus_unicolor_swi      TAATAAAGATTGTAAACAACGCATTCATTGACCTCCCAGCCCCATCAAAT
NC_004563.1_Muntiacus_muntjak       TAATAAAAATTGTAAACAACGCATTCATTGACCTCCCAGCCCCATCAAAC
NC_008491.1_Muntiacus_reevesi       TAATAAAAATTGTAAACAACGCATTTATTGACCTCCCAGCCCCGTCAAAT
                                    ******* ***************** ***********  **** *****


NC_006973.1_Cervus_nippon_yeso      ATTTCATCCTGATGAAATTTCGGCTCCCTACTAGGAATTTGTCTAATCCT
EF058308.1_Cervus_nippon_taiou      ATTTCATCCTGATGAAATTTCGGCTCCTTACTAGGAATTTGTCTAATCCT
NC_007704.1_Cervus_elaphus          ATTTCATCCTGATGAAATTTCGGCTCATTACTAGGAGTCTGTCTAATCCT
EF035448.1_Cervus_unicolor_swi      ATTTCATCCTGATGAAATTTCGGCTCCTTACTAGGAATTTGTCTAATCCT
NC_004563.1_Muntiacus_muntjak       ATTTCATCTTGATGAAACTTTGGCTCCCTACTAGGAATTTGCTTAATTCT
NC_008491.1_Muntiacus_reevesi       ATTTCATCTTGATGAAACTTTGGCTCCCTACTAGGAATTTGCTTAATTCT
                                    ******** ******** ** *****  ******** * **  **** **


NC_006973.1_Cervus_nippon_yeso      ACAAATCCTTACAGGCCTATTCCTAGCAATACACTATACATCTGACACAA
EF058308.1_Cervus_nippon_taiou      ACAAATTCTCACAGGCCTATTCCTAGCAATACACTATACATCCGACACAA
NC_007704.1_Cervus_elaphus          ACAAATCCTCACAGGCCTATTCCTAGCGATACACTATACATCTGATACAA
EF035448.1_Cervus_unicolor_swi      ACAAATCATCACAGGCCTATTCCTAGCAATACACTATACATCCGATACAA
NC_004563.1_Muntiacus_muntjak       ACAAATCCTCACAGGCCTATTCCTAGCAATACACTACACATCCGACACAA
NC_008491.1_Muntiacus_reevesi       ACAAATCCTCACAGGCCTATTTCTAGCAATACACTATACATCCGATACAA
                                    ******  * *********** ***** ******** ***** ** ****


NC_006973.1_Cervus_nippon_yeso      TAACAGCATTTTCCTCTGTCACCCATATCTGTCGAGATGTCAACTATGGT
EF058308.1_Cervus_nippon_taiou      TAACAGCATTCTCCTCTGTCACCCATATCTGTCGAGATGTCAATTATGGT
NC_007704.1_Cervus_elaphus          TAACAGCATTCTCCTCTGTCACCCATATCTGTCGAGATGTCAATTATGGC
EF035448.1_Cervus_unicolor_swi      TAACAGCATTTTCCTCTGTTACCCATATCTGCCGAGATGTCAATTATGGC
NC_004563.1_Muntiacus_muntjak       TAACAGCATTCTCCTCAGTTACCCATATCTGCCGAGACGTCAACTATGGC
NC_008491.1_Muntiacus_reevesi       TAACAGCATTCTCCTCGGTTACCCATATCTGCCGAGACGTCAACTATGGC
                                    ********** ***** ** *********** ***** ***** *****


NC_006973.1_Cervus_nippon_yeso      TGAATTATCCGATACATACACGCAAACGGGGCATCAATATTTTTCATCTG
EF058308.1_Cervus_nippon_taiou      TGAATTATTCGATACATACACGCAAACGGGGCGTCAATATTCTTCATCTG
NC_007704.1_Cervus_elaphus          TGAATTATTCGATATATACACGCAAACGGGGCATCAATATTTTTCATCTG
EF035448.1_Cervus_unicolor_swi      TGAATCATTCGATATATACACGCAAACGGGGCATCAATATTTTTCATCTG
NC_004563.1_Muntiacus_muntjak       TGAATTATCCGATATATACACGCGAACGGAGCATCAATATTTTTTATTTG
NC_008491.1_Muntiacus_reevesi       TGAATTATCCGATATATACACGCAAACGGAGCATCTATATTTTTTATTTG
                                    ***** ** ***** ******** ***** ** ** ***** ** ** **
```

Figure 18 (Continued)

```
NC_006973.1_Cervus_nippon_yeso      CCTATTCATACATGTAGGACGAGGCCTGTACTACGGATCATATACTTTTC
EF058308.1_Cervus_nippon_taiou      CCTATTCATACATGTAGGACGAGGCCTGTACTACGGATCATATACTTTTC
NC_007704.1_Cervus_elaphus          TCTATTCATACATGTAGGGCGAGGCCTGTACTACGGATCATATACTTTTC
EF035448.1_Cervus_unicolor_swi      TCTATTTATACACGTAGGACGAGGCCTGTACTACGGATCATACACCTTTC
NC_004563.1_Muntiacus_muntjak       CCTATTTATTCACGTAGGACGAGGCCTATACTATGGATCATATACCTTTC
NC_008491.1_Muntiacus_reevesi       CCTATTTATCCACGTAGGACGAGGCCTATACTATGGATCATACACCTTTC
                                     ***** ** ** ***** ******** ***** ******** ** ****

NC_006973.1_Cervus_nippon_yeso      TAGAGACATGAAACATCGGAGTAATTCTCCTATTTACAGTTATAGCCACA
EF058308.1_Cervus_nippon_taiou      TAGAGACATGAAACATCGGAGTAATCCTCCTATTTACAGTTATAGCCACA
NC_007704.1_Cervus_elaphus          TAGAGACGTGAAACATCGGAGTAGTTCTTCTATTTACAGTTATAGCCACA
EF035448.1_Cervus_unicolor_swi      TAGAGACATGAAACATCGGAGTAATCCTCCTATTTACAGTTATAGCCACA
NC_004563.1_Muntiacus_muntjak       TAGAAACATGAAATATTGGAGTAATCCTCCTATTTACAGTTATAGCCACA
NC_008491.1_Muntiacus_reevesi       TAGAAACATGAAATATTGGAGTAATTCTCCTATTTACAGTTATAGCCACA
                                    **** ** ***** ** ****** * ** *********************

NC_006973.1_Cervus_nippon_yeso      GCATTCGTAGGATATGTCCTACCATGAGGACAAATATCATTCTGAGGAGC
EF058308.1_Cervus_nippon_taiou      GCATTCGTAGGATATGTCCTACCATGAGGACAAATATCATTCTGAGGAGC
NC_007704.1_Cervus_elaphus          GCATTCGTAGGATATGTCCTACCATGAGGACAAATATCATTCTGAGGAGC
EF035448.1_Cervus_unicolor_swi      GCATTCGTAGGGTATGTTCTACCATGAGGACAAATATCATTCTGAGGAGC
NC_004563.1_Muntiacus_muntjak       GCATTCGTAGGTTATGTTCTACCATGAGGACAAATATCATTTTGAGGAGC
NC_008491.1_Muntiacus_reevesi       GCATTCGTAGGATATGTTCTACCATGAGGACAAATATCATTTTGAGGGGC
                                    *********** ***** *********************** ***** **

NC_006973.1_Cervus_nippon_yeso      AACAGTCATTACCAACCTCCTCTCAGCAATTCCATATATTGGCACAAACC
EF058308.1_Cervus_nippon_taiou      AACAGTCATCACCAACCTCCTCTCAGCAATTCCATACATTGGCACAAACC
NC_007704.1_Cervus_elaphus          AACAGTCATCACCAACCTTCTCTCAGCAATTCCATATATTGGGACAAACC
EF035448.1_Cervus_unicolor_swi      AACAGTCATTACCAACCTTCTCTCAGCAATTCCATATATTGGTACAAACC
NC_004563.1_Muntiacus_muntjak       AACAGTTATCACTAATCTCCTTTCAGCAATTCCATATATTGGCACAAACT
NC_008491.1_Muntiacus_reevesi       AACAGTTATTACTAACCTCCTTTCAGCAATCCCATATATTGGCACAAATT
                                    ****** ** ** ** ** ** ******** ***** ***** *****

NC_006973.1_Cervus_nippon_yeso      TAGTCGAATGGATCTGAGGGGGCTTCTCAGTAGATAAAGCAACCCTAACC
EF058308.1_Cervus_nippon_taiou      TAGTCGAATGAATCTGAGGGGGCTTTTCAGTAGATAAAGCAACCCTAACC
NC_007704.1_Cervus_elaphus          TAGTCGAATGGATCTGAGGAGGCTTTTCAGTAGACAAAGCAACCCTAACC
EF035448.1_Cervus_unicolor_swi      TAGTCGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCCTAACC
NC_004563.1_Muntiacus_muntjak       TAGTTGAATGAATCTGGGGAGGCTTTTCAGTAGATAAAGCGACCCTGACC
NC_008491.1_Muntiacus_reevesi       TGGTCGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCAACCCTAACC
                                    * ** ***** ***** ** ***** ******** ***** ***** ***

NC_006973.1_Cervus_nippon_yeso      CGATTTTTCGCTTTCCACTTTATTCTTCCATTTATCATCGCAGCACTTGC
EF058308.1_Cervus_nippon_taiou      CGATTTTTCGCTTTCCACTTTATTTTTCCATTCATCATCGCAGCACTCGC
NC_007704.1_Cervus_elaphus          CGATTTTTCGCTTTCCACTTTATTCTCCCATTTATCATCGCAGCACTCGC
EF035448.1_Cervus_unicolor_swi      CGATTTTTTGCTTTCCACTTTATTCTCCCATTTATCATCGCGGCACTCGC
NC_004563.1_Muntiacus_muntjak       CGATTCTTCGCCTTCCATTTTATTCTCCCATTTATTATCGCAGCACTCGC
NC_008491.1_Muntiacus_reevesi       CGATTCTTCGCCTTCCACTTTATTCTCCCATTTATTATTGCAGCACTTGC
                                    ***** ** ** ***** ****** * ***** ** ** ** ***** **

NC_006973.1_Cervus_nippon_yeso      TATAGTACACTTACTCTTCCTTCACGAGACAGGATCCAACAACCCAACAG
EF058308.1_Cervus_nippon_taiou      TATAGTACATTTACTCTTCCTTCACGAGACAGGATCCAACAACCCAACAG
NC_007704.1_Cervus_elaphus          TATAGTACACTTACTCTTCCTTCACGAAACAGGATCTAATAACCCAACAG
EF035448.1_Cervus_unicolor_swi      TATAGTACATTTACTCTTCCTTCACGAGACAGGATCCAATAACCCAACAG
NC_004563.1_Muntiacus_muntjak       TATAGTCCACCTGCTCTTTCTCCACGAAACAGGATCCAACAACCCAACAG
NC_008491.1_Muntiacus_reevesi       TATAGTCCACCTACTTTTCCTCCACGAAACAGGATCCAACAATCCAACAG
                                    ****** **  * ** ** ** ***** ******** ** ** *******

NC_006973.1_Cervus_nippon_yeso      GAATCCCATCGGACGCAGACAAAATCCCCTTCCATCCTTACTACACCATT
EF058308.1_Cervus_nippon_taiou      GAATCCCATCGGACGCAGACAAAATCCCCTTCCACCCTTACTATACTATC
NC_007704.1_Cervus_elaphus          GAATCCCATCAGACGCAGACAAAATCCCCTTTCATCCTTATTATACCATT
EF035448.1_Cervus_unicolor_swi      GAATCCCATCAGACGCAGATAAAATTCCTTTCCATCCTTACTATACCATT
NC_004563.1_Muntiacus_muntjak       GAATCCCATCAGATGCAGATAAGATTCCCTTCCACCCCTACTACACCATC
NC_008491.1_Muntiacus_reevesi       GAATCCCATCAGATGCAGACAAAATCCCTTTCCACCCCTACTATACCATC
                                    ********** ** ***** ** ** ** ** ** ** ** ** ** **

NC_006973.1_Cervus_nippon_yeso      AAAGATATCTTAGGCATCTTACTTCTAGTACTCTTCCTAATATTACTAGT
EF058308.1_Cervus_nippon_taiou      AAAGATATCTTAGGCATCTTACTTCTAATACTCTTCCTAATATTACTAGT
NC_007704.1_Cervus_elaphus          AAAGATATCTTAGGCATCTTACTTCTTGTACTCTTCTTAATATTACTAGT
EF035448.1_Cervus_unicolor_swi      AAAGATATCTTAGGCATCTTACTTATAGTACTCTTCTTAATATTACTAGT
NC_004563.1_Muntiacus_muntjak       AAAGATATTCTAGGTGTCCTACTTCTAATTCTCTTCCTAATATCACTAGT
NC_008491.1_Muntiacus_reevesi       AAAGATATTCTGGGTGCCTTACTTCTAACTCTCTCTCTAATATTACTAGT
                                    ********  * **   * ***** *    ****   ****** ******
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Figure 18 (Continued)

```
NC_006973.1_Cervus_nippon_yeso      ATTATTCGCACCAGACCTGCTTGGAGATCCAGACAACTATACCCCAGCAA
EF058308.1_Cervus_nippon_taiou      ATTATTCGCACCAGACCTGCTTGGAGATCCAGACAACTATACCCCAGCAA
NC_007704.1_Cervus_elaphus          ATTATTCGCACCAGACCTACTTGGAGATCCAGATAACTACACCCCAGCAA
EF035448.1_Cervus_unicolor_swi      ATTATTCGCACCGGACCTGCTTGGAGACCCAGACAACTACACCCCAGCAA
NC_004563.1_Muntiacus_muntjak       ATTATTCGTACCAGACCTGCTTGGAGACCCCGACAACTACACCCCAGCAA
NC_008491.1_Muntiacus_reevesi       ATTATTCGCACCAGACCTGCTAGGAGACCCCGACAACTATACCCCAGCAA
                                    ******** *** ***** ** ***** ** ** ***** **********

NC_006973.1_Cervus_nippon_yeso      ATCCACTCAACACACCCCCTCACATCAAACCTGAATGATACTTCCTATTT
EF058308.1_Cervus_nippon_taiou      ATCCACTCAGCACACCCCCTCACATCAAACCCGAATGATACTTCCTATTT
NC_007704.1_Cervus_elaphus          ACCCACTCAACACACCCCCTCATATTAAACCTGAATGATATTTCCTATTT
EF035448.1_Cervus_unicolor_swi      ATCCACTCAACACACCCCCTCATATCAAACCTGAATGGTATTTCCTATTC
NC_004563.1_Muntiacus_muntjak       ACCCACTCAATACACCTCCCCATATTAAGCCCGAATGATATTTCCTATTT
NC_008491.1_Muntiacus_reevesi       ATCCACTCAATACACCTCCCCATATTAAACCCGAATGATATTTCCTATTT
                                    * *******  ***** ** ** ** ** ** ***** ** ********

NC_006973.1_Cervus_nippon_yeso      GCATACGCAATCCTACGATCAATTCCCAACAAACTAGGAGGAGTCTTAGC
EF058308.1_Cervus_nippon_taiou      GCATACGCAATCCTACGATCAATTCCCAACAAACTAGGAGGAGTCTTAGC
NC_007704.1_Cervus_elaphus          GCATACGCAATCCTACGATCAATTCCCAACAAACTAGGAGGAGTCTTAGC
EF035448.1_Cervus_unicolor_swi      GCATACGCAATCCTACGATCAATTCCCAACAAACTAGGAGGAGTCCTAGC
NC_004563.1_Muntiacus_muntjak       GCATACGCAATTCTACGATCAATTCCTAATAAACTAGGAGGAGTCCTAGC
NC_008491.1_Muntiacus_reevesi       GCATATGCAATTCTACGATCAATCCCCAATAAACTAGGAGGAGTTCTAGC
                                    ***** ***** *********** ** ** **************  ****

NC_006973.1_Cervus_nippon_yeso      CCTAGTCTCATCCATCCTAATCTTGATTCTCATGCCTCTTCTTCACACGT
EF058308.1_Cervus_nippon_taiou      CCTAGTCTCATCTATCCTAATCTTGATTCTCATGCCTTTTCTTCACACGT
NC_007704.1_Cervus_elaphus          CCTAGTCTCATCCATCCTAGTCTTAATTCTCATGCCTCTTCTTCACACAT
EF035448.1_Cervus_unicolor_swi      CTTAGTCTCATCTATCCTAATCTTAATTTTCATGCCTCTTCTCCACACAT
NC_004563.1_Muntiacus_muntjak       CCTAATTTCATCCATCCTAATCCTAATCCTTATACCCCTTCTCCATACGT
NC_008491.1_Muntiacus_reevesi       TCTAATCTCATCCATCCTAGTCCTAATCCTCATACCCCTCCTTCATACAT
                                      ** * ***** ****** ** * **  * ** **  * ** ** ** *

NC_006973.1_Cervus_nippon_yeso      CCAAACAACGCAGCATGATATTCCGACCATTCAGCCAATGCCTATTCTGA
EF058308.1_Cervus_nippon_taiou      CCAAACAACGCAGCATGATATTCCGACCATTCAGCCAATGCCTATTCTGA
NC_007704.1_Cervus_elaphus          CCAAACAACGCAGCATGATATTCCGACCATTCAGTCAATGCCTATTCTGA
EF035448.1_Cervus_unicolor_swi      CCAAACAACGCAGCATGATATTCCGACCATTCAGCCAATGCCTATTCTGA
NC_004563.1_Muntiacus_muntjak       CCAAACAACGTAGCATAATTTTCCGACCACTCAGTCAATGCCTATTCTGA
NC_008491.1_Muntiacus_reevesi       CCAAACAACGCAGCATAATTTTTCGACCGCTCAGTCAATGCTTATTCTGA
                                    ********** ***** ** ** *****  **** ****** ********

NC_006973.1_Cervus_nippon_yeso      ATCTTAGTAGCAGACCTACTAACACTCACATGAATTGGAGGACAACCAGT
EF058308.1_Cervus_nippon_taiou      ATCTTAGTAGCAGACCTACTAACACTCACATGAATTGGAGGACAACCAGT
NC_007704.1_Cervus_elaphus          ATCTTAGTAGCAGATCTACTAACACTTACATGAATCGGAGGACAACCAGT
EF035448.1_Cervus_unicolor_swi      ATCTTAGTAGCAGACCTACTAACACTCACATGAATCGGAGGACAACCAGT
NC_004563.1_Muntiacus_muntjak       ATCCTAGTAGCAGATCTGCTAACACTTACATGAATTGGAGGACAACCAGT
NC_008491.1_Muntiacus_reevesi       ATTCTAGTAGCAGACCTGCTAACACTCACATGAATTGGAGGACAGCCAGT
                                    **  ********** ** ******** ******** ******** *****

NC_006973.1_Cervus_nippon_yeso      TGAATATCCCTTTATTATTATTGGACAACTAGCATCTGTTCTATACTTTT
EF058308.1_Cervus_nippon_taiou      TGAGTACCCTTTTATTATTATTGGACAACTAGCATCTATCCTATACTTTT
NC_007704.1_Cervus_elaphus          CGAATACCCCTTTATCATTATTGGACAACTAGCATCTGTCTTATATTTCT
EF035448.1_Cervus_unicolor_swi      TGAATACCCCTTTATTATTATTGGACAACTAGCATCTATCTTATACTTTT
NC_004563.1_Muntiacus_muntjak       TGAGCACCCCTTCATCATTATTGGACAACTAGCATCCATTCTATATTTCC
NC_008491.1_Muntiacus_reevesi       TGAACACCCCTTCATTATTATTGGGCAACTAGCATCCATTTTATATTTCC
                                     **  * ** ** ** ******** ***********  *  **** **

                                                         CerCF
NC_006973.1_Cervus_nippon_yeso      TCATTATCCTAGTCCTTATACCGATTACCAGCACAATCGAAAATAACCTC
EF058308.1_Cervus_nippon_taiou      TCATTATTCTGGTCCTTATACCGATCACCAGCACAATCGAAAATAACCTC
NC_007704.1_Cervus_elaphus          TCATTATCCTAGTCCTCATACCAATCACCAGCACAATCGAAAACAACCTC
EF035448.1_Cervus_unicolor_swi      TCATTATCCTGGTCCTTATACCAATCACCAGCACAATCGAAAACAACCTC
NC_004563.1_Muntiacus_muntjak       TCATTATTCTTGTACTTATGCCAATCATCAGTACAATCGAAAATAACCTC
NC_008491.1_Muntiacus_reevesi       TCATTATTCTTGTACTTATACCAATCATCAGCACAATTGAAAATAATCTC
                                    ******* ** ** ** ** ** ** * *** ***** ***** ** ***
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Figure 18 (Continued)

```
                                 3′ Cyt.b <    > 5′tRNA-Thr
NC_006973.1_Cervus_nippon_yeso      CTAAAATGAAGACAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAA
EF058308.1_Cervus_nippon_taiou      CTAAAATGAAGATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAA
NC_007704.1_Cervus_elaphus          CTAAAATGAAGATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAA
EF035448.1_Cervus_unicolor_swi      CTAAAATGAAGACAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAA
NC_004563.1_Muntiacus_muntjak       CTAAAATGAAGACAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAA
NC_008491.1_Muntiacus_reevesi       CTAAAATGAAGATAAGTCTTTGTAGTACATTTAATACACTGGTCTTGTAA
                                    ************ ************** * * ******************
                                                     3′tRNA-Thr <> 5′tRNA-Pro
NC_006973.1_Cervus_nippon_yeso      ACCAGAAAAGGAGAACTACCAACCTCCCCAAGACTCAAGGAAGAAGCCAT
EF058308.1_Cervus_nippon_taiou      ACCAGAAAAGGAGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCAT
NC_007704.1_Cervus_elaphus          ACCAGAAAAGGAGAGCAACTAACCTCCCTAAGACTCAAGGAAGAAGCCAT
EF035448.1_Cervus_unicolor_swi      ACCAGAAAAGGAGAGCAACCAACCTCCCTAAGACTCAAGGAAGAAGCCAT
NC_004563.1_Muntiacus_muntjak       ACCAGAAAAGGAGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCAT
NC_008491.1_Muntiacus_reevesi       ACCAGAAAAGGAGGACAACCAATCTCCCTAAGACTCAAGGAAGAAGCCAC
                                    *************  * ** ** ***** ********************

                                                                               CCyt 2
                                                                         3′tRNA-Pro <
NC_006973.1_Cervus_nippon_yeso      AGCCCCACTATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
EF058308.1_Cervus_nippon_taiou      AGCCCCACTATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
NC_007704.1_Cervus_elaphus          AGCCCCACTATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
EF035448.1_Cervus_unicolor_swi      AGCCCCACTATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
NC_004563.1_Muntiacus_muntjak       AGCCCCACCATCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
NC_008491.1_Muntiacus_reevesi       AGCCCCACCGTCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTG
                                    ********  ****************************************

                                     > 5′ Control region (D-loop)
NC_006973.1_Cervus_nippon_yeso      ACGCTTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAA
EF058308.1_Cervus_nippon_taiou      ACGCTTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAA
NC_007704.1_Cervus_elaphus          ATGCTTATTAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAA
EF035448.1_Cervus_unicolor_swi      ATGCTTATTAATATAGTTCCATAAAAATCAAGAACCTTATCAGTATTAAA
NC_004563.1_Muntiacus_muntjak       ACGCATATTAATATAGCTCCATAAAAATCAAGAACCTTATCAGTATTAAA
NC_008491.1_Muntiacus_reevesi       ACGCATATTAATATAGCTTCACAAAAATCAAGAACCTTATTAGTATTAAA
                                    * ** *********** * ** ************* **** *********

NC_006973.1_Cervus_nippon_yeso      TTTCCAAAAA-TTTTAATATTTTAATACAGTTTTCT-ACTCAACACCCAA
EF058308.1_Cervus_nippon_taiou      TTTCCAAAAA-TTTTAATATTTTAATACAGCTTTCT-ACTCAACATCCAA
NC_007704.1_Cervus_elaphus          TTTCCAAAAAGTTTTAATATTTCAATACAGCTTTCC-ACTCAACACCCAT
EF035448.1_Cervus_unicolor_swi      TTTCCAAAAAATTTTAATATTTCAATACAGCTTTCT-ACTCAACATCCAA
NC_004563.1_Muntiacus_muntjak       TTTCCAAAAACCTTTAGTAATTTAACACAGCTTTCTCACTCAACAGCCAA
NC_008491.1_Muntiacus_reevesi       TTTCCAAAAATCTTTAGTAATTTAACACAGCTTTTTCACTCAACAGCCAA
                                    **********  **** ** ** ** **** ***   ******** ***

NC_006973.1_Cervus_nippon_yeso      TTTACATTT-ATGTCCTACTAATTACACAA----CAAAACA-CGTG-A--
EF058308.1_Cervus_nippon_taiou      TTTACATTTTATGTCCTACTAATTACACAG----CAAAACA-CGTG-A--
NC_007704.1_Cervus_elaphus          TTTACATTTTACATCC-ACCAACCACACAA----CAAAATA-TGTA-A--
EF035448.1_Cervus_unicolor_swi      TTTACATTTTACATCCCACTAACTAAACAA----CATAACA-TGTG-ACA
NC_004563.1_Muntiacus_muntjak       TTTACATTTTACATACCATTATCTACACAAGCCACATGATAAGGTATATA
NC_008491.1_Muntiacus_reevesi       TTTAAACTTTACATATAATCAACTACACAAACCACATAATAATATGTATG
                                    **** * ** *  *   *  *   * ***     **  * *   *  *

NC_006973.1_Cervus_nippon_yeso      -TATAACCTTATGCACTTGTAGCACATAAAATTAATGCGTTAAGACATAC
EF058308.1_Cervus_nippon_taiou      -TACAACCTTATGTGTTTGTAATACATAAAATTAGTGCATTAAGACACAC
NC_007704.1_Cervus_elaphus          -TAACACCTTATGCGCTTATAGTACATAGAATTA----------------
EF035448.1_Cervus_unicolor_swi      GTATAACCTCGCGCACTCGCAGTACGTAAAATTAGTGTGTTAAGACATAT
NC_004563.1_Muntiacus_muntjak       ATCTAACCATATGCGCTTATAGTACATAAAATTA----------------
NC_008491.1_Muntiacus_reevesi       ATTTAATTTTATGCGCTTATAGTACATAGAATTA----------------
                                     *   *      *   *   *  ** ** *****

NC_006973.1_Cervus_nippon_yeso      CATGTACAACAGCACATAAACCGGTGCGTAAGATACATTATGTATGATAG
EF058308.1_Cervus_nippon_taiou      --------------------------------------------------
NC_007704.1_Cervus_elaphus          --------------------------------------------------
EF035448.1_Cervus_unicolor_swi      TATATATAATAGTACATAAA------------------------------
NC_004563.1_Muntiacus_muntjak       --------------------------------------------------
NC_008491.1_Muntiacus_reevesi       --------------------------------------------------
```

Figure 18 (Continued)

```
NC_006973.1_Cervus_nippon_yeso      TACATAAATTAATGTGTTAGGACATATTATGTATAATAGTACATAAATTA
EF058308.1_Cervus_nippon_taiou      ----------------------------ATGTATAACAGTACATAAA---
NC_007704.1_Cervus_elaphus          ------------------------------------------A-------
EF035448.1_Cervus_unicolor_swi      -------ATTAATGTATTAGGACATATCATGTATAATAGTACATAAA---
NC_004563.1_Muntiacus_muntjak       --------------------------------------------------
NC_008491.1_Muntiacus_reevesi       --------------------------------------------------


NC_006973.1_Cervus_nippon_yeso      ATGTATTAGGACATACTATGTATAATAGTACATAAATTAATGTACTAGGA
EF058308.1_Cervus_nippon_taiou      -----------------------------------ATCGGTGTA-TAGGA
NC_007704.1_Cervus_elaphus          --------------------------------------------------
EF035448.1_Cervus_unicolor_swi      -----------------------------------ATTAATGTATTAGGA
NC_004563.1_Muntiacus_muntjak       --------------------------------------------------
NC_008491.1_Muntiacus_reevesi       --------------------------------------------------


NC_006973.1_Cervus_nippon_yeso      CATATTATGTATAATAGTACATAAA-TTAATGTATTAGGACATACTATGT
EF058308.1_Cervus_nippon_taiou      CATATTATGTATAATAGTACATAAA-TTAATGTATTAGGACATATTATGT
NC_007704.1_Cervus_elaphus          ------------------------------TGTACTAGGACATACTATGT
EF035448.1_Cervus_unicolor_swi      CATATCATGTATAATAGTACATAAAATTAATGTATTAGGACATATTATGT
NC_004563.1_Muntiacus_muntjak       -----------------------------ATGTATTAGGACATATTATGT
NC_008491.1_Muntiacus_reevesi       -----------------------------ATGTAATAGGACATACTATGT
                                                                  **** ********* *****

NC_006973.1_Cervus_nippon_yeso      ATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTACCCTCT
EF058308.1_Cervus_nippon_taiou      ATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTATTTTCT
NC_007704.1_Cervus_elaphus          ATAATAGTACATTATATTATATGCCCCATGCATATAAGCATGTACTTTCT
EF035448.1_Cervus_unicolor_swi      ATAATAGTACATTATATTATATGCCCCATGCTTATAAGCATGTATTTTCT
NC_004563.1_Muntiacus_muntjak       ATAATAGTACATTACATTACATGCCCCATGCTTATAAGCACGTATATTCC
NC_008491.1_Muntiacus_reevesi       ATAATAGTACATTACATTATATGCCCCATGCTTATAAGCACGTACATTCT
                                    ************** **** *********** ******** ***   ** 

NC_006973.1_Cervus_nippon_yeso      ACCATTTACAGTACATAGCACATGATGTTATTT-ATCGTACATAGCGCAT
EF058308.1_Cervus_nippon_taiou      ATCATTTATAGTACATAGTACATGATGTTGTCC-ATCGTACATAGTGCAT
NC_007704.1_Cervus_elaphus          ATTATTTATAGTACATAGTACATGATGTTGTTC-ATCGTACATAGCGCAT
EF035448.1_Cervus_unicolor_swi      GTCATTTACAGTACATAGTACATGATGTTGTTC-ATCGTACATAGTGCAT
NC_004563.1_Muntiacus_muntjak       ATTATTTACAGTACATGGTACATATCCTTGCTTGATAGTACATAGCACAT
NC_008491.1_Muntiacus_reevesi       ATTATTTACAGTACATAGTACATGTCCCTGCTTAATCGTACATAGCACAT
                                       ***** ******* * ****     *     ** ********  ***

NC_006973.1_Cervus_nippon_yeso      T-AAGTCAAATCAGTCCTCGTCAACATGCATATCCCGTCCCCTAGATCAC
EF058308.1_Cervus_nippon_taiou      C-AAGTCAAATCAGTCCTCGTCAACATGCGTATCCCGTCCCCTAGATCAC
NC_007704.1_Cervus_elaphus          T-AAGTCAAATCAGTCCCTGTCAACATGCGTATCCCGTCCCCTAGATCAC
EF035448.1_Cervus_unicolor_swi      T-AAGTCAAATCAATCCTCGTCAACATGCGTATCCCGTCCGCTAGATCAC

NC_004563.1_Muntiacus_muntjak       TTAAGTCAAATCAATTCTTGCGAACATGCGTATCCCGTCCCTTAGATCAC
NC_008491.1_Muntiacus_reevesi       TTAAGTCAAATCAATCCTCGCCAGCATGCGTATCCCGTCCACTAGATCAC
                                      *********** * *  *  * ***** **********  ********

NC_006973.1_Cervus_nippon_yeso      GAGCTTAATCACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATC
EF058308.1_Cervus_nippon_taiou      GAGCTTGATCACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATC
NC_007704.1_Cervus_elaphus          GAGCTTGATCACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATC
EF035448.1_Cervus_unicolor_swi      GAGCTTAATCACCATGCCGCGTGAAACCAGCAACCCGCTGGGCAGGGATC
NC_004563.1_Muntiacus_muntjak       GAGCTTAATTACCATGCCGCGTGAAACCAGCAACCCGCTTGGCAGGGATC
NC_008491.1_Muntiacus_reevesi       GAGCTTAGTCACCATGCCGCGTGAAACCAGCAACCCGCTTGGCAGGGATC
                                    ******  * ***************************** **********

NC_006973.1_Cervus_nippon_yeso      CCTCTTCTCGCTCCGGGCCCATGAACCGTGGGGGTAGCTATTTAATGAAT
EF058308.1_Cervus_nippon_taiou      CCTCTTCTCGCTCCGGGCCCATGAATTGTGGGGGTAGCTATTTAATGAAT
NC_007704.1_Cervus_elaphus          CCTCTTCTCGCTCCGGGCCCATGAACTGTGGGGGTAGCTATTTAATGAAC
EF035448.1_Cervus_unicolor_swi      CCTCTTCTCGCTCCGGGCCCATGAATTGTGGGGGTAGCTACTTAATGAAC
NC_004563.1_Muntiacus_muntjak       CTTGTTCTCGCTCCGGGCCCATGCACCGTGGGGGTAGCTATTTAATGAAC
NC_008491.1_Muntiacus_reevesi       CTTGTTCTCGCTCCGGGCCCATGTATTGTGGGGGTAGCTATTTAATGAAC
                                    * * ******************* *  ************* ******** 
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Figure 18 (Continued)

```
NC_006973.1_Cervus_nippon_yeso      TTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCACCTAAAATCGCC
EF058308.1_Cervus_nippon_taiou      TTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCACCTAAAATCGCC
NC_007704.1_Cervus_elaphus          TTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCACCTAAAATCGCC
EF035448.1_Cervus_unicolor_swi      TTTATCAGACATCTGGTTCTTTTTTCAGGGCCATCTCACCTAAAATCGCC
NC_004563.1_Muntiacus_muntjak       TTTATCAGACATCTGGTTCTTTCTTCAGGGCCATCTCACCTAAAATCGCC
NC_008491.1_Muntiacus_reevesi       TTTATCAGACATCTGGTTCTTTCTTCAGGGCCATCTCACCTAAAATCGCC
                                    ********************** ***************************

NC_006973.1_Cervus_nippon_yeso      CACCA---CTTGTAATATAAGACATCTCGATGGACTAATGACTAATCAGG
EF058308.1_Cervus_nippon_taiou      CACTC---CTTGCAATATAAGACATCTCGATGGACTAATGACTAATCAGC
NC_007704.1_Cervus_elaphus          CACTC---CTTGCAATATAAGACATCTCGATGGACTAATGACTAATCAGC
EF035448.1_Cervus_unicolor_swi      CACTC---CTTGTAGTATAAGACATCTCGATGGACTAATGACTAATCAGC
NC_004563.1_Muntiacus_muntjak       CACTCGTTCCTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGC
NC_008491.1_Muntiacus_reevesi       CACTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGACTAATCAGC
                                    ***     * *     *********************************

NC_006973.1_Cervus_nippon_yeso      CCATGCTCACACATAACTGTGGTGTCAAACATTTGGTATTTTTAATTTTT
EF058308.1_Cervus_nippon_taiou      CCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTAATTTTT
NC_007704.1_Cervus_elaphus          CCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTAATTTTT
EF035448.1_Cervus_unicolor_swi      CCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTAATTTTT
NC_004563.1_Muntiacus_muntjak       CCATGCTCACACATAACTGTGGTGTCATACATTTGGTATTTTTATTTTTT
NC_008491.1_Muntiacus_reevesi       CCATGCTCACACATAACTGTGATGTCATACATTTGGTATTTTTAATTTTT
                                    ********************* ***** **************** *****

NC_006973.1_Cervus_nippon_yeso      GGGGGGATGCTTGGACTCAGCAATGGCCGTTTGA-GGCCCCGTCCCGGAG
EF058308.1_Cervus_nippon_taiou      GGGGGGATGCTTGGACTCAGCAATGGCCGTCTGA-GGCCCCGTCTCGGAG
NC_007704.1_Cervus_elaphus          GGGGGGATGCTTGGACTCAGCAATGACCGTCTGGCGGTCCCGTCCCGGAG
EF035448.1_Cervus_unicolor_swi      GGGGGGATGCTTGGACTCAGCAATGGCCGTCTGA-GGCCCCGTCCCGGAG
NC_004563.1_Muntiacus_muntjak       GGGGG-ATGCTTGGACTCAGCTATGGCCGTCTGA-GGCCCCGACCCGGAG
NC_008491.1_Muntiacus_reevesi       GGGGGGATGCTTGGACTCAGCTATGGCCGTCTGA-GGCCCCGACCCGGAG
                                    ***** *************** *** **** **  ** **** * *****

NC_006973.1_Cervus_nippon_yeso      CATAAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
EF058308.1_Cervus_nippon_taiou      CATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
NC_007704.1_Cervus_elaphus          CATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
EF035448.1_Cervus_unicolor_swi      CATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
NC_004563.1_Muntiacus_muntjak       CATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
NC_008491.1_Muntiacus_reevesi       CATGAATTGTAGCTGGACTTAACTGCATCTTGAGCATCCCCATAATGGTA
                                    *** **********************************************

NC_006973.1_Cervus_nippon_yeso      GGCATAGGGCATTGCAGTCAATGGTCACAGGACATAATTATTATTTCATG
EF058308.1_Cervus_nippon_taiou      GGCGCAGGGCATTGCAGTCAATGGTCACAGGACATAGTTATTATTCCATG
NC_007704.1_Cervus_elaphus          GGCAT-GGGCATGGCAGTCAATGGTCACAGGACATAATCATTATTTCATG
EF035448.1_Cervus_unicolor_swi      GGCAT-GGGCATTGCAGTCAATGGTCACAGGACATAGTCATTATTTCATG
NC_004563.1_Muntiacus_muntjak       GGCGCAGGGCATTGCAGTCAATGGTTACAGGACATAGTTATTATTTCAAG
NC_008491.1_Muntiacus_reevesi       GGCGCAGGGCATTACAGTCAATGGTCACAGGACATAGTCATTATTTCAAG
                                    ***   ******  *********** ********** * ****** ** *

NC_006973.1_Cervus_nippon_yeso      AATCAACCCTAAGATCTAT--TTCCCCCCCCTT-CTTATTTTT-CCCCCT
EF058308.1_Cervus_nippon_taiou      AATCAACCCTAAGATCTAT--TTCCCCCCCCTT-CTTATTTTTTCCCCCT
NC_007704.1_Cervus_elaphus          AGTCAACCCTAAGATCTAT--TTTCCCCCCCCTGCTAATTTTTTCCCCCT
EF035448.1_Cervus_unicolor_swi      AGTCAACCCTAAGACCTATATTTCCCCCCCCTCCATTAATTTTTCCCCCT
NC_004563.1_Muntiacus_muntjak       ACTCAACTTTATAATCCATTATTCCCCCCCCCT--------------CCT
NC_008491.1_Muntiacus_reevesi       ATTCAACTTTACAATCC-TTATCCCCCCCCCC----------------CT
                                    * *****  **  * *  *  *  *******                 **

NC_006973.1_Cervus_nippon_yeso      TATATAGTTATCATCATTTTTAACACACTTTCCCCTAGATATTATTTTAA
EF058308.1_Cervus_nippon_taiou      TATATAGTTATCACCTTTTTTAACACACTTTTCCCTAGATATTATTTTAA
NC_007704.1_Cervus_elaphus          TATATAGTTATCACCATTTTTAACACACTTTTCCCTAAATATTATTTTAA
EF035448.1_Cervus_unicolor_swi      TATATAGTTATCACCATTTTTAACACACTTTTCCCTAGATATTATTTCAA
NC_004563.1_Muntiacus_muntjak       TATATAGTTATCACCTTTTTTAACACGCTTTTCCCTAGATAGTATTTTAA
NC_008491.1_Muntiacus_reevesi       TATATAGTTACCATCTTTTTTAACACGCTTTTCCCTAGATAATATTTTAA
                                    ********** ** * ********** **** ***** *** ***** **
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Figure 18 (Continued)

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NC_006973.1_Cervus_nippon_yeso      ATTTATCACATTTTCAATACTCAAATTAGCACTCCAGAGGGAGGTAAGTA
EF058308.1_Cervus_nippon_taiou      ATTTATCACATTTCCAATACTCAAATTAGCACTCCAGAGGGAGGTAAGTA
NC_007704.1_Cervus_elaphus          ATTTATCACATTTCCAATACTCAAATCAGCACTCCAGGGGGTGGTAACTA
EF035448.1_Cervus_unicolor_swi      ATTTATCACATTTCCAATACTCAAATTAGCACTCCAGGGGGAGGTAAGTA
NC_004563.1_Muntiacus_muntjak       ATTTATCGCATTTTCAATACTCAAATTAGCACTCCAGAAGGAGGTAAGTA
NC_008491.1_Muntiacus_reevesi       ATTTATCGCATTTTCAATACTCAAAT-AGCACTCCAGGGGGAGGTAACTA
                                    ******* ***** ************ **********  ** ***** **

                                     3' Control region (D-loop) <    > 5'tRNA-Phe
NC_006973.1_Cervus_nippon_yeso      TATAAACGCCAATTTTTCCCTAATTACGC-ATAGTTAATGTAGCTTAAAC
EF058308.1_Cervus_nippon_taiou      TATAAACGCCAATTTTTCCCTAATTACGC-ATAGTTAATGTAGCTTAAAC
NC_007704.1_Cervus_elaphus          TATAAACGCCAATTTTTCCCTAATTATGT-ACAGTTGATGTAGCTTAAAT
EF035448.1_Cervus_unicolor_swi      TATAAGCGCCAATTTTTCCCTAATTATGC-ATAGTTGATGTAGCTTAAAC
NC_004563.1_Muntiacus_muntjak       TATAAGCGCCAATTCTTCATCAATCGCACCACAGTTAATGTAGCTTAAAC
NC_008491.1_Muntiacus_reevesi       TATAAGCGCCAATCCTCCATCAATCGCACCACAGTTAATGTAGCTTAAGC
                                    ***** *******  * *   ***      * **** ***********

                                                                   3'tRNA-Phe <
NC_006973.1_Cervus_nippon_yeso      AACAAAGCAAGGCACTGAAAATGCCTAGATGAGTATATCAACTCCATAAA
EF058308.1_Cervus_nippon_taiou      AGCAAAGCAAGGCACTGAAAATGCCTAGATGAGTGTATTAACTCCATAAA
NC_007704.1_Cervus_elaphus          GGCAAAGCAAGGCACTGAAAATGCCTAGATGAGTATATTAACTCCATAAA
EF035448.1_Cervus_unicolor_swi      AGCAAAGCAAGGCACTGAAAATGCCTAGATGAGTATATTAACTCCATAAA
NC_004563.1_Muntiacus_muntjak       AATAAAGCAAGGCACTGAAAATGCCTAGATGAGTGTATTAACTCCATAAA
NC_008491.1_Muntiacus_reevesi       AATAAAGCAAGGCACTGAAAATGCCTAGATGAGTGTACTAGCTCCATAAA
                                       ******************************* **  * *********

                                    > 5'12S rRNA
NC_006973.1_Cervus_nippon_yeso      CACACAGGTTTGGTCCCAGCCTTCCTATTGACCCTTAATAGACTTACACA
EF058308.1_Cervus_nippon_taiou      CACACAGGTTTGGTCCCAGCCTTCCTATTGACCCTTAATAGACTTACACA
NC_007704.1_Cervus_elaphus          CATATAGGTTTGGTCCCAGCCTTCCTATTAACCCTTAATAGACTTACACA
EF035448.1_Cervus_unicolor_swi      CATACAGGTTTGGTCCCAGCCTTCCTATTAACCCTTAATAGACTTACACA
NC_004563.1_Muntiacus_muntjak       CATATAGGTCTGGTCCCAGCCTTCCTATTAGCCTTTAATAGACTTACACA
NC_008491.1_Muntiacus_reevesi       CATATAGGTTTGGTCCCAGCCTTCCTATTAACCCTTAATAGACTTACACA
                                    ** * **** *******************  ** ****************

NC_006973.1_Cervus_nippon_yeso      TGCAAGCATCCACACCCCAGTGAAAATGCCCTCCAAATTAA-TAAGACTA
EF058308.1_Cervus_nippon_taiou      TGCAAGCATCCACACCCCAGTGAAAATGCCCTCCAAGTTAACTAAGACTA
NC_007704.1_Cervus_elaphus          TGCAAGCATCCGCACCCCGGTGAAAATGCCCTCCAAGTTAA-TAAGACTA
EF035448.1_Cervus_unicolor_swi      TGCAAGCATCCGCGCCCCAGTGAAAATGCCCCCCAAGTTAA-TAAGACTA
NC_004563.1_Muntiacus_muntjak       TGCAAGCATCCACGCCCCAGTGAAAATGCCCTCCAAGTTAA-TAAGACCA
NC_008491.1_Muntiacus_reevesi       TGCAAGCATCCACGTCCCAGTGAAAATGCCCTCTAAGTTAA-TAAGACTA
                                    *********** *  *** ************ * ** **** ****** *

                                          CerCR
NC_006973.1_Cervus_nippon_yeso      AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACACCTTGCATAG
EF058308.1_Cervus_nippon_taiou      AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACACCTTGCATAG
NC_007704.1_Cervus_elaphus          AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACACCTTGCACAG
EF035448.1_Cervus_unicolor_swi      AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACACCTTGCACAG
NC_004563.1_Muntiacus_muntjak       AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACACCTTGCTCAG
NC_008491.1_Muntiacus_reevesi       AGAGGAGCTGGTATCAAGCACACATCCGTAGCTCACGACGCCTTGCTCAG
                                    *************************************** ******  **

NC_006973.1_Cervus_nippon_yeso      CCACACCCCCACGGGAGACAGCAGTGATAAAAATTAAGCCATAAA
EF058308.1_Cervus_nippon_taiou      CCACACCCCCACGGGAGACAGCAGTGATAAAAATTAAGCCATAAA
NC_007704.1_Cervus_elaphus          CCACACCCCCACGGGAGACAGCAGTGATAAAAATTAAGCCATAAA
EF035448.1_Cervus_unicolor_swi      CCACACCCCCACGGGAGACAGCAGTGATAAAAATTAAGCCATAAA
NC_004563.1_Muntiacus_muntjak       CCACACCCCCACGGGAAACAGCAGTGATAAAAATTAAGCCATAAA
NC_008491.1_Muntiacus_reevesi       CCACACCCCCACGGGAGACAGCAGTGACAAAAATTAAGCCATAAA
                                    **************** ********** *****************
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Figure 18 (Continued)

2. Phylogenetic Relationship Analysis Inferred from Cytochrome *b* Gene

A. DNA amplification

Whole genomic DNA from the blood samples was extracted using Aquapure Genomic Blood kit (Bio-Rad). Each DNA extract was used as a template for PCR amplification with the primer pair of CCyt1 and CCyt2, the newly constructed primer from this study. The best quality of PCR products were amplified in a 50 μl reaction containing 5 μl of 10X KCl Taq buffer, 4 μl of 2.5 mM $MgCl_2$, 0.25 μl of Taq DNA polymerase (Fermentas), 4 μl of 2.0 mM dNTP mixture, 2.5 μl of 5 pmol/μl of each primer and 4 μl of 100 ng/μl DNA template solution, with the PCR temperature profile consists of an initial denaturation step at 94°C for 4 min, 35 cycles of a denaturation step at 94°C for 20s, an annealing step at 50°C for 30s, and an polymerization step at 72°C for 1 min, and a final polymerization step at 72°C for 5 min. The expected single band of PCR products of 1,440 bp corresponded to the size of DNA target fragment as shown in Figure 19.

B. Samples for phylogenetic analysis

Twenty-one sample sequences from cytochrome *b* target PCR products from this study including hog deer (*Cervus* or *Axis porcinus*), sambar deer (*Cervus unicolor equinus*), thamin Eld's deer (*C. eldii thamin*), common barking deer (*Muntiacus muntjak*), Fea's barking deer (*M. feae*) and one cervid species which are not native to Thailand; spotted deer (*Axis axis*) were aligned using ClustalX version 1.82 (Thompson *et al.* 1997) along with nine cervid sequences obtained from GenBank. Before launching to PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis, multiple sequences alignment on ClustalX was saved as nexus file (*.nxs). Sample names and accession number of all sequences for phylogenetic analysis as shown in Table 5.

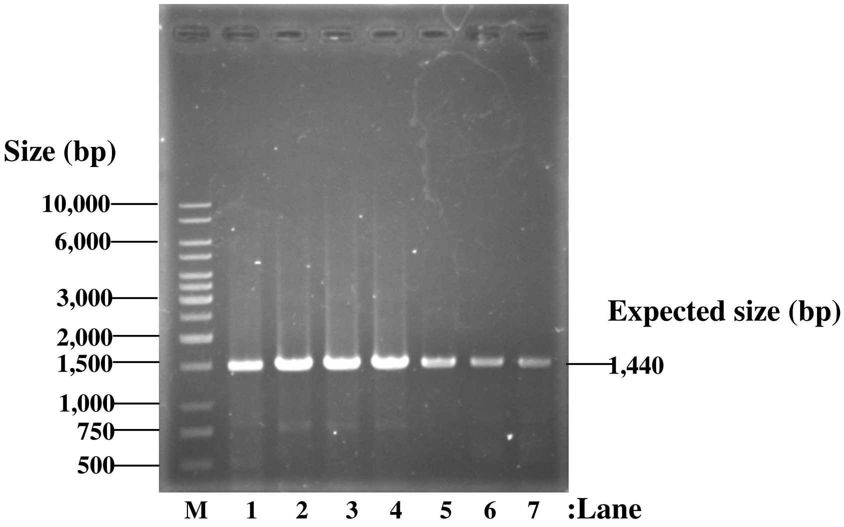

Figure 19 PCR products of Cervidae's cytochrome *b* gene target (including partial sequence of NADH dehydrogenase subunit 6, complete sequence of tRNA^{Glu} gene, complete sequence of cytochrome *b* gene, complete sequence tRNA^{Thr} gene and partial sequence of tRNA^{Pro} gene). Each lane indicated the yield of DNA band as below:

- Lane M = the size standard marker
- Lane 1 = *Muntiacus muntjak*
- Lane 2-4 = *Cervus* or *Axis porcinus*
- Lane 5 = *C. unicolor*
- Lane 6-7 = *C. eldii thamin*

Table 5 Cervid samples for cytochrome *b* phylogenetic analysis used in this study.

Common name	Zoological name	Sample name /Accession no.	Source	No. of sample
Common barking deer	*Muntiacus muntjak*	CBM2, CBM3	Pattalung Wildlife Station[a]	3
		NC_004563	GenBank	
Fea's barking deer	*Muntiacus feae*	CBF1, CBF2	Ton Nga-Chang Wildlife[a] Station	2
Reeves's barking deer	*Muntiacus reevesi micrurus*	NC_008491	GenBank	1
Hog deer	*Cervus* or *Axis porcinus*	CBP1, CBP5, CBP6	Hauy Sai Wildlife Station[a]	6
		CBP9		
		CBP11	Dusit Zoo[b]	
		CBP12	Korat Zoo[b]	
			Songkhla Zoo[b]	
Sambar deer	*Cervus unicolor equinus*	CBU2, CBU3, CBU4	Hauy Sai Wildlife Station[a]	7
		CBU5	Songkhla Zoo[b]	
		CBU8	Khao Kho Wildlife station[a]	
	Cervus unicolor swinhoei	EF035448	GenBank	
	Cervus unicolor cambojensis	AF423201	GenBank	

Table 5 (Continued)

Common name	**Zoological name**	**Sample name /Accession no.**	**Source**	**No. of sample**
Thamin Eld’s deer	*Cervus eldii thamin*	CBT1	Hauy Sai Wildlife Station[a]	3
		CBT9	Dusit Zoo[b]	
		EU878390	GenBank	
Schomburgk’s deer	*Cervus schomburgki*	AY607036	GenBank	1
Swamp deer	*Cervus duvaucelii*	AY607041	GenBank	1
Sika deer	*Cervus nippon taiouanus*	EF058308	GenBank	1
Red deer or wapiti	*Cervus elaphus*	NC_007704	GenBank	1
Spotted deer	*Axis axis*	CBA1,CBA2	Songkhla Zoo[b]	4
		CBA3, CBA4	Korat Zoo[b]	
Total				30

[a] Wildlife Propagation Division, DNP

[b] The Zoological Park Organization under the Royal Patronage of His Majesty the King

C. Cytochrome *b* gene, with additional NADH dehydrogenase subunit 6 gene and tRNA gene characteristics

After ClustalX multiple alignments were completed, the excessive nucleotide of two flank side from each sequence was cut off. Therefore, the final alignment of complete cytochrome *b* gene, with supplement of partial NADH dehydrogenase subunit 6 gene, complete tRNA^{Glu} gene and complete tRNA^{Thr} gene sequences of these 30 deer taxa was still remained 1,328 base characters. The nexus file of sequence was launched into PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis. Of these 1,328 characters, 985 were constant and 290 were parsimony informative. The cytochrome *b* gene sequences of all deer taxa was 1,140 bp long, which was located at position (pos.) 114 to pos. 1,253. These gene sequences was started with ATG codon and stopped with TGA codon. It was found no gap throughout this gene. GC content of this gene was approximately 40 %. The base composition was calculated by averaging base composition values from individuals of each species as shown in Table 6.

Table 6 Percentage of average base composition of cytochrome *b* gene, with additional NADH dehydrogenase subunit 6 gene and tRNA gene of each cervid species examined by PAUP* version 4.010b.

Zoological name	**Base composition (%)**			
	A	**C**	**G**	**T**
Muntiacus muntjak	32.35	26.10	12.65	28.89
M. feae	32.38	26.81	12.65	28.16
M. reevesi micrurus	32.38	25.83	12.73	29.07
Cervus unicolor equinus	32.35	26.49	12.98	28.18
C. unicolor swinhoei	32.38	26.13	13.03	28.46
C. eldii thamin	32.61	26.17	12.80	28.43
C. elaphus	32.30	25.98	13.10	28.61
C. nippon taiouanus	32.23	26.81	13.03	27.94

Table 6 (Continued)

Zoological name	**Base composition (%)**			
	A	**C**	**G**	**T**
C. or *Axis porcinus*	32.91	25.51	12.44	29.08
Axis axis	32.46	24.32	12.80	30.42
All taxa	32.43	26.02	12.82	28.72

D. Phylogenetic analysis

In the phylogenetic analyses, six taxa of the subfamily Muntiacinae; *Muntiacus muntjak*, *M. feae* and *M. reevesi micrurus* were used as outgroups. The phylogenetic trees were executed as 2 types, which were (1) neighbor-joining (NJ) analysis and (2) maximum parsimony (MP) analysis using PAUP* version 4.010b (Swofford, 2002). For the NJ tree, non-parametric bootstrap analyzes with 1,000 replicates were performed to obtain estimates of support for each node of the NJ tree as shown in Figure 20. For the MP tree, the informative sites were analyzed using equally weighted characters and were searched by heuristic option with a stepwise starting tree, a random stepwise addition of 1,000 replicates, tree-bisection-reconnection (TBR), and steepest descent branch-swapping algorithm. Gaps were treated as missing data. With this heuristic search option, the most four parsimonious trees were generated and the semi-strict consensus of them was shown in Figure 21. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

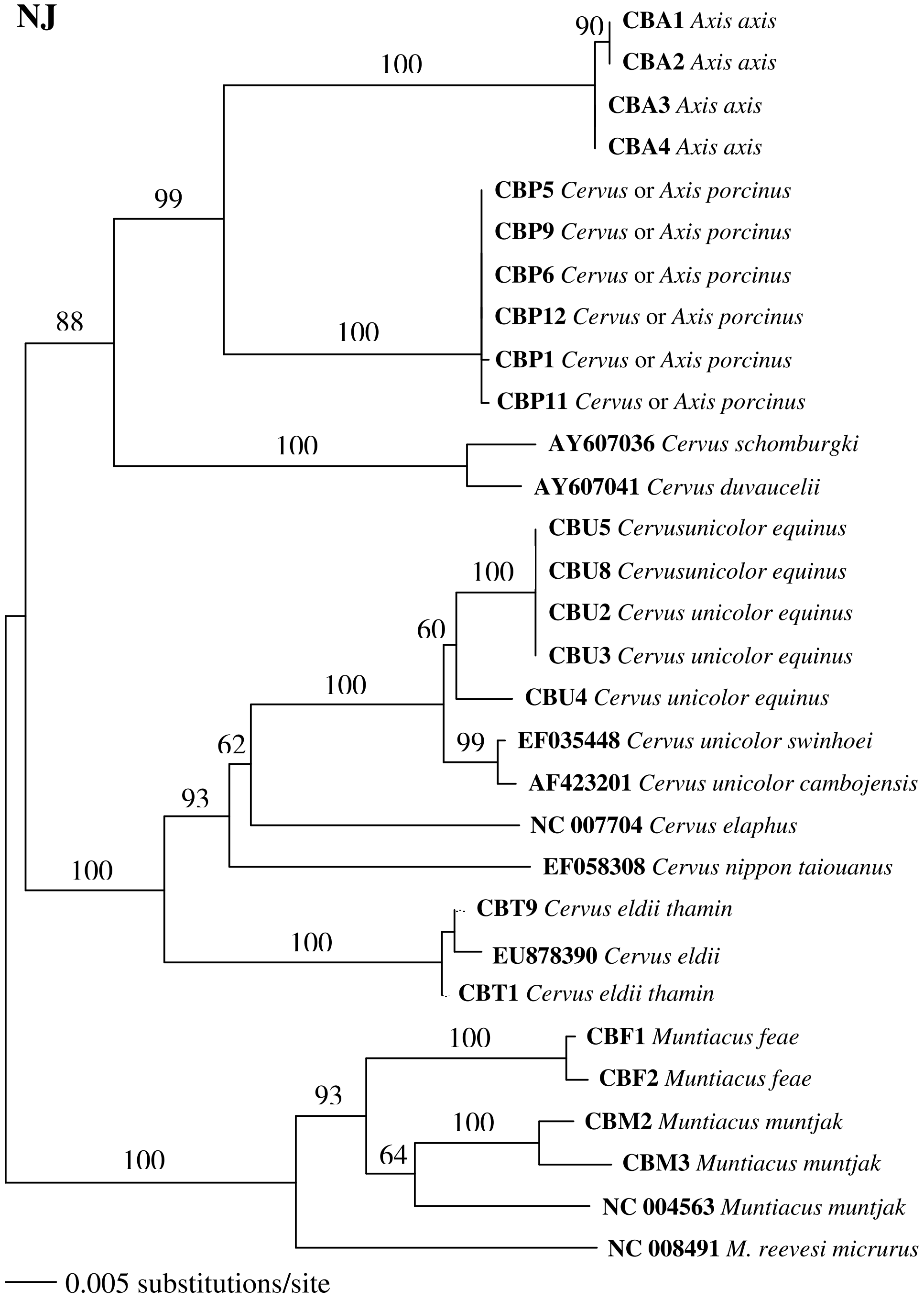

Figure 20 Neighbor-joining tree from cytochrome *b* gene, with supplement of partial NADH dehydrogenase subunit 6 gene, complete tRNA^{Glu} gene and complete tRNA^{Thr} gene sequences of 30 deer taxa. Numbers above branches indicate % bootstrap support (only >50 %) generated from 1,000 replications.

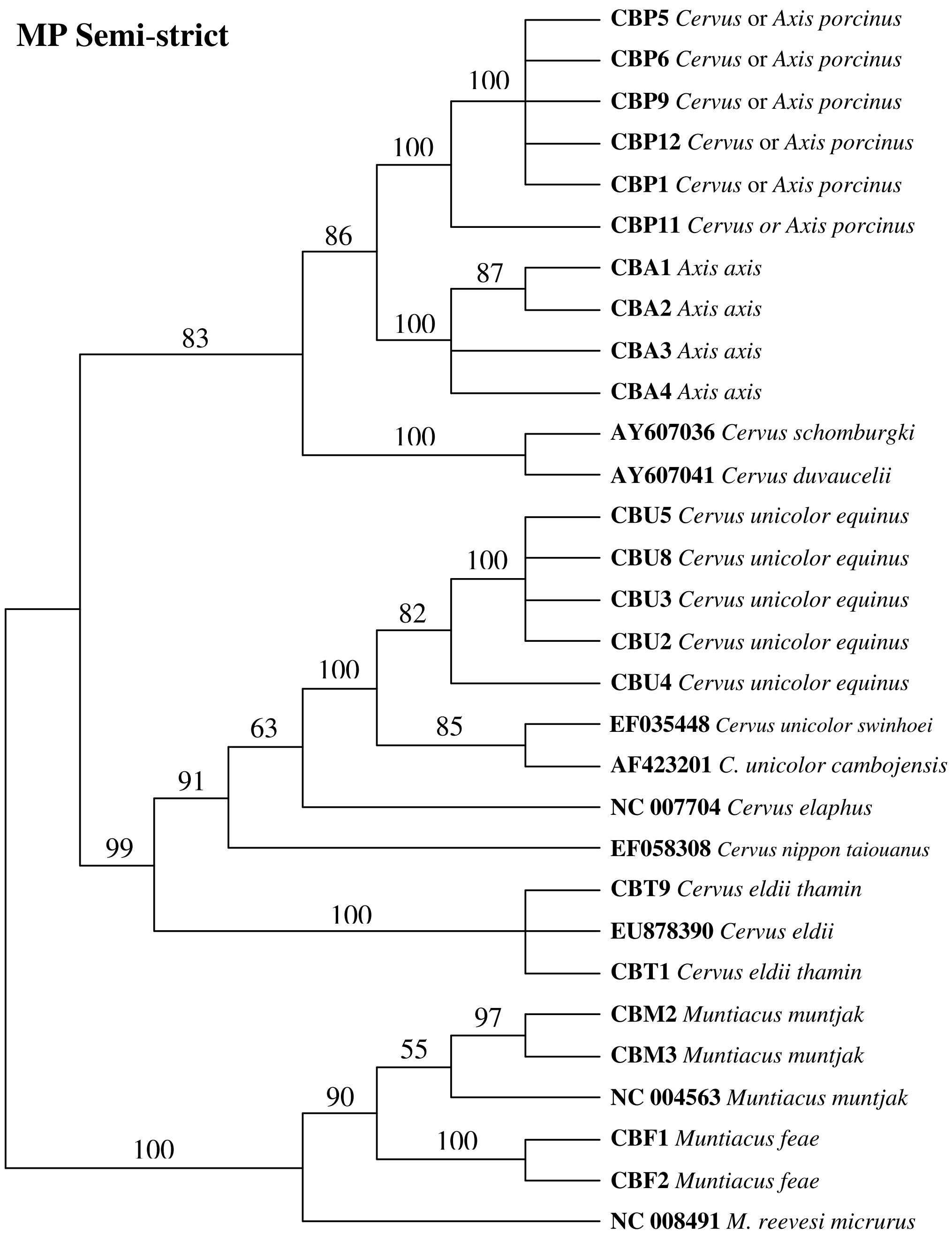

Figure 21 Semi-strict consensus tree of the most four parsimonious trees from cytochrome *b* gene, with supplement of partial NADH dehydrogenase subunit 6 gene, complete tRNA^{Glu} gene and complete tRNA^{Thr} gene sequences of 30 deer taxa. Numbers below branches indicate % bootstrap support (only >50 %) based on 1,000 replications.

E. Phylogenetic relationship result

All phylogenetic analyses yielded the similar topology of tree. It found that hog deer (*Cervus* or *Axis porcinus*) were grouped monophyletically in the same clade of spotted deer (*Axis axis*) (assumption as genus *Axis* clade) with very strong statistical bootstrap support (99 % on NJ tree and 86 % on MP tree). Unexpected result was also found, in which Schomburgk's deer (*Cervus schomburgki*) and swamp deer (*Cervus duvaucelii*) were joined in the *Axis* clade of hog deer and spotted deer, not to the *Cervus* clade with strong statistical bootstrap support (88 % on NJ tree and 83 % on MP tree). In the case of the genus *Cervus* (except Schomburgk's deer and swamp deer), it was formed as clearly monophyletic clade with very strong statistical bootstrap support (100 % on NJ tree and 99 % on MP tree). Among *Cervus* clade, Eld's deer (*Cervus eldii*) were found as former sister lineage of all *Cervus* taxa, while sambar deer (*Cervus unicolor*) was the latter of clade. Among sambar deer, *Cervus unicolor swinhoei* from China and *Cervus unicolor cambojensis* from Cambodia were sister lineage of Thai sambar deer (*Cervus unicolor equinus*)

To distinguish the genus *Axis* clade (include 2 taxa of Schomburgk's deer and swamp deer) from the genus *Cervus* clade (not include *Cervus* or *Axis porcinus*), 129 base change were needed. These 129 synapomorphic nucleotide characters were counted from 48 base changes of the *Axis* clade, which could group spotted deer and hog deer together, and 81 base synapomorphic characters between *Cervus unicolor*, *Cervus elaphus*, *Cervus nippon* and *Cervus eldii*. It could be noticed that Schomburgk's deer and swamp deer were differed a lot of base changes (129 base changes) from genus *Cervus* clade, therefore, they should not be placed in the genus *Cervus*. For hog deer, it was placed in the genus *Axis* clade, with 145 base changes if compared with spotted deer. These 145 synapomorphic nucleotide characters were counted from 87 base changes of spotted deer and 59 base changes of hog deer. The parsimonious tree that shows numbers of base changes is shown in Figure 22.

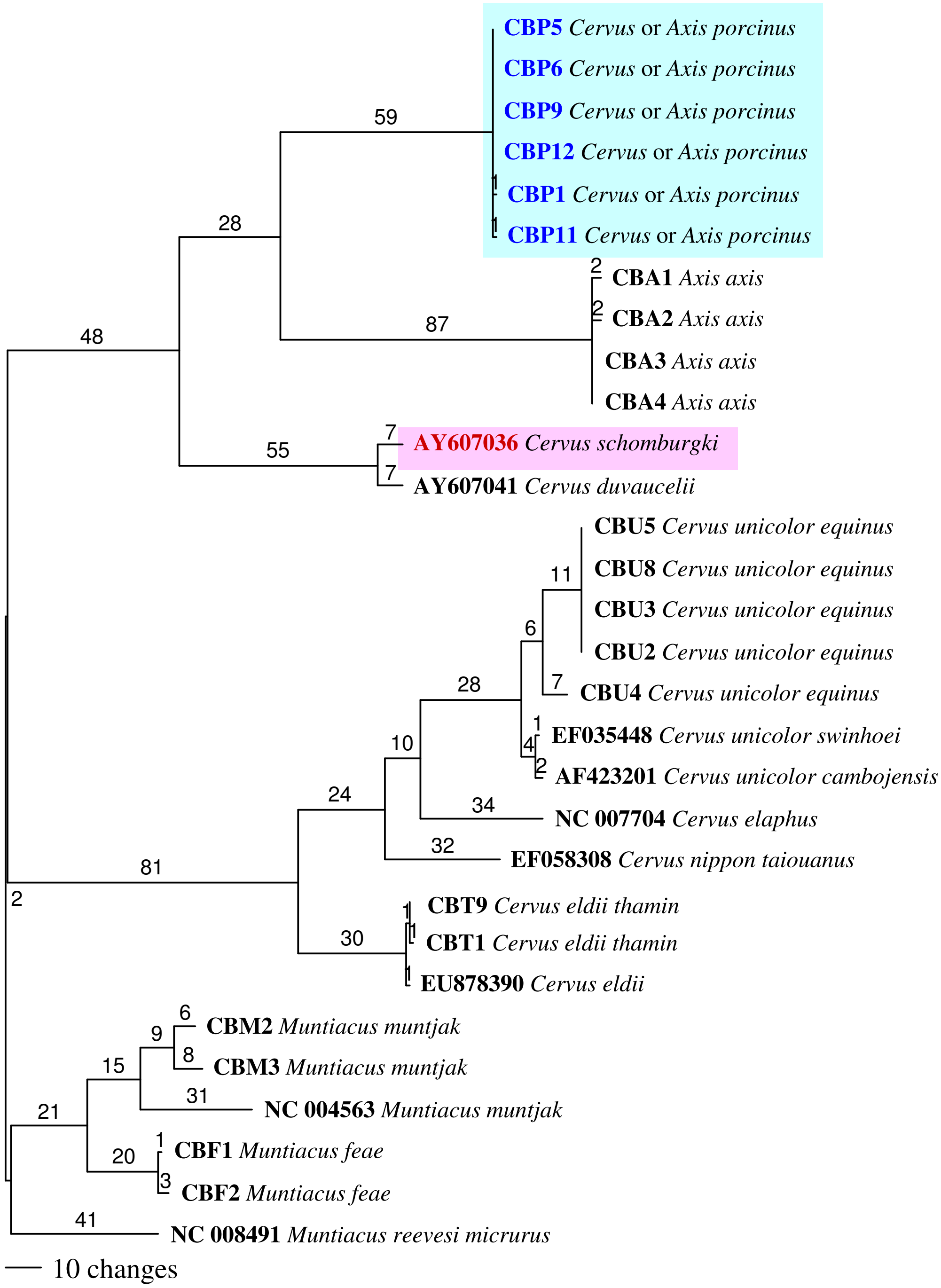

Figure 22 One of the most four parsimonious trees from cytochrome *b* gene, with supplement of partial NADH dehydrogenase subunit 6 gene, complete tRNA^{Glu} gene and complete tRNA^{Thr} gene sequences of 30 deer taxa. Number above branches show numbers of base changes.

3. Phylogenetic Relationship Analysis Inferred from Control Region

A. DNA amplification

Whole genomic DNA from the blood samples was extracted using Aquapure Genomic Blood kit (Bio-Rad). Each DNA extract was used as a template for PCR amplification with the primer pair of CerCF and CerCR, the newly constructed primer from this study. The best quality of PCR products were amplified in a 50 µl reaction containing 5 µl of 10X KCl Taq buffer, 4 µl of 2.5 mM $MgCl_2$, 0.25 µl of Taq DNA polymerase (Fermentas), 4 µl of 2.0 mM dNTP mixture, 2.5 µl of 5 pmol/µl of each primer and 4 µl of 100 ng/µl DNA template solution, with the PCR temperature profile consists of an initial denaturation step at 94°C for 4 min, 35 cycles of a denaturation step at 94°C for 20s, an annealing step at 52°C for 30s, and an polymerization step at 72°C for 1 min, and a final polymerization step at 72°C for 5 min. The expected single band of PCR products of approximately 1,000 up to 1,260 bp corresponded to the size of DNA target fragment as shown in Figure 23.

B. Samples for phylogenetic analysis

Eleven sample sequences from control region target PCR products from this study including hog deer (*Cervus* or *Axis porcinus*), sambar deer (*Cervus unicolor equinus*), thamin Eld's deer (*C. eldii thamin*), common barking deer (*Muntiacus muntjak*), Fea's barking deer (*M. feae*) and one cervid species which not native to Thailand; spotted deer (*Axis axis*) were aligned using ClustalX version 1.82 (Thompson *et al*. 1997) along with five cervid sequences obtained from GenBank. Before launching to PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis, multiple sequences alignment on ClustalX was saved as nexus file (*.nxs). Sample names and accession number of all sequences for phylogenetic analysis as shown in Table 7.

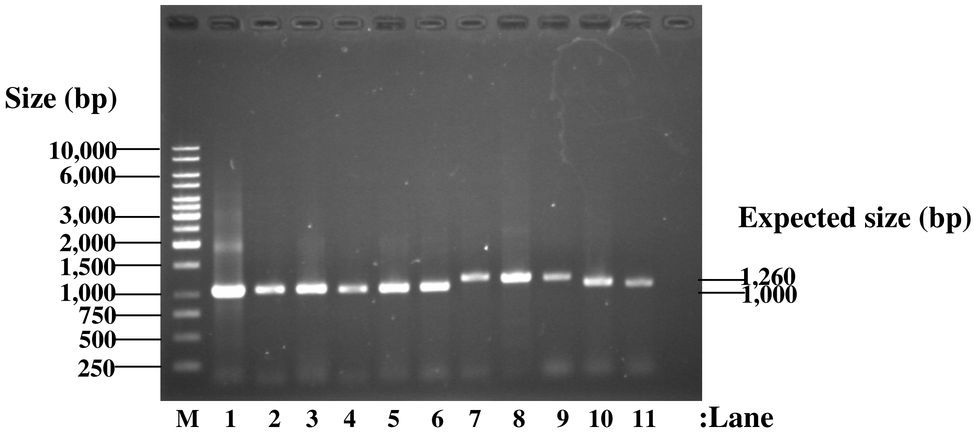

Figure 23 PCR products of Cervidae's control region portion target (including of partial sequence of cytochrome *b*, complete sequence of tRNA^{Thr} gene, complete sequence of tRNA^{Pro} gene, complete sequence of control region, complete sequence of tRNA^{Phe} gene and partial sequence of 12S ribosomal RNA gene). Each lane indicated the yield of DNA band as below:

- Lane M = the size standard marker
- Lane 1 = *Muntiacus muntjak*
- Lane 2-6 = *Cervus* or *Axis porcinus*,
- Lane 7-9 = *C. unicolor*
- Lane 10-11 = *C. eldii thamin*

Table 7 Cervid samples for control region phylogenetic analysis used in this study.

Common name	Zoological name	Sample name /Accession no.	Source	No. of sample
Common barking deer	*Muntiacus muntjak*	CRM2, CRM3	Pattalung Wildlife Station[a]	3
		NC_004563	GenBank	
Fea's barking deer	*Muntiacus feae*	CRF1	Ton Nga-Chang Wildlife Station[a]	1
Reeves's barking deer	*Muntiacus reevesi micrurus*	NC_008491	GenBank	1
Hog deer	*Cervus* or *Axis porcinus*	CRP5, CRP6	Hauy Sai Wildlife Station[a]	4
		CRP9	Dusit Zoo[b]	
		CRP12	Songkhla Zoo[b]	
Sambar deer	*Cervus unicolor equinus*	CRU4	Hauy Sai Wildlife Station[a]	2
	Cervus unicolor swinhoei	EF035448	GenBank	
Thamin Eld's deer	*Cervus eldii thamin*	CRT1	Hauy Sai Wildlife Station[a]	2
		CRT9	Dusit Zoo[b]	
Sika deer	*Cervus nippon taiouanus*	EF058308	GenBank	1
Red deer or wapiti	*Cervus elaphus*	NC_007704	GenBank	1

Table 7 (Continued)

Common name	**Zoological name**	**Sample name /Accession no.**	**Source**	**No. of sample**
Spotted deer	*Axis axis*	CRA1	Songkhla Zoo[b]	1
Total				16

[a] Wildlife Propagation Division, DNP

[b] The Zoological Park Organization under the Royal Patronage of His Majesty the King

C. Control region portion, with additional tRNA gene and 12S ribosomal RNA gene characteristics

For control region, sambar deer (*Cervus unicolor*) were found very long insertion in 5' left peripheral domain. They have to be cut off for better alignment. After ClustalX multiple alignment was completed, the excessive nucleotide of two flank side from each sequence also very long insertion region in 5' left peripheral domain of sambar deer were removed. Therefore, the final alignment of partial control region portion, with supplement of complete sequence of tRNA^{Phe} gene and partial 12S ribosomal RNA gene sequences of these 16 deer taxa was still remained 923 base characters. The nexus file of sequence was launched into PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis. Of these 923 characters, 731 were constant and 149 were parsimony informative. It was found several gaps also some insertions and deletions throughout this portion. GC content in this portion was approximately 40 %. The base composition was calculated by averaging base composition values from individuals of each species as shown in Table 8.

Table 8 Percentage of average base composition of control region, with additional tRNA gene and 12S ribosomal RNA gene of cervid species examined by PAUP* version 4.010b.

Zoological name	**Base composition (%)**				**Amount**
	A	**C**	**G**	**T**	
Muntiacus muntjak	28.61	23.69	17.12	30.57	887.66
M. feae	29.22	23.55	16.33	30.89	900.00
M. reevesi micrurus	28.51	24.66	17.31	29.52	884.00
Cervus unicolor equinus	29.11	23.96	16.68	30.23	893.00
C. unicolor swinhoei	28.52	23.26	17.45	30.76	894.00
C. eldii thamin	28.12	24.27	16.81	30.79	898.00
C. elaphus	28.19	23.60	17.45	30.76	894.00
C. nippon taiouanus	28.28	23.34	17.28	31.09	891.00

Table 8 (Continued)

Zoological name	Base composition (%)				Amount
	A	**C**	**G**	**T**	
C. or *Axis porcinus*	28.46	28.46	15.85	32.09	916.25
Axis axis	28.22	24.19	17.24	30.35	893.00
All taxa	28.50	23.79	16.75	30.96	898.31

PCR products of control region portion target (including of partial sequence of cytochrome *b*, complete sequence of tRNA^{Thr} gene, complete sequence of tRNA^{Pro} gene, complete sequence of control region, complete sequence of tRNA^{Phe} gene and partial sequence of 12S ribosomal RNA gene) which obtained from this study were approximately more than 1,000 up to 1,260 bp long. The complete sequences of cervid control region were derived from this study, nevertheless, they were removed some partial of 5' left peripheral domain (domain I) for phylogenetic analysis. The length of complete sequences of cervid control region and complete sequence of adjacent tRNA gene which were obtained from this study are shown in Table 9.

Table 9 The length of complete sequences of control region (CR) and adjacent tRNA gene of Cervidae which were obtained from this study (the name of sequences in row were arranged by their order in the sequences).

Zoological name	Sample name	tRNA^{Thr} (bp)	tRNA^{Pro} (bp)	CR (bp)	tRNA^{Phe} (bp)
Muntiacus muntjak	CRM2	70	71	921	66
	CRM3	70	71	923	66
Muntiacus feae	CRF1	70	71	934	66
Cervus or *Axis porcinus*	CRP5	70	71	943	66
	CRP6	70	71	942	66
	CRP9	70	71	941	66
	CRP12	70	71	940	66

Table 9 (Continued)

Zoological name	Sample name	$tRNA^{Thr}$ (bp)	$tRNA^{Pro}$ (bp)	CR (bp)	$tRNA^{Phe}$ (bp)
Cervus unicolor equinus	CRU4	70	71	1,041	66
Cervus eldii thamin	CRT1	70	71	921	66
	CRT9	70	71	922	66
Axis axis	CRA1	70	72	917	66

D. Occurrence of multiple repeats in control region

The control region (CR) or D-loop of mitochondrial DNA (mtDNA) is noncoding, which have function for replication of the H-strand and transcription of mtDNA molecule. CR is divided into three domain, domain II; a conserved central region (CCR) which flanked by domain I; 5’ left peripheral domain and domain III; 3’ right peripheral domain (Figure 24). The peripheral domains (both of domain I; 5’ left peripheral domain and domain III; 3’ right peripheral domain) were found highly variable number of tandem repeats (VNTRs), especially in several species of mammals. (Clayton, 1992; Douzery and Randi, 1997; Wilkinson *et al.*, 1997). The extensive length differences VNTRs can be produced among species. Because of their high rate of base change in peripheral regions, CR sequences are widely used in population and evolutionary genetics study in mammals, while the more slowly evolving CCR has been used to reconstruct phylogenies of placental order (Saccone *et al.*, 1991; Douzery and Randi, 1997).

For better understanding of cervid control region and its nearby tRNAs gene, the complete sequences control region of all cervid obtained from this study, with complete sequences of $tRNA^{Thr}$, $tRNA^{Pro}$ and $tRNA^{Phe}$ genes were aligned in ClustalW again as shown in Figure 25. The control region sequence of all deer taxa was spanned from 917 bp of spotted deer; *Axis axis* and up to 1,041 bp of sambar deer; *Cervus unicolor equinus*, which were located at position (pos.) 142 to pos. 1,221. These portions were started with TGC or CGC characters and were stopped with TACA, CACA or CATA characters. Domain I of 5’ left peripheral domain was approximately located at pos. 142-628, domain II of a conserved central region (CCR) was approximately located at pos. 629-861 and domain III of 3' right peripheral domain was approximately located at pos. 862-1,221, respectively as shown in Figure 24. The position (bp) of all portions location was also described in Figure 25.

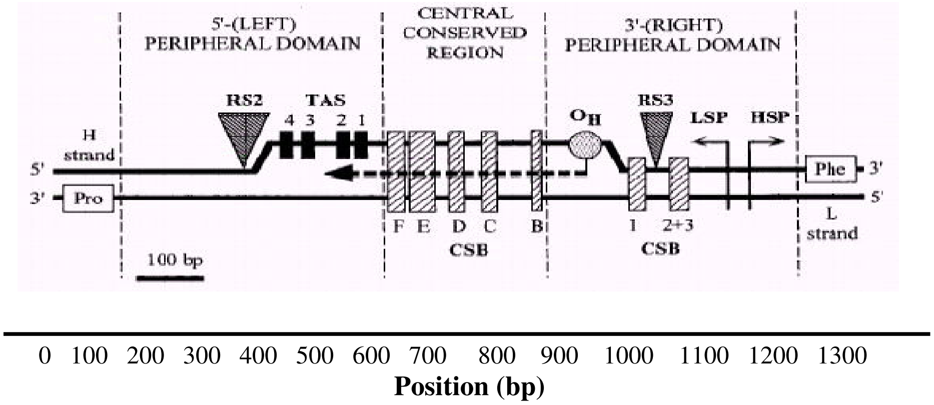

Figure 24 Schematic diagram of the organization of the mammalian mitochondrial DNA control region (CR). It consists of domain I; 5' left variable peripheral domain, domain II; a conserved central region and domain III; 3' right variable peripheral domain. The variable domain I contains termination association sequences (TAS). The variable domain III contains conserved sequences blocks (CSBs). Letters F to B denote conserved sequences in the conserved domain II.

Source: Modified from Douzery and Randi (1997)

```
CLUSTAL W (1.81) Multiple Sequence Alignments

Sequence 1: CRA1_A_axis                 1124 bp
Sequence 2: CRF1_M_feae                 1140 bp
Sequence 3: CRM2_M_muntjak              1127 bp
Sequence 4: CRM3_M_muntjak              1129 bp
Sequence 5: CRP5_A_porcinus             1149 bp
Sequence 6: CRP6_A_porcinus             1148 bp
```

Figure 25 The position of cervid control region and adjacent tRNA genes from this study. (The gray label presents the location of the 5' left variable domain I and the 3' right variable domain III, and the yellow label presents the copied of tandem repeats (VNTRs) in their regions).

```
Sequence 7: CRP9_A_porcinus                  1147 bp
Sequence 8: CRP12_A_porcinus                 1146 bp
Sequence 9: CRT1_C_eldi                      1127 bp
Sequence 10: CRT9_C_eldi                     1128 bp
Sequence 11: CRU4_C_unicolor                 1247 bp
Sequence 12: EF035448_C_unicolor_swinhoei    1248 bp
Sequence 13: NC_007704_C_elaphus             1123 bp
Sequence 14: EF058308_C_nippon_taiouanus     1199 bp
Sequence 15: NC_008491_M_reevesi_micrurus    1124 bp
Sequence 16: NC_004563_M_muntjak             1127 bp
Start of Pairwise alignments aigning...

                                  > 5'tRNA-Thr
CRP9_A_porcinus                ATAAGTCTTTGTAGTATACTAAATACACTGGTCTTGTAAACCAGAAAAGG 50
CRP12_A_porcinus               ATAAGTCTTTGTAGTATACTAAATACACTGGTCTTGTAAACCAGAAAAGG
CRP6_A_porcinus                ATAAGTCTTTGTAGTATACTAAATACACTGGTCTTGTAAACCAGAAAAGG
CRP5_A_porcinus                ATAAGTCTTTGTAGTATACTAAATACACTGGTCTTGTAAACCAGAAAAGG
CRA1_A_axis                    ATAAGTCTTTGTAGTATACTGAATACACTGGTCTTGTAAATCAGAAAAGG
CRU4_C_unicolor                ACAAGTCTTTGTAGTACACTCAATACACTGGTCTTGTAAACCAGAAAAGG
EF035448_C_unicolor_swinhoei   ACAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
NC_007704_C_elaphus            ATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
EF058308_C_nippon_taiouanus    ATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
CRT1_C_eldi                    ATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
CRT9_C_eldi                    ATAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
CRM2_M_muntjak                 ACAAGTCTTTGTAGTATACTCAATACACTGGTCCTGTAAACCAGAAAAGG
NC_004563_M_muntjak            ACAAGTCTTTGTAGTATACTCAATACACTGGTCTTGTAAACCAGAAAAGG
CRM3_M_muntjak                 ACAAGTCTTTGTAGTATACTCAATACACTGGTCCTGTAAACCAGAAAAGG
NC_008491_M_reevesi_micrurus   ATAAGTCTTTGTAGTACATTTAATACACTGGTCTTGTAAACCAGAAAAGG
CRF1_M_feae                    ACAAGTCTTTGTAGTATACTCAATACACTGGTCCTGTAAACCAGAAAAGG
                               * ************** * * ************ ****** *********

                                      3'tRNA-Thr <> 5'tRNA-Pro
CRP9_A_porcinus                AGAATAATTAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA 100
CRP12_A_porcinus               AGAATAATTAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRP6_A_porcinus                AGAATAATTAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRP5_A_porcinus                AGAATAATTAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRA1_A_axis                    AGAGTAATTAACCTCCCTAAGACTCAAGGAAGAAGCAATAGCCCCACGTA
CRU4_C_unicolor                AGAGCAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-CA
EF035448_C_unicolor_swinhoei   AGAGCAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
NC_007704_C_elaphus            AGAGCAACTAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
EF058308_C_nippon_taiouanus    AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRT1_C_eldi                    AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRT9_C_eldi                    AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-TA
CRM2_M_muntjak                 AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-CA
NC_004563_M_muntjak            AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-CA
CRM3_M_muntjak                 AGAACAACCAACCTCCCTAAGACTCAAGGAAGAAGCCATAGCCCCAC-CA
NC_008491_M_reevesi_micrurus   AGGACAACCAATCTCCCTAAGACTCAAGGAAGAAGCCACAGCCCCAC-CG
CRF1_M_feae                    AGAACAACCAACCTCCCTAAGACTCAAAATCGATTCGTCAGTCTTAC-CA
                               **   **  ** ***************    **  *   ** *  **

                                                          3'tRNA-Pro <  > 5'
CRP9_A_porcinus                TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTAT 150
CRP12_A_porcinus               TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTAT
CRP6_A_porcinus                TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTAT
CRP5_A_porcinus                TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTAT
CRA1_A_axis                    TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTGT
CRU4_C_unicolor                TCAACCCCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--TTAT
EF035448_C_unicolor_swinhoei   TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGC--TTAT
NC_007704_C_elaphus            TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGC--TTAT
EF058308_C_nippon_taiouanus    TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--TTAT
CRT1_C_eldi                    TCAACACCCAAAGCTGAAGTTCTATTTAAACTACTCCTTGATGC--TTAT
CRT9_C_eldi                    TCAACACCCAAAGCTGAAGTTCTATTTAAACTACTCCTTGATGC--TTAT
CRM2_M_muntjak                 TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--ATAT
NC_004563_M_muntjak            TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--ATAT
CRM3_M_muntjak                 TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--ATAT
NC_008491_M_reevesi_micrurus   TCAACACCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGACGC--ATAT
CRF1_M_feae                    TCAACCCCCAAAGCTGAAGTTCTATTTAAACTATTCCCTGATGC--ATAT
                               ***** *************************** *** *** **   * *
```

Figure 25 (Continued)

```
                               Control region (D-loop)
CRP9_A_porcinus                TAATATAGTTCCATAAAAATCAAGAACTTTATTAGTATTAAATTTCCAAA 200
CRP12_A_porcinus               TAATATAGTTCCATAAAAATCAAGAACTTTATTAGTATTAAATTTCCAAA
CRP6_A_porcinus                TAATATAGTTCCATAAAAATCAAGAACTTTATTAGTATTAAATTTCCAAA
CRP5_A_porcinus                TAATATAGTTCCATAAAAATCAAGAACTTTATTAGTATTAAATTTCCAAA
CRA1_A_axis                    TAATATAGTTCCATAAAAATCAAGAACTTTATTAGTATTAAATTTCCAAA
CRU4_C_unicolor                TAATATAGTTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
EF035448_C_unicolor_swinhoei   TAATATAGTTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
NC_007704_C_elaphus            TAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCAAA
EF058308_C_nippon_taiouanus    TAATATAGTTCCATAAAAATCAAGAACTTTATCAGTATTAAATTTCCAAA
CRT1_C_eldi                    TAATATAGCTCCATAAAAACCAAGAACTTTATCAGTATTAAATTTCCAAA
CRT9_C_eldi                    TAATATAGCTCCATAAAAACCAAGAACTTTATCAGTATTAAATTTCCAAA
CRM2_M_muntjak                 TAATATAGCTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
NC_004563_M_muntjak            TAATATAGCTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
CRM3_M_muntjak                 TAATATAGCTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
NC_008491_M_reevesi_micrurus   TAATATAGCTTCACAAAAATCAAGAACCTTATTAGTATTAAATTTCCAAA
CRF1_M_feae                    TAATATAGCTCCATAAAAATCAAGAACCTTATCAGTATTAAATTTCCAAA
                               ******** * ** ***** ******* **** *****************

CRP9_A_porcinus                AAAA-TTAATAATTCAATACAGCTTTCT-ACTCAACACCCAAATTTACAT 250
CRP12_A_porcinus               AAAA-TTAATAATTCAATACAGCTTTCT-ACTCAACACCCAAATTTACAT
CRP6_A_porcinus                AAAA-TTAATAATTCAATACAGCTTTCT-ACTCAACACCCAAATTTACAT
CRP5_A_porcinus                AAAAATTAATAATTCAATACAGCTTTCT-ACTCAACACCCAAATTTACAT
CRA1_A_axis                    AATT-TTAACAATTCAATACAGCTTTCT-ACTCAACATCCAA-TTTACAC
CRU4_C_unicolor                AAATTTTAATATTTTAATACAGCTTTCT-ACTCAACACCCAA-TTTACAT
EF035448_C_unicolor_swinhoei   AAATTTTAATATTTCAATACAGCTTTCT-ACTCAACATCCAA-TTTACAT
NC_007704_C_elaphus            AAGTTTTAATATTTCAATACAGCTTTCC-ACTCAACACCCAT-TTTACAT
EF058308_C_nippon_taiouanus    AA-TTTTAATATTTTAATACAGCTTTCT-ACTCAACATCCAA-TTTACAT
CRT1_C_eldi                    AAATTACAATAATTTAATAC-GCTTTCT-ACTCAAGACCCAA-TTTACAT
CRT9_C_eldi                    AAATTACAATAATTTAATACAGCTTTCT-ACTCAAGACCCAA-TTTACAT
CRM2_M_muntjak                 AACCTTTAGTAATTTAACACAGCTTTCTCACTCAACAGCCAA-TTTACAT
NC_004563_M_muntjak            AACCTTTAGTAATTTAACACAGCTTTCTCACTCAACAGCCAA-TTTACAT
CRM3_M_muntjak                 AACCTTTAGTAATTTAACACAGCTTTCTCACTCAACAGCCAA-TTTACAT
NC_008491_M_reevesi_micrurus   AATCTTTAGTAATTTAACACAGCTTTTTCACTCAACAGCCAA-TTTAAAC
CRF1_M_feae                    AACCTTTAGTAATTTAACACAGCTTTCTCACTCAACAGCTAA-TTTATAC
                               **     *  * ** ** ** *****   ****** * * *  **** *

CRP9_A_porcinus                TTTATATAGTATTTCCCACACGA----CATAACATATAATGCA------C 300
CRP12_A_porcinus               TTTATATAGTATTTCCCACACGA----CATAACATATAATGCA------C
CRP6_A_porcinus                TTTATATAGTATTTCCCACACGA----CATAACATATAATGCA------C
CRP5_A_porcinus                TTTATATAGTATTTCCCACACGA----CATAACATATAATGCA------C
CRA1_A_axis                    CCAACATACCATTTTCTACACAA----CATGACATGCAATGCA------T
CRU4_C_unicolor                CTTACATCCCACTAACTAAACAA----CATAACATGTGATATTAT--AAC
EF035448_C_unicolor_swinhoei   TTTACATCCCACTAACTAAACAA----CATAACATGTGACAGTAT--AAC
NC_007704_C_elaphus            TTTACATCC-ACCAACCACACAA----CAAAATATGTAATAACA-----C
EF058308_C_nippon_taiouanus    TTTATGTCCTACTAATTACACAG----CAAAACACGTGATACAA-----C
CRT1_C_eldi                    CTTACGTCCCACTAACTACACGA----TAAAGCATGCACTACAA-----C
CRT9_C_eldi                    CTTACGTCCCACTAACTACACGA----TAAAGCATGCACTACAA-----C
CRM2_M_muntjak                 TTTACATACCATTATCTACACAAGCCACATGATAAGGTATATAATCTAAC
NC_004563_M_muntjak            TTTACATACCATTATCTACACAAGCCACATGATAAGGTATATAATCTAAC
CRM3_M_muntjak                 TTTACATACCATTATCTACACAAGCCACATGATAAGGTATATAATCTAAC
NC_008491_M_reevesi_micrurus   TTTACATATAATCAACTACACAAACCACATAATAATATGTATGATTTAAT
CRF1_M_feae                    ATTACATACCATTAACTACACGAACCACATGATGAAATATATAATTTAGC
                                     *  *   *      * **       *
                                             >>> TS2 region
CRP9_A_porcinus                TTCATGCGCTT--------------------------------------- 350
CRP12_A_porcinus               TTCATGCGCTT---------------------------------------
CRP6_A_porcinus                TTCATGCGCTT---------------------------------------
CRP5_A_porcinus                TTCATGCGCTT---------------------------------------
CRA1_A_axis                    TGCATGCGCTT---------------------------------------
CRU4_C_unicolor                CTTGCGTACTCGCGATACATAAAATTAATGTATTAAGACATATTGCGTAT
EF035448_C_unicolor_swinhoei   CTCGCGCACTCGCAGTACGTAAAATTAGTGTGTTAAGACATATTATATAT
NC_007704_C_elaphus            CTTATGCGCTT---------------------------------------
EF058308_C_nippon_taiouanus    CTTATGTGTTTGTAATACATAAAATTAGTGCATTAAGACACA--------
CRT1_C_eldi                    CTCATGCGCTT---------------------------------------
CRT9_C_eldi                    CTCATGCGCTT---------------------------------------
CRM2_M_muntjak                 CATATGCGCTT---------------------------------------
```

Figure 25 (Continued)

```
NC_004563_M_muntjak            CATATGCGCTT---------------------------------------
CRM3_M_muntjak                 CATATGCGCTT---------------------------------------
NC_008491_M_reevesi_micrurus   TTTATGCGCTT---------------------------------------
CRF1_M_feae                    CTTATGCGCTT---------------------------------------
                                     *   *

CRP9_A_porcinus                -------------------------------------------------- 400
CRP12_A_porcinus               --------------------------------------------------
CRP6_A_porcinus                --------------------------------------------------
CRP5_A_porcinus                --------------------------------------------------
CRA1_A_axis                    --------------------------------------------------
CRU4_C_unicolor                AATAGCACATAAAATTAGTGTGTTAGGACATACTATGTATGATAGTACAT
EF035448_C_unicolor_swinhoei   AATAGTACATAAAATTAATGTATTAGGACATATCATGTATAATAGTACAT
NC_007704_C_elaphus            --------------------------------------------------
EF058308_C_nippon_taiouanus    ---------------------------------CATGTATAACAGTACAT
CRT1_C_eldi                    --------------------------------------------------
CRT9_C_eldi                    --------------------------------------------------
CRM2_M_muntjak                 --------------------------------------------------
NC_004563_M_muntjak            --------------------------------------------------
CRM3_M_muntjak                 --------------------------------------------------
NC_008491_M_reevesi_micrurus   --------------------------------------------------
CRF1_M_feae                    --------------------------------------------------

                                             TS2 region <<< >    TAS-4     <
CRP9_A_porcinus                -------------------------------ATAGTACATAAAATTAATG 450
CRP12_A_porcinus               -------------------------------ATAGTACATAAAATTAATG
CRP6_A_porcinus                -------------------------------ATAGTACATAAAATTAATG
CRP5_A_porcinus                -------------------------------ATAGTACATAAAATTAATG
CRA1_A_axis                    -------------------------------ATAGTACATAAAATTAATG
CRU4_C_unicolor                GAAATTAATGTATTAGGACATATTATGTATAATAGTACATAAAATTAATG
EF035448_C_unicolor_swinhoei   AAAATTAATGTATTAGGACATATCATGTATAATAGTACATAAAATTAATG
NC_007704_C_elaphus            -------------------------------ATAGTACATAGAATTAATG
EF058308_C_nippon_taiouanus    AAAATCGGTGTAT-AGGACATATTATGTATAATAGTACATA-AATTAATG
CRT1_C_eldi                    -------------------------------ATAGTACATAAAATTAATG
CRT9_C_eldi                    -------------------------------ATAGTACATAAAATTAATG
CRM2_M_muntjak                 -------------------------------ATAGTACATAAAATTAATG
NC_004563_M_muntjak            -------------------------------ATAGTACATAAAATTAATG
CRM3_M_muntjak                 -------------------------------ATAGTACATAAAATTAATG
NC_008491_M_reevesi_micrurus   -------------------------------ATAGTACATAGAATTAATG
CRF1_M_feae                    -------------------------------ATAGTACATAAAATTAATG
                                                              ********** ********
                               >                TAS-3                <>stop<
CRP9_A_porcinus                TATTAGGACATATTATGTATAATAGTACATTACATTATATTCCCCATGCT 500
CRP12_A_porcinus               TATTAGGACATATTATGTATAATAGTACATTACATTATATTCCCCATGCT
CRP6_A_porcinus                TATTAGGACATATTATGTATAATAGTACATTACATTATATTCCCCATGCT
CRP5_A_porcinus                TATTAGGACATATTATGTATAATAGTACATTACATTATATTCCCCATGCT
CRA1_A_axis                    TATTAGGACATACTATGTATAATAGTACATTACATTACATTCCCCATGCT
CRU4_C_unicolor                TATTAGGACATACTATGTATAATAGTACATTATATCATATGCCCCATGCT
EF035448_C_unicolor_swinhoei   TATTAGGACATATTATGTATAATAGTACATTATATTATATGCCCCATGCT
NC_007704_C_elaphus            TACTAGGACATACTATGTATAATAGTACATTATATTATATGCCCCATGCA
EF058308_C_nippon_taiouanus    TATTAGGACATATTATGTATAATAGTACATTATATTATATGCCCCATGCT
CRT1_C_eldi                    TATTAGGACATACTATGTATAATAGTACATTACATTATATGCCCCATGCT
CRT9_C_eldi                    TATTAGGACATACTATGTATAATAGTACATTACATTATATGCCCCATGCT
CRM2_M_muntjak                 TATCAAGACATATTATGTATAATAGTACATTACATTATATGCCCCATGCT
NC_004563_M_muntjak            TATTAGGACATATTATGTATAATAGTACATTACATTACATGCCCCATGCT
CRM3_M_muntjak                 TATCAAGACATATTATGTATAATAGTACATTACATTATATGCCCCATGCT
NC_008491_M_reevesi_micrurus   TAATAGGACATACTATGTATAATAGTACATTACATTATATGCCCCATGCT
CRF1_M_feae                    TATTAGGACATACTATGTATAATAGTACATTATATTACATACCCCATGCT
                               **  * ****** ******************* ** * ** ********

CRP9_A_porcinus                TATAAGCATGTACATCCTATTATTTATAGTACATAGTACATAATATTGTC 550
CRP12_A_porcinus               TATAAGCATGTACATCTTATTATTTATAGTACATAGTACATAATATTGTC
CRP6_A_porcinus                TATAAGCATGTACATCTTATTATTTATAGTACATAGTACATAGTATTGTC
CRP5_A_porcinus                TATAAGCATGTACATCTTATTATTTATAGTACATAGTACATAATATTGTC
CRA1_A_axis                    TATAAGCATGTACCCCTCATCATTTATAGTACATAGTACATAGTGTTGTT
CRU4_C_unicolor                TATAAGCATGTATACTCTATCATTTACAGTACATAGTACATGATGTTGTT
EF035448_C_unicolor_swinhoei   TATAAGCATGTATTTTCTGTCATTTACAGTACATAGTACATGATGTTGTT
```

Figure 25 (Continued)

```
NC_007704_C_elaphus            TATAAGCATGTACTTTCTATTATTTATAGTACATAGTACATGATGTTGTT
EF058308_C_nippon_taiouanus    TATAAGCATGTATTTTCTATCATTTATAGTACATAGTACATGATGTTGTC
CRT1_C_eldi                    TATAAGCATGTATTTCCTATTATTTACAGTACATAGTACATACTATTGTT
CRT9_C_eldi                    TATAAGCATGTATTTCCTATTATTTACAGTACATAGTACATACTATTGTT
CRM2_M_muntjak                 TATAAGCACGTATATTCCATTATTTACAGTACATAGTACATATCCTTGCT
NC_004563_M_muntjak            TATAAGCACGTATATTCCATTATTTACAGTACATGGTACATATCCTTGCT
CRM3_M_muntjak                 TATAAGCACGTATATTCCATTATTTACAGTACATAGTACATATCCTTGCT
NC_008491_M_reevesi_micrurus   TATAAGCACGTACATTCTATTATTTACAGTACATAGTACATGTCCCTGCT
CRF1_M_feae                    TATAAGCATGTACATTATATTATTTATAGTACATAGTACATGCTCTTGCT
                               ******** ***       * ***** ******* ******     **

CRP9_A_porcinus                C-ATCGTACATGGCACATT-GAGTCAAATCAGTCCTTGTCAACATGCGTA 600
CRP12_A_porcinus               C-ATCGTACATGGCACATT-GAGTCAAATCAGTCCTTGTCAACATGCGTA
CRP6_A_porcinus                C-ATCGTACATGGCACATT-GAGTCAAATCAGTCCTTGTCAACATGCGTA
CRP5_A_porcinus                C-ATCGTACATGGCACATT-GAGTCAAATCAGTCCTTGTCAACATGCGTA
CRA1_A_axis                    C-ATCGTACATAGCACATT-GAGTCAAATCAATCCTTGTCAACATGCGTA
CRU4_C_unicolor                C-ATCGTACATAGTACATT-AAGTCAAATCAGTCCTTGTCAACATGCGTA
EF035448_C_unicolor_swinhoei   C-ATCGTACATAGTGCATT-AAGTCAAATCAATCCTCGTCAACATGCGTA
NC_007704_C_elaphus            C-ATCGTACATAGCGCATT-AAGTCAAATCAGTCCCTGTCAACATGCGTA
EF058308_C_nippon_taiouanus    C-ATCGTACATAGTGCATC-AAGTCAAATCAGTCCTCGTCAACATGCGTA
CRT1_C_eldi                    C-ATCGTACATGGCGCATT-GAGTCAAATCAACTCTTGTCAACATGCGTA
CRT9_C_eldi                    C-ATCGTACATGGCGCATT-GAGTCAAATCAACTCTTGTCAACATGCGTA
CRM2_M_muntjak                 TGGTAGTACATAGCACATTTAAGTCAAATCAATTCTCGCGAACATGCGTA
NC_004563_M_muntjak            TGATAGTACATAGCACATTTAAGTCAAATCAATTCTTGCGAACATGCGTA
CRM3_M_muntjak                 TGGTAGTACATAGCACATTTAAGTCAAATCAATTCTCGCGAACATGCGTA
NC_008491_M_reevesi_micrurus   TAATCGTACATAGCACATTTAAGTCAAATCAATCCTCGCCAGCATGCGTA
CRF1_M_feae                    TAGCAGTACATAGCACATTTAAGTCAAATCAATTCTTGCGAACATGCGTA
                                    ****** *  ***   **********   *  *  * ********
                                                            > CSB-F
CRP9_A_porcinus                TCCCGTCCCCTAGATCACGAGCTTAACTACCATGCCGCGTGAAACCAACA 650
CRP12_A_porcinus               TCCCGTCCCCTAGATCACGAGCTTAACTACCATGCCGCGTGAAACCAACA
CRP6_A_porcinus                TCCCGTCCCCTAGATCACGAGCTTAACTACCATGCCGCGTGAAACCAACA
CRP5_A_porcinus                TCCCGTCCCCTAGATCACGAGCTTAACTACCATGCCGCGTGAAACCAACA
CRA1_A_axis                    TCCCGTCCCCTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAACA
CRU4_C_unicolor                TCCCGTCCACTAGATCACGAGCTTAATCACCATGCCGCGTGAAACCAGCA
EF035448_C_unicolor_swinhoei   TCCCGTCCGCTAGATCACGAGCTTAATCACCATGCCGCGTGAAACCAGCA
NC_007704_C_elaphus            TCCCGTCCCCTAGATCACGAGCTTGATCACCATGCCGCGTGAAACCAGCA
EF058308_C_nippon_taiouanus    TCCCGTCCCCTAGATCACGAGCTTGATCACCATGCCGCGTGAAACCAGCA
CRT1_C_eldi                    TCCCGTCCCCTAGATCGCGAGCTTAATGACCATGCCGCGTGAAACCAGCA
CRT9_C_eldi                    TCCCGTCCCCTAGATCGCGAGCTTAATGACCATGCCGCGTGAAACCAGCA
CRM2_M_muntjak                 TCCCGTCCCTTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCA
NC_004563_M_muntjak            TCCCGTCCCTTAGATCACGAGCTTAATTACCATGCCGCGTGAAACCAGCA
CRM3_M_muntjak                 TCCCGTCCCTTAGATCGCGAGCTTAATTACCATGCCGCGTGAAACCAGCA
NC_008491_M_reevesi_micrurus   TCCCGTCCACTAGATCACGAGCTTAGTCACCATGCCGCGTGAAACCAGCA
CRF1_M_feae                    TCCCGTCCCCTAGATCACGAGCTTAATCACCATGCCGCGTGAAACCAACA
                               ********  ****** *******    ******************  **

CRP9_A_porcinus                ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATATATTGTGGG 700
CRP12_A_porcinus               ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATATATTGTGGG
CRP6_A_porcinus                ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATATATTGTGGG
CRP5_A_porcinus                ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATATATTGTGGG
CRA1_A_axis                    ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATAAACTGTGGG
CRU4_C_unicolor                ACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGAATCGTGGG
EF035448_C_unicolor_swinhoei   ACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGAATTGTGGG
NC_007704_C_elaphus            ACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGAACTGTGGG
EF058308_C_nippon_taiouanus    ACCCGCTGGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGAATTGTGGG
CRT1_C_eldi                    ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGTATTGTGGG
CRT9_C_eldi                    ACCCGCTAGGCAGGGATCCCTCTTCTCGCTCCGGGCCCATGTATTGTGGG
CRM2_M_muntjak                 ACCCGCTTGGCAGGGATCCTTGTTCTCGCTCCGGGCCCATACACCGTGGG
NC_004563_M_muntjak            ACCCGCTTGGCAGGGATCCTTGTTCTCGCTCCGGGCCCATGCACCGTGGG
CRM3_M_muntjak                 ACCCGCTTGGCAGGGATCCTTGTTCTCGCTCCGGGCCCATACATTGTGGG
NC_008491_M_reevesi_micrurus   ACCCGCTTGGCAGGGATCCTTGTTCTCGCTCCGGGCCCATGTATTGTGGG
CRF1_M_feae                    ACCCGCTTGGCAGGGATCCTTGTTCTCGCTCCGGGCCCATATACCGTGGG
                               ******* *********** * ******************  *  *****

CRP9_A_porcinus                GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTTCAGGGCC 750
CRP12_A_porcinus               GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRP6_A_porcinus                GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
```

Figure 25 (Continued)

```
CRP5_A_porcinus                GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRA1_A_axis                    GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRU4_C_unicolor                GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTTTTCAGGGCC
EF035448_C_unicolor_swinhoei   GGTAGCTACTTAATGAACTTTATCAGACATCTGGTTCTTTTTTCAGGGCC
NC_007704_C_elaphus            GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTTTTCAGGGCC
EF058308_C_nippon_taiouanus    GGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTTTTCAGGGCC
CRT1_C_eldi                    GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRT9_C_eldi                    GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRM2_M_muntjak                 GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
NC_004563_M_muntjak            GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRM3_M_muntjak                 GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
NC_008491_M_reevesi_micrurus   GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
CRF1_M_feae                    GGTAGCTATTTAATGAACTTTATCAGACATCTGGTTCTTTCTTCAGGGCC
                               ******** ******** ********************** *********

CRP9_A_porcinus                ATCTCATCTAAAATCGCCCACTCTTTCCCCTTAAATAAGACATCTCGATG 800
CRP12_A_porcinus               ATCTCACCTAAAATCGCCCACTCTTTCCCCTTAAATAAGACATCTCGATG
CRP6_A_porcinus                ATCTCACCTAAAATCGCCCACTCTTTCCCCTTAAATAAGACATCTCGATG
CRP5_A_porcinus                ATCTCACCTAAAATCGCCCACTCTTTCCCCTTAAATAAGACATCTCGATG
CRA1_A_axis                    ATCTCACCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATG
CRU4_C_unicolor                ATCTCACCTAAAATCGCCCACTCCTTGCAAT---ATAAGACATCTCGATG
EF035448_C_unicolor_swinhoei   ATCTCACCTAAAATCGCCCACTCCTTGTAGT---ATAAGACATCTCGATG
NC_007704_C_elaphus            ATCTCACCTAAAATCGCCCACTCCTTGCAAT---ATAAGACATCTCGATG
EF058308_C_nippon_taiouanus    ATCTCACCTAAAATCGCCCACTCCTTGCAAT---ATAAGACATCTCGATG
CRT1_C_eldi                    ATCTCATCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATG
CRT9_C_eldi                    ATCTCATCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATG
CRM2_M_muntjak                 ATCTCACCTAAAATCGCCCACTCGTTCCTCTTAAATAAGACATCTCGATG
NC_004563_M_muntjak            ATCTCACCTAAAATCGCCCACTCGTTCCTCTTAAATAAGACATCTCGATG
CRM3_M_muntjak                 ATCTCACCTAAAATCGCCCACTCGTTCCTCTTAAATAAGACATCTCGATG
NC_008491_M_reevesi_micrurus   ATCTCACCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATG
CRF1_M_feae                    ATCTCACCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATG
                               ****** **************** **    *   ****************

CRP9_A_porcinus                GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT 850
CRP12_A_porcinus               GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRP6_A_porcinus                GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRP5_A_porcinus                GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRA1_A_axis                    GACTAATGACTAATCAGCCCATGCTCACACATAACTGGGGGGTCATACAT
CRU4_C_unicolor                GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGCGTCATACAT
EF035448_C_unicolor_swinhoei   GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
NC_007704_C_elaphus            GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
EF058308_C_nippon_taiouanus    GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRT1_C_eldi                    GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRT9_C_eldi                    GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRM2_M_muntjak                 GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
NC_004563_M_muntjak            GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
CRM3_M_muntjak                 GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
NC_008491_M_reevesi_micrurus   GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGATGTCATACAT
CRF1_M_feae                    GACTAATGACTAATCAGCCCATGCTCACACATAACTGTGGTGTCATACAT
                               ************************************* *  *********
                                 CSB-B < > OH
CRP9_A_porcinus                TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCATTGGCCGTCT 900
CRP12_A_porcinus               TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCATTGGCCGTCT
CRP6_A_porcinus                TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCATTGGCCGTCT
CRP5_A_porcinus                TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCATTGGCCGTCT
CRA1_A_axis                    TTGGTATTTTTAATTTTTGGGGG-ATGCTTGGACTCAGCATTGGCCGTCT
CRU4_C_unicolor                TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGGCCGTCT
EF035448_C_unicolor_swinhoei   TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGGCCGTCT
NC_007704_C_elaphus            TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGACCGTCT
EF058308_C_nippon_taiouanus    TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGGCCGTCT
CRT1_C_eldi                    TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGACCGTCT
CRT9_C_eldi                    TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGACCGTCT
CRM2_M_muntjak                 TTGGTATTTTTATTTTTTGGGGG-ATGCTTGGACTCAGCTATGGCCGTCT
NC_004563_M_muntjak            TTGGTATTTTTATTTTTTGGGGG-ATGCTTGGACTCAGCTATGGCCGTCT
CRM3_M_muntjak                 TTGGTATTTTTATTTTTTGGGGG-ATGCTTGGACTCAGCTATGGCCGTCT
NC_008491_M_reevesi_micrurus   TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCTATGGCCGTCT
CRF1_M_feae                    TTGGTATTTTTAATTTTTGGGGGGATGCTTGGACTCAGCAATGGCCGTCT
                               ************ ********** ***************  ** ******
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Figure 25 (Continued)

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CRP9_A_porcinus                GA-GGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG 950
CRP12_A_porcinus               GA-GGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
CRP6_A_porcinus                GA-GGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
CRP5_A_porcinus                GA-GGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
CRA1_A_axis                    GA-GGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
CRU4_C_unicolor                GA-GGCCCCGTCCCAGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
EF035448_C_unicolor_swinhoei   GA-GGCCCCGTCCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
NC_007704_C_elaphus            GGCGGTCCCGTCCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
EF058308_C_nippon_taiouanus    GA-GGCCCCGTCTCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
CRT1_C_eldi                    GGCGGTCCCGTCCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
CRT9_C_eldi                    GGCGGTCCCGTCCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
CRM2_M_muntjak                 GA-GGCCCCGACCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
NC_004563_M_muntjak            GA-GGCCCCGACCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
CRM3_M_muntjak                 GA-GGCCCCGACCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
NC_008491_M_reevesi_micrurus   GA-GGCCCCGACCCGGAGCATGAATTGTAGCTGGACTTAACTGCATCTTG
CRF1_M_feae                    GA-GGCCCCGTCCCAGAGCATAAATTGTAGCTGGACTTAACTGCATCTTG
                               *  ** **** * * ****** ****************************
                                                        OH <> CSB-1
CRP9_A_porcinus                AGCATCCCCATAATGGTAGGCGTGGGGCATGGCAGTCAATGGTCACAGGA 1000
CRP12_A_porcinus               AGCATCCCCATAATGGTAGGCGTGGGGCATGGCAGTCAATGGTCACAGGA
CRP6_A_porcinus                AGCATCCCCATAATGGTAGGCGTGGGGCATGGCAGTCAATGGTCACAGGA
CRP5_A_porcinus                AGCATCCCCATAATGGTAGGCGTGGGGCATGGCAGTCAATGGTCACAGGA
CRA1_A_axis                    AGCATCCCCATAATGGTAGGCGCGGGGCATGGCAGTCAATGGTCCCAGGA
CRU4_C_unicolor                AGCATCCCCATAATGATAGGCAT-GGACATTGCAGTCAATGGTCACAGGA
EF035448_C_unicolor_swinhoei   AGCATCCCCATAATGGTAGGCAT-GGGCATTGCAGTCAATGGTCACAGGA
NC_007704_C_elaphus            AGCATCCCCATAATGGTAGGCAT-GGGCATGGCAGTCAATGGTCACAGGA
EF058308_C_nippon_taiouanus    AGCATCCCCATAATGGTAGGCGCAGGGCATTGCAGTCAATGGTCACAGGA
CRT1_C_eldi                    AGCACCCCCATAATGGTAGGCAT-GGGCATTGCTGTCAATGGTCACAGGA
CRT9_C_eldi                    AGCACCCCCATAATGGTAGGCAT-GGGCATTGCAGTCAATGGTCACAGGA
CRM2_M_muntjak                 AGCATCCCCATAATGGTAGGCGCAGGGCATTGCAGTCAATGGTTACAGGA
NC_004563_M_muntjak            AGCATCCCCATAATGGTAGGCGCAGGGCATTGCAGTCAATGGTTACAGGA
CRM3_M_muntjak                 AGCATCCCCATAATGGTAGGCGCAGGGCATTACAGTCAATGGTCACAGGA
NC_008491_M_reevesi_micrurus   AGCATCCCCATAATGGTAGGCGCAGGGCATTACAGTCAATGGTCACAGGA
CRF1_M_feae                    AGCATCCCCATAATGATAGGCAT-GGGCATTGCAGTCAATGGTCACAGGA
                               **** ********** *****   ** ***  * *********  *****

                               CSB-1 <>      RS3 region     < > CSB-2+3
CRP9_A_porcinus                CATAATTATTATTTCATGAATTAATCCCATAGTATTTATTTTT-CCCCCC 1050
CRP12_A_porcinus               CATAATTATTATTTCATGAATTAATCCCATAGTATTTATTTTT-CCCCCC
CRP6_A_porcinus                CATAATTATTATTTCATGAATTAATCCCATAGTATTTATTTTT-CCCCCC
CRP5_A_porcinus                CATAATTATTATTTCATGAATTAATCCCATAGTATTTATTTTTTCCCCCC
CRA1_A_axis                    CATAGTTATTATTTCAGGGGTCAACCCTTTA-------------------
CRU4_C_unicolor                CATAATTATTATTTCATGAGTCAACCCTAAGGC-----------------
EF035448_C_unicolor_swinhoei   CATAGTCATTATTTCATGAGTCAACCCTAAGAC-----------------
NC_007704_C_elaphus            CATAATCATTATTTCATGAGTCAACCCTAAGAT-----------------
EF058308_C_nippon_taiouanus    CATAGTTATTATTCCATGAATCAACCCTAAGAT-----------------
CRT1_C_eldi                    CATAATTATTATTTCATGAATCAACCCTAAGAT-----------------
CRT9_C_eldi                    CATAATTATTATTTCATGAATCAACCCTAAGAT-----------------
CRM2_M_muntjak                 CATAGTTATTATTTCAAGACTCAACTTTATAAT-----------------
NC_004563_M_muntjak            CATAGTTATTATTTCAAGACTCAACTTTATAAT-----------------
CRM3_M_muntjak                 CATAGTTATTATTTCAAGACTCAACTTTATAAT-----------------
NC_008491_M_reevesi_micrurus   CATAGTCATTATTTCAAGATTCAACTTTACAAT-----------------
CRF1_M_feae                    CATAATTATTATTTCATGAGTCAACCCTAAGAC-----------------
                               **** * ****** ** *  * **
                                                          CSB-2+3  <
CRP9_A_porcinus                CTTATTTTTTCCCCCCCCTTTTTTATTTTT-CCCCCTTATATAGCTACC 1100
CRP12_A_porcinus               CTTATTTTTTCCCCCCC-TTTTTTATTTTT-CCCCCTTATATAGCTACC
CRP6_A_porcinus                CTTATTTTTTCCCCCCCCTTTTTTTATTTTTTCCCCCTTATATAGCTACC
CRP5_A_porcinus                CTTATTTTTTCCCCCCCCTTTTTTTATTTTT-CCCCCTTATATAGCTACC
CRA1_A_axis                    ---ATTTTTTTTCCCCCCCCCTCATATTTTC--CCCCTTATATAGTTACC
CRU4_C_unicolor                ---CTATATTTCCCCCCCCTCCATTAATTTTTCCCCCTTATATAGTTATC
EF035448_C_unicolor_swinhoei   ---CTATATTTCCCCCCCCTCCATTAATTTTTCCCCCTTATATAGTTATC
NC_007704_C_elaphus            ---CTATTTTCCCCCCCCTGCTA--ATTTTTTCCCCCTTATATAGTTATC
EF058308_C_nippon_taiouanus    ---CTATTTCCCCCCCCTTCTTA---TTTTTTCCCCCTTATATAGTTATC
CRT1_C_eldi                    ---CCTATTTCCCCCCCCTTCCATTATTTTT-CCCCCTTATATAGTCATC
CRT9_C_eldi                    ---CCTATTTCCCCCCCCTTCCATTATTTTT-CCCCCTTATATAGTCATC
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Figure 25 (Continued)

```
CRM2_M_muntjak                 ---CCATTATTCCCCCCCCCTCCT--------------TATATAGTTATC
NC_004563_M_muntjak            ---CCATTATTCCCCCCCCCTCCT--------------TATATAGTTATC
CRM3_M_muntjak                 ---CCATTATTCCCCCCCCCTCCTC------------TTATATAGTTATC
NC_008491_M_reevesi_micrurus   ---CC-TTATCCCCCCCCCCCT----------------TATATAGTTACC
CRF1_M_feae                    ---CTATATTTCCCCCCCCTCCATTAATTTTTCCCCCTTATATAGTTATC
                                           *****                     *******  * *

CRP9_f_porcinus                ACTATTTTTAACACACTTTCCCCTAGATATTATTTCAAATTTATCACATT 1150
CRP12_A_porcinus               ACTATTTTTAACACACTTTCCCCTAGATATTATTTCAAATTTATCACATT
CRP6_A_porcinus                ACTATTTTTAACACACTTTCCCCTAGATATTATTTCAAATTTATCACATT
CRP5_A_porcinus                ACTATTTTTAACACACTTTCCCCTAGATATTATTTCAAATTTATCACATT
CRA1_A_axis                    ACCATTTTTAACACGCTTTCCCCTAGATATTATTTTAAATTTATCACATT
CRU4_C_unicolor                ACCATTTTTAACACACTTTTCCCTAGATATTATTTTAAATTTATCACATT
EF035448_C_unicolor_swinhoei   ACCATTTTTAACACACTTTTCCCTAGATATTATTTCAAATTTATCACATT
NC_007704_C_elaphus            ACCATTTTTAACACACTTTTCCCTAAATATTATTTTAAATTTATCACATT
EF058308_C_nippon_taiouanus    ACCTTTTTTAACACACTTTTCCCTAGATATTATTTTAAATTTATCACATT
CRT1_C_eldi                    ACCATTTTTAACACGCTTCCCCCTAGATATAATTTTAAATTTATCACGTT
CRT9_C_eldi                    ACCATTTTTAACACGCTTCCCCCTAGATATAATTTTAAATTTATCACGTT
CRM2_M_muntjak                 ACCTTTTTCAACACGCTTTTCCCTAGATAGTATTTTAAATTTATCGCATT
NC_004563_M_muntjak            ACCTTTTTTAACACGCTTTTCCCTAGATAGTATTTTAAATTTATCGCATT
CRM3_M_muntjak                 ACCTTTTTTAACACGCTTTTCCCTAGATAGTATTTTAAATTTATCGCATT
NC_008491_M_reevesi_micrurus   ATCTTTTTTAACACGCTTTTCCCTAGATAATATTTTAAATTTATCGCATT
CRF1_M_feae                    ACCATTTTTAACACACTTTTCCCTAGATATTATTTTAAATTTATCACATT
                               *   **** ***** ***  ***** ***  **** ********* * **

CRP9_A_porcinus                TCCAATACTCAAAT-AGCACTCCAGAAGAAGGTAAGTATATAAGCGCCAA 1200
CRP12_A_porcinus               TCCAATACTCAAAT-AGCACTCCAGAAGAAGGTAAGTATATAAGCGCCAA
CRP6_A_porcinus                TCCAATACTCAAAT-AGCACTCCAGAAGAAGGTAAGTATATAAGCGCCAA
CRP5_A_porcinus                TCCAATACTCAAAT-AGCACTCCAGAAGAAGGTAAGTATATAAGCGCCAA
CRA1_A_axis                    TCCAATACTCAAAT-GGCACTCCATAAGGAGGTAAGTATATAAGCGCCTA
CRU4_C_unicolor                TCCAATACTCAAATTAGCACTCCAGGGGG-GGTAAGTATATAAGCGCCAA
EF035448_C_unicolor_swinhoei   TCCAATACTCAAATTAGCACTCCAGGGGGAGGTAAGTATATAAGCGCCAA
NC_007704_C_elaphus            TCCAATACTCAAATCAGCACTCCAGGGGGTGGTAACTATATAAACGCCAA
EF058308_C_nippon_taiouanus    TCCAATACTCAAATTAGCACTCCAGAGGGAGGTAAGTATATAAACGCCAA
CRT1_C_eldi                    TCCAATACTCAAATTGGCACTCCAGGAGCAGGTAACTATATAAGCGCCAA
CRT9_C_eldi                    TCCAATACTCAAATTGGCACTCCAGGAGCAGGTAACTATATAAGCGCCAA
CRM2_M_muntjak                 TTCAATACTCAAATTAGCACTCCAGAAGGAGGTAAGTATATAAGCGCCAA
NC_004563_M_muntjak            TTCAATACTCAAATTAGCACTCCAGAAGGAGGTAAGTATATAAGCGCCAA
CRM3_M_muntjak                 TTCAATACTCAAATTAGCACTCCAGAAGGAGGTAAGTATATAAGCGCCAA
NC_008491_M_reevesi_micrurus   TTCAATACTCAAAT-AGCACTCCAGGGGGAGGTAACTATATAAGCGCCAA
CRF1_M_feae                    TCCAATACTCAAATTAGCACTCCAGGGGGGGGTAAGTATATAAGCGCCAA
                               * ************  ********   *  ***** ******* **** *

                        3’ Control region (D-loop) <     > 5’tRNA-Phe
CRP9_A_porcinus                TTTTTTCCTAATTCCAT-ACAGTTAATGTAGCTTAAATAATAAAGCAAGG 1250
CRP12_A_porcinus               TTTTTTCCTAATTCCAT-ACAGTTAATGTAGCTTAAATAATAAAGCAAGG
CRP6_A_porcinus                TTTTTTCCTAATTCCAT-ACAGTTAATGTAGCTTAAATAATAAAGCAAGG
CRP5_A_porcinus                TTTTTTCCTAATTCCAT-ACAGTTAATGTAGCTTAAATAATAAAGCAAGG
CRA1_A_axis                    TTTTTTCCTAATTACGC-ACAGTTAATGTAGCTTAAACAATAAAGCAAGG
CRU4_C_unicolor                TTTTTCCCTAATTATGC-ATAGTTGATGTAGCTTAAACAGCAAAGCAAGG
EF035448_C_unicolor_swinhoei   TTTTTCCCTAATTATGC-ATAGTTGATGTAGCTTAAACAGCAAAGCAAGG
NC_007704_C_elaphus            TTTTTCCCTAATTATGT-ACAGTTGATGTAGCTTAAATGGCAAAGCAAGG
EF058308_C_nippon_taiouanus    TTTTTCCCTAATTACGC-ATAGTTAATGTAGCTTAAACAGCAAAGCAAGG
CRT1_C_eldi                    TTTTTCTCTAATTATAC-ACAGTTAATGTAGCTTAAACAGTAAAGCAAGG
CRT9_C_eldi                    TTTTTCTCTAATTATAC-ACAGTTAATGTAGCTTAAACAGTAAAGCAAGG
CRM2_M_muntjak                 TTCTTCATCAATCGCACCACAGTTAATGTAGCTTAAACAATAAAGCAAGG
NC_004563_M_muntjak            TTCTTCATCAATCGCACCACAGTTAATGTAGCTTAAACAATAAAGCAAGG
CRM3_M_muntjak                 TTCTCCATCAATCGCACCACAGTTAATGTAGCTTAAACAATAAAGCAAGG
NC_008491_M_reevesi_micrurus   TCCTCCATCAATCGCACCACAGTTAATGTAGCTTAAGCAATAAAGCAAGG
CRF1_M_feae                    TTTTTCCCTAATTATGC-ATAGTTGATGTAGCTTAAACAGCAAAGCAAGG
                               *  *     ***      * **** ***********     *********
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Figure 25 (Continued)

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                                                   3’tRNA-Phe <
CRP9_A_porcinus               CACTGAAAATGCCTAGATGAGTGTATCAACTCCATAA 1,287
CRP12_A_porcinus              CACTGAAAATGCCTAGATGAGTGTATCAACTCCATAA
CRP6_A_porcinus               CACTGAAAATGCCTAGATGAGTGTATCAACTCCATAA
CRP5_A_porcinus               CACTGAAAATGCCTAGATGAGTGTATCAACTCCATAA
CRA1_A_axis                   CACTGAAAATGCCTAGATGAGTATATTAACTCCATAA
CRU4_C_unicolor               CACTGAAAATGCCTAGATGAGTCTCCCAACTCCATAA
EF035448_C_unicolor_swinhoei  CACTGAAAATGCCTAGATGAGTATATTAACTCCATAA
NC_007704_C_elaphus           CACTGAAAATGCCTAGATGAGTATATTAACTCCATAA
EF058308_C_nippon_taiouanus   CACTGAAAATGCCTAGATGAGTGTATTAACTCCATAA
CRT1_C_eldi                   CACTGAAAATGCCTAGATGAGTGTACTAACTCCATAA
CRT9_C_eldi                   CACTGAAAATGCCTAGATGAGTGTACTAACTCCATAA
CRM2_M_muntjak                CACTGAAAATGCCTAGATGAGTGTATTAACTCCATAA
NC_004563_M_muntjak           CACTGAAAATGCCTAGATGAGTGTATTAACTCCATAA
CRM3_M_muntjak                CACTGAAAATGCCTAGATGAGTCTCCCAACTCCATAA
NC_008491_M_reevesi_micrurus  CACTGAAAATGCCTAGATGAGTGTACTAGCTCCATAA
CRF1_M_feae                   CACTGAAAATGCCTAGATGAGTATATTAACTCCATAA
                              ********************** *   * ********
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Figure 25 (Continued)

From the ClustalW multiple alignment result (Figure 25), 3 copies of 120 bp tandem repeats (VNTRs) was found in RS2 region of domain I; 5’ left variable peripheral domain of sambar deer (*Cervus unicolor*), while sika deer (*Cervus nippon*) was found only 2 copies of 78 bp long. The characters pattern of these copies is TAS-4 and TAS-3-like (-TAAAATTAATG- or -TAAAATTAGTG - elements) which each copy consists of approximately 40 base characters. Insertions of these long VNTRs are located at pos. 312-431. These insertions are not found in other *Cervus* or *Muntiacus*, which were obtained from this study.

To focus on domain III; 3’ right variable peripheral domain, the unexpected result was occurred in which 2 copies of 52-54 bp CSB-2+3-like were found in hog deer (*Cervus* or *Axis porcinus*) and 1 copies in Fea’s barking deer (*Muntiacus feae*), spotted deer (*Axis axis*), sambar deer (*Cervus unicolor*), thamin Eld’s deer (*Cervus eldii thamin*), red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*). No copied was found in common barking deer (*Muntiacus muntjak*) and Reeves’s barking deer (*Muntiacus reevesi*). The characters pattern of CSB-2+3-like copies is approximately - (2-8) Ts (5-11) Cs - which base amount of each copy is not consistent. Insertion of these copies is located at pos. 1,033-1,088. It could be noticed that Fea’s barking deer (*Muntiacus feae*) has shared this characters with all of *Cervus* deer and *Axis* deer (except hog deer; *Cervus* or *Axis porcinus*).

For domain II; the conserved domain, it was found some indels in this region. The one obvious deletion is deletion of TAA at pos. 782-784 in sambar deer (*Cervus unicolor*), red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*).

E. Phylogenetic analysis

In the phylogenetic analyses, four taxa of the subfamily Muntiacinae; *Muntiacus muntjak* and *M. reevesi micrurus* were used as outgroups. For Fea's barking deer (*M. feae*), it could not be joined as outgroups after ClustalX multiple alignment was complete. It was found that *M. feae* was grouped nearly the clade of genus *Cervus*. To solve this problem, 2 kinds of data sets were made as (1) the data set of *M. feae* was not included and (2) the data set which was included *M. feae*, but not set it as outgroups.

The phylogenetic trees were executed as 2 types, which were (1) neighbor-joining (NJ) analysis and (2) maximum parsimony (MP) analysis using PAUP* version 4.010b (Swofford, 2002). For the NJ tree, non-parametric bootstrap analyzes with 1,000 replicates were performed to obtain estimates of support for each node of the NJ tree as shown in Figure 26 (not included *M. feae*) and Figure 27 (included *M. feae*). For the MP tree, the informative sites were analyzed using equally weighted characters and were searched by branch-and-bound search option. Gaps were treated as missing data. With this branch-and-bound search option, only one parsimonious tree was found if *M. feae* was not included in the data set as shown in Figure 28, and the most eight parsimonious trees were generated if *M. feae* was included in the data set. The semi-strict consensus of eight most parsimonious trees is shown in Figure 29. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

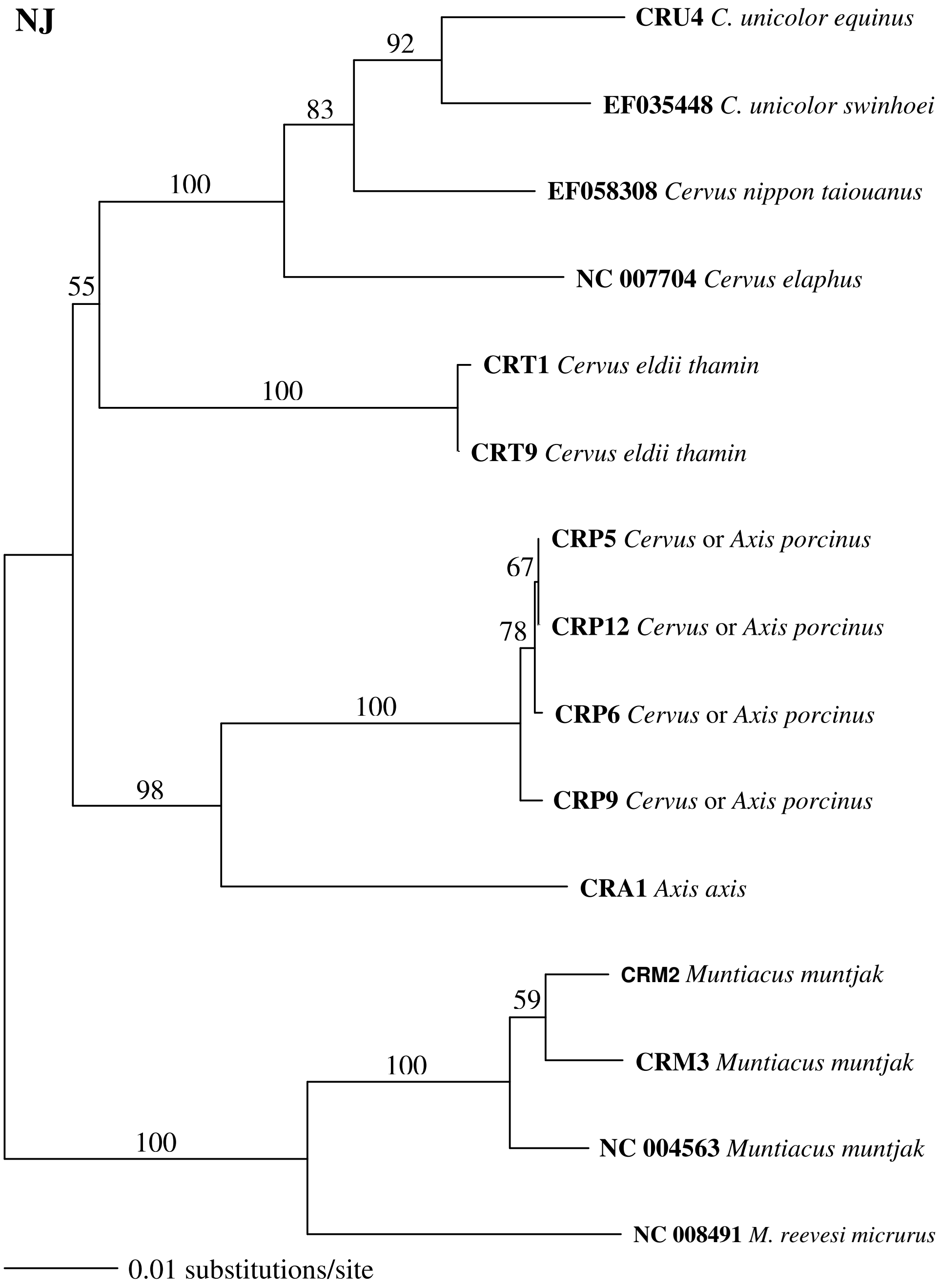


Figure 26 Neighbor-joining tree from partial control region, with supplement of complete tRNA^{Phe} gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50 %) generated from 1,000 replications. (This NJ tree is presented by not included *M. feae* in data set).

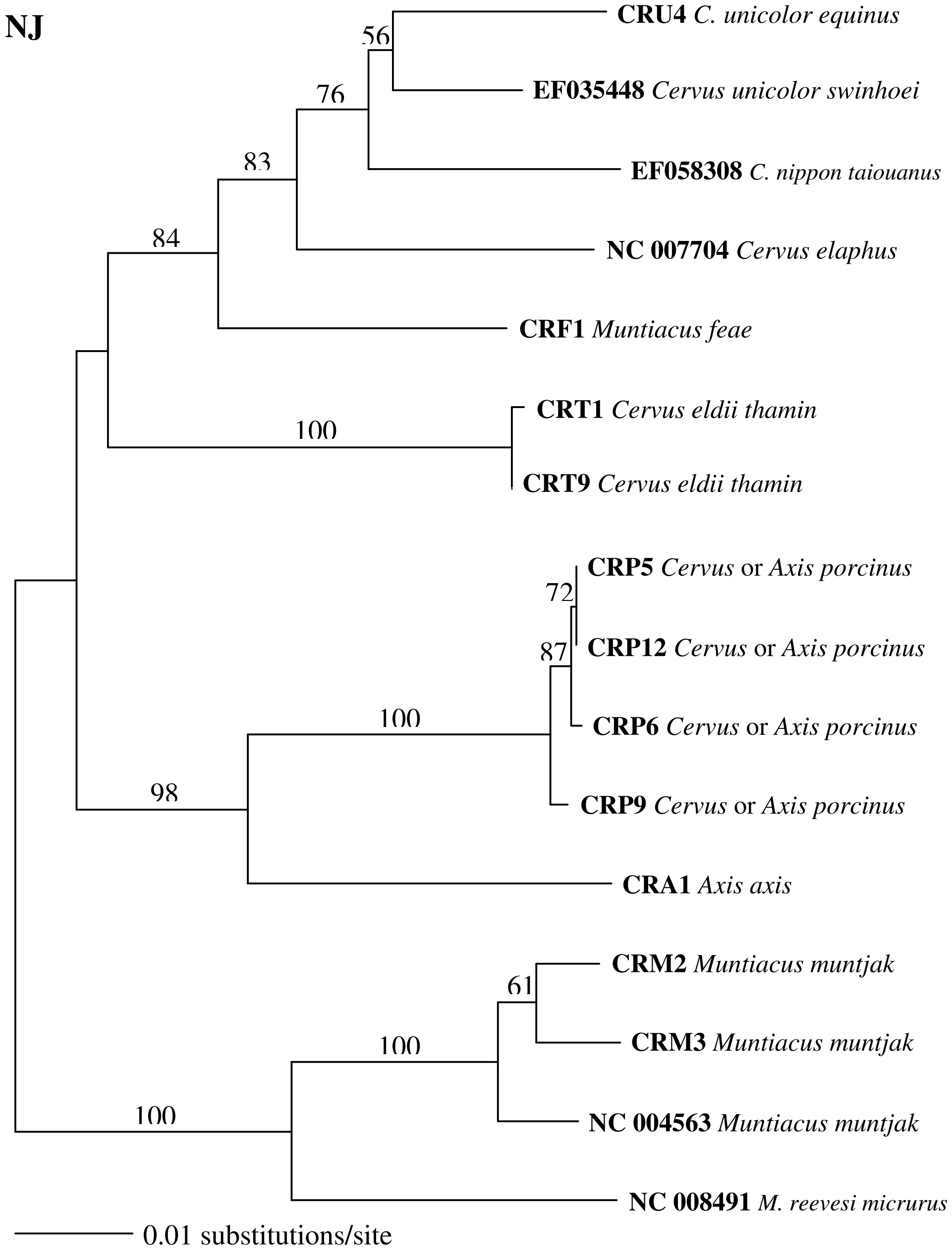


Figure 27 Neighbor-joining tree from partial control region, with supplement of complete tRNA^{Phe} gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50 %) generated from 1,000 replications. (This NJ tree is presented by included *M. feae* in data set).

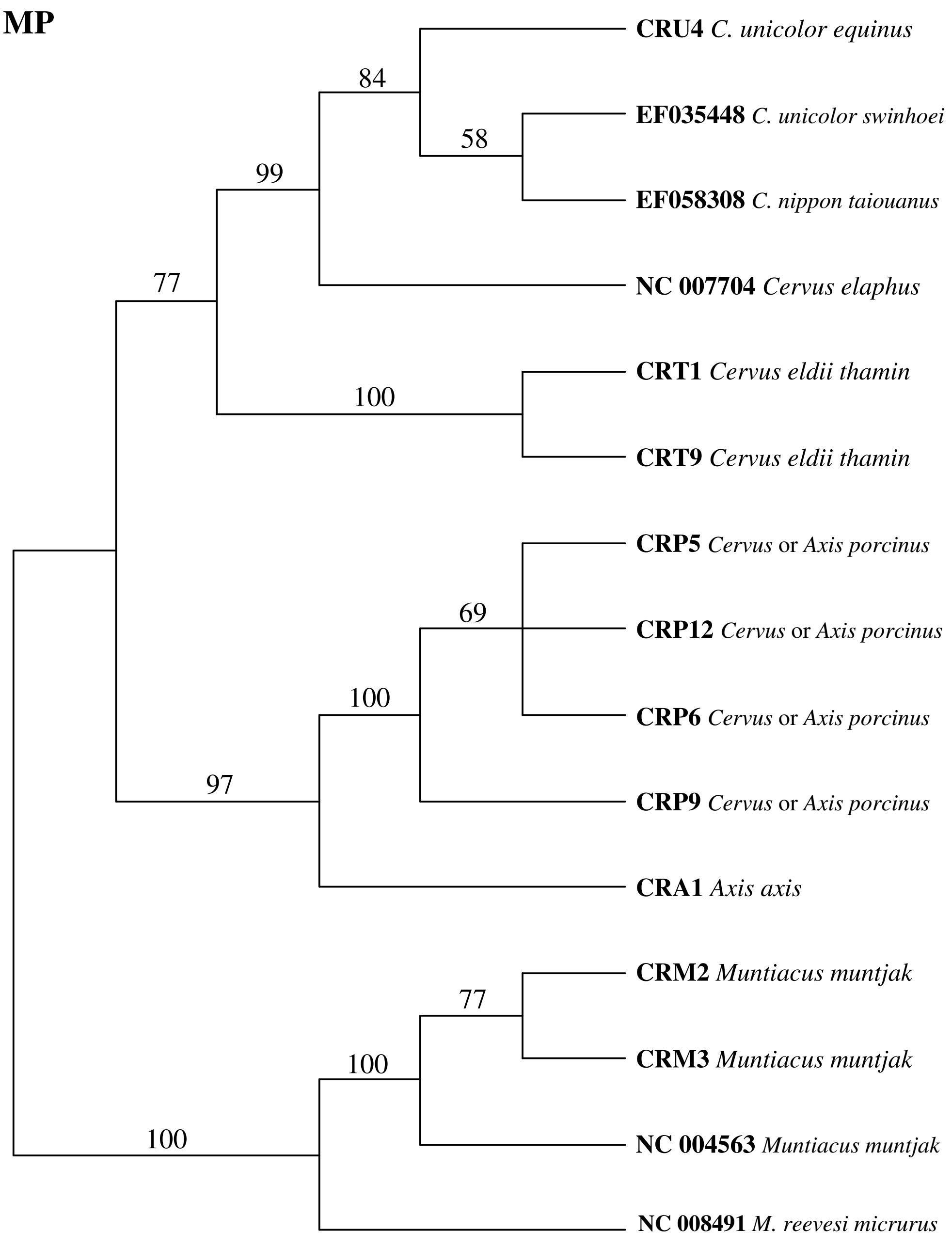


Figure 28 The consensus parsimonious tree from partial control region, with supplement of complete $tRNA^{Phe}$ gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50 %) generated from 1,000 replications. (This MP tree is presented by not included *M. feae* in data set).

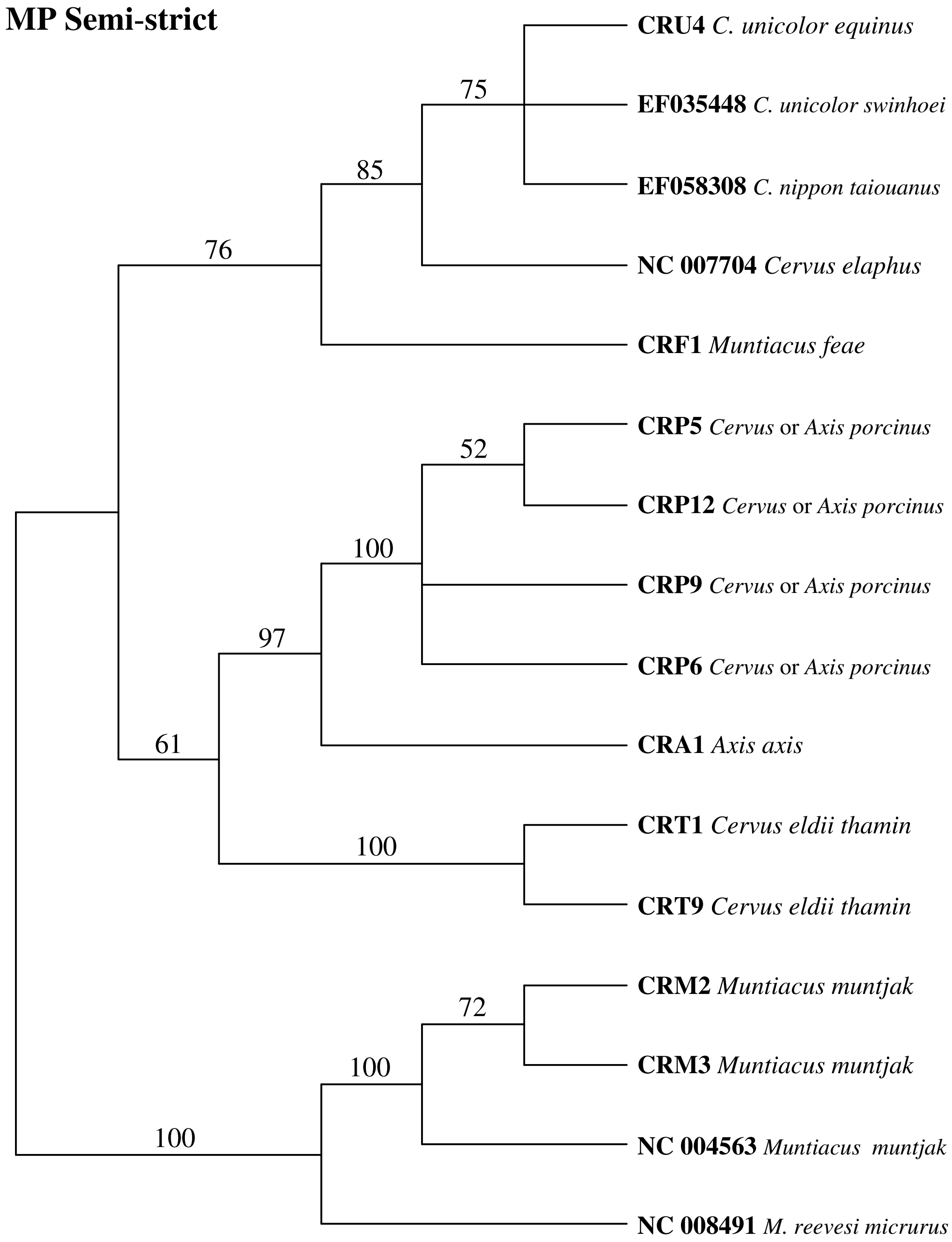


Figure 29 Semi-strict consensus tree of the most eight parsimonious trees from partial control region, with supplement of complete tRNA^{Phe} gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50 %) generated from 1,000 replications. (This NJ tree is presented by included *M. feae* in data set).

F. Phylogenetic relationship result

For the data set which Fea’s barking deer (*M. feae*) was not included, all phylogenetic analyses yielded the similar topology of tree. It was found that hog deer (*Cervus* or *Axis porcinus*) were grouped monophyletically in the same clade of spotted deer (*Axis axis*) (assumption as genus *Axis* clade) with very strong statistical bootstrap support (98 % on NJ tree and 97 % on MP tree). For the genus *Cervus*, it was formed as monophyletic clade with moderate to strong statistical bootstrap support (55 % on NJ tree and 77 % on MP tree). Among Cervus clade, Eld’s deer (*Cervus eldii*) were found as former sister lineage of all *Cervus* taxa. To distinguish the genus *Axis* clade from the genus *Cervus* clade (not include *Cervus* or *Axis porcinus*), 77 base change were needed. These 77 synapomorphic nucleotide characters were counted from 46 base changes of the *Axis* clade, which could group spotted deer and hog deer together, and 31 base synapomorphic characters between *Cervus unicolor*, *Cervus elaphus*, *Cervus nippon* and *Cervus eldii*. For hog deer, it was placed in the genus *Axis* clade, with 51 base changes if compared with spotted deer. These 51 synapomorphic nucleotide characters were counted from 26 base changes of spotted deer and 25 base changes of hog deer. The parsimonious tree that shows numbers of base changes is shown in Figure 30.

If Fea’s barking deer (*M. feae*) was included to the data set, it was found that phylogenetic analyses yielded different topology of tree, but hog deer (*Cervus* or *Axis porcinus*) were still grouped in the same clade of spotted deer (*Axis axis*) with very strong statistical bootstrap support (98 % on NJ tree and 97 % on MP tree). The different topology was among in genus *Cervus*, in which thamin Eld’s deer (*Cervus eldii thamin*) were still joined in the genus *Cervus* clade on NJ tree, but were changed to join nearby the genus *Axis* clade on MP tree. For Fea’s barking deer (*M. feae*), all phylogenetic analyzed yielded the similar topology, in which it was grouped among the genus *Cervus* clade with strong statistical bootstrap support (84 % on NJ tree and 76 % on MP tree). This incredible result will be explained in discussion part. For base changes consideration focus on Fea’s barking deer, 56 base changes were needed to distinguish Fea’s barking deer from the *Cervus* species. These 56 synapomorphic nucleotide characters were counted from 36 base changes of Fea’s barking deer, and 20 base synapomorphic characters between the *Cervus* species as shown in Figure 31.

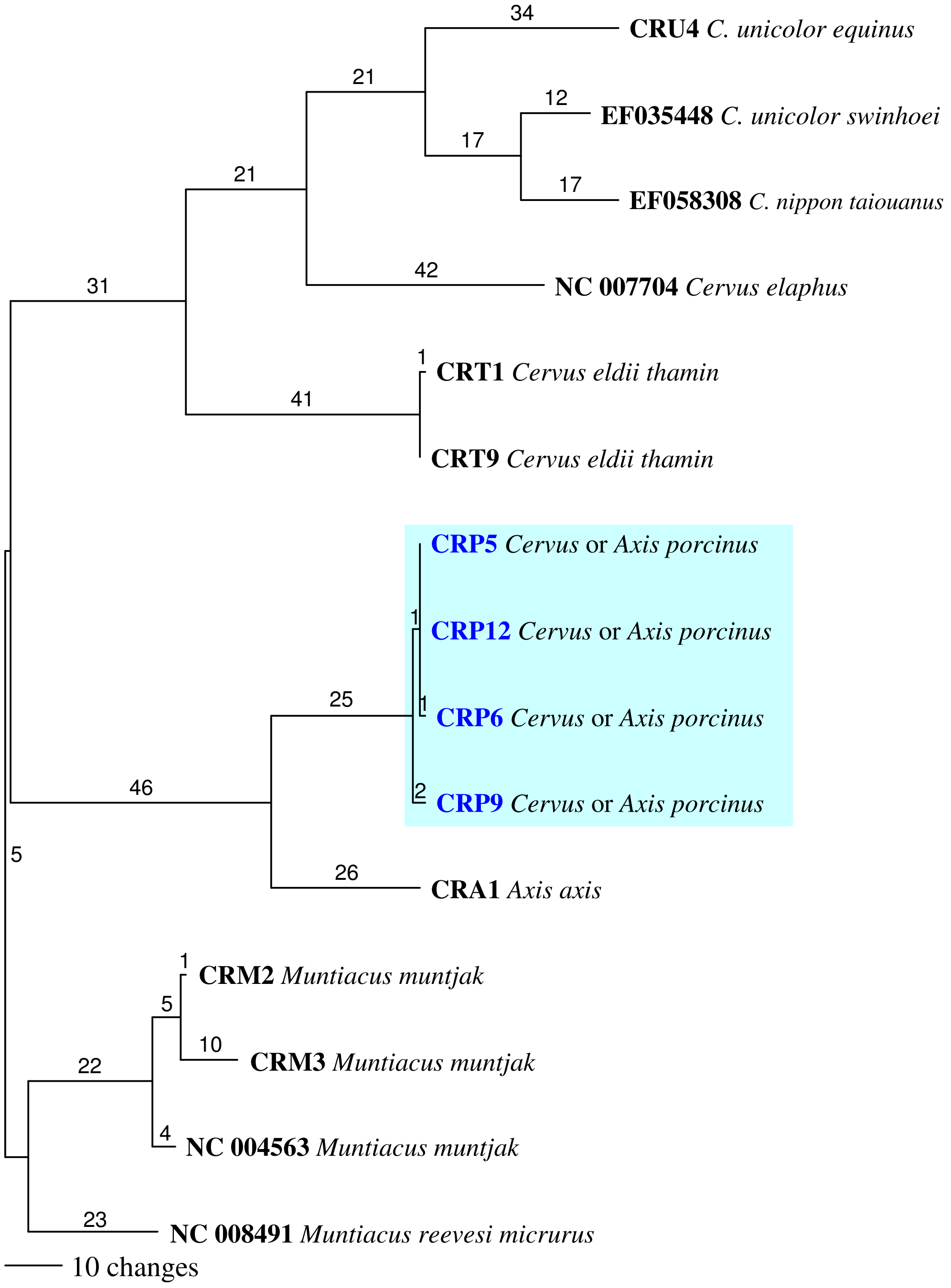


Figure 30 The most parsimonious tree from partial control region, with supplement of complete tRNA$^{\mathrm{Phe}}$ gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Number above branches shows number of base changes. (This MP tree is presented by not included *M. feae* in data set).

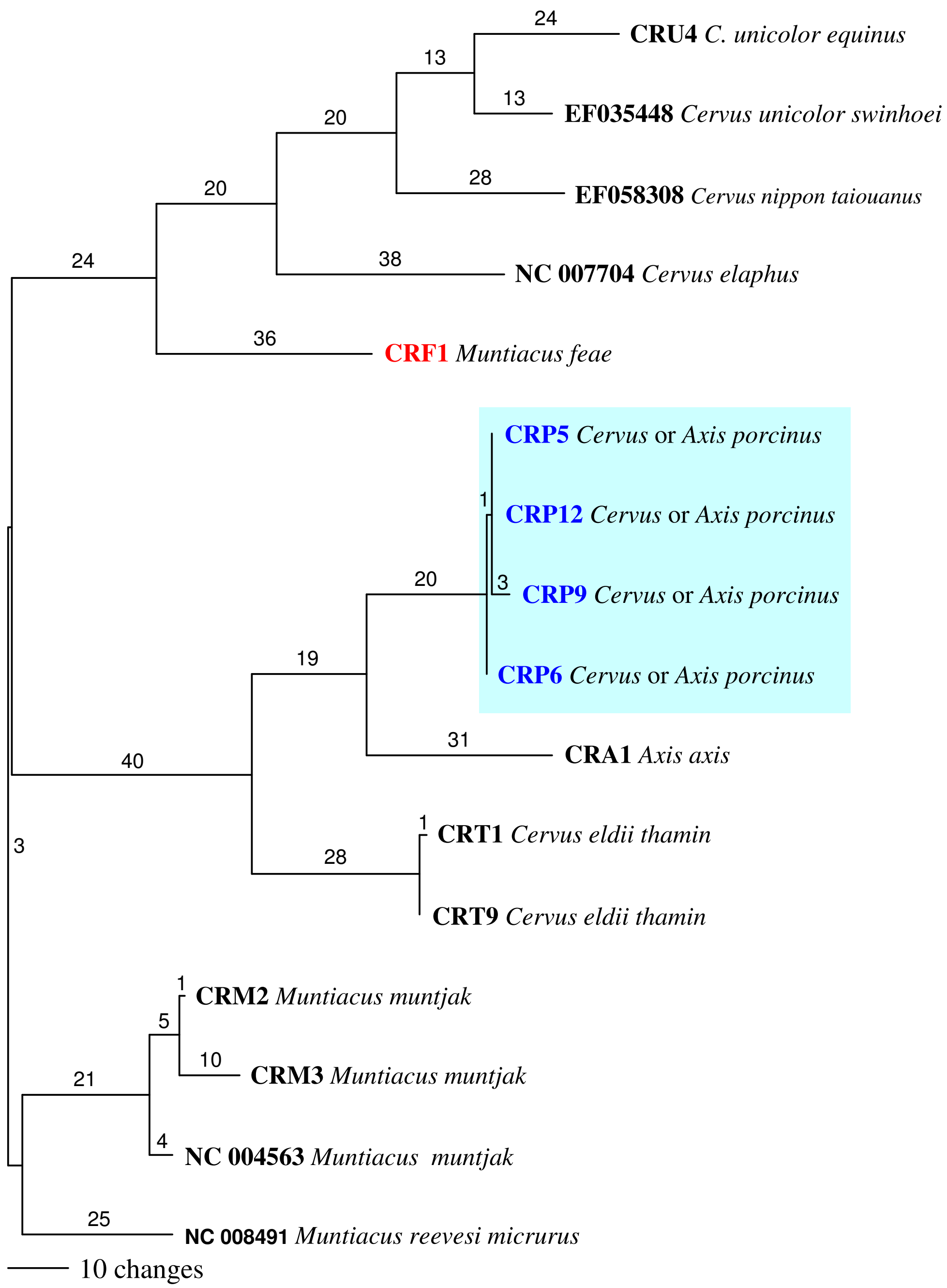


Figure 31 One of the most eight parsimonious trees from partial control region, with supplement of complete tRNA^{Phe} gene and partial 12S ribosomal RNA gene sequence of 16 deer taxa. Number above branches shows number of base changes. (This MP tree is presented by included *M. feae* in data set).

4. Phylogenetic relationship analysis inferred from intron1 of protein kinase C, iota gene (PRKCI)

A. DNA amplification

Whole genomic DNA from the blood samples was extracted using Aquapure Genomic Blood kit (Bio-RAD). The extracted DNA in total volume of 100 µl of TE buffer was stored at –20°C in a refrigerator. Each DNA extract was used as a template for PCR amplification, with the primer pair U26 and L748 from the previous study of Ropiquet and Hassanin (2005). PCR products of PRKCI intron1 were amplified in a 15 µl reaction containing 1.5 µl of 10X Tag buffer, 1.5 µl of 2.5 mM $MgCl_2$, 0.075 µl of *Taq* DNA polymerase (Fermentas), 1.2 µl of 2.0 mM dNTP mixture, 0.75 µl of 5 pmol/µl of each primer and 2 µl of 100 ng/µl DNA template solution. Thermal cycling program consisted of an initial denaturation step at 94°C for 4 min, 35 cycles of a denaturation step at 94°C for 20s, an annealing step at 53°C for 30s, and an extension step at 72°C for 1 min, and a final extension step at 72°C for 5 min. The expected single band of PCR products of approximately 600 bp corresponded to the size of DNA target fragment as shown in Figure 32.

B. Samples for phylogenetic analysis

Twenty-five sample sequences from PCR products of PRKCI gene intron1 from this study (Appendix C), including hog deer (*Cervus* or *Axis porcinus*), sambar deer (*Cervus unicolor equinus*), thamin Eld's deer (*C. eldii thamin*), siamensis Eld's deer (*C. eldii siamensis*) common barking deer (*Muntiacus muntjak*), Fea's barking deer (*M. feae*) and one cervid species which not native to Thailand; spotted deer (*Axis axis*) were aligned using ClustalX version 1.82 (Thompson *et al.* 1997) along with five of 514 bp cervid sequences obtained from GenBank. Before launching to PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis, multiple sequences alignment on ClustalX was saved as nexus file (*.nxs). For unknown character at the two flank side of 514 bp cervid sequences from GenBank were replaced as "?" character and were saved as nexus file again. Sample name and accession number of all sequences for phylogenetic analysis as shown in Table 10.

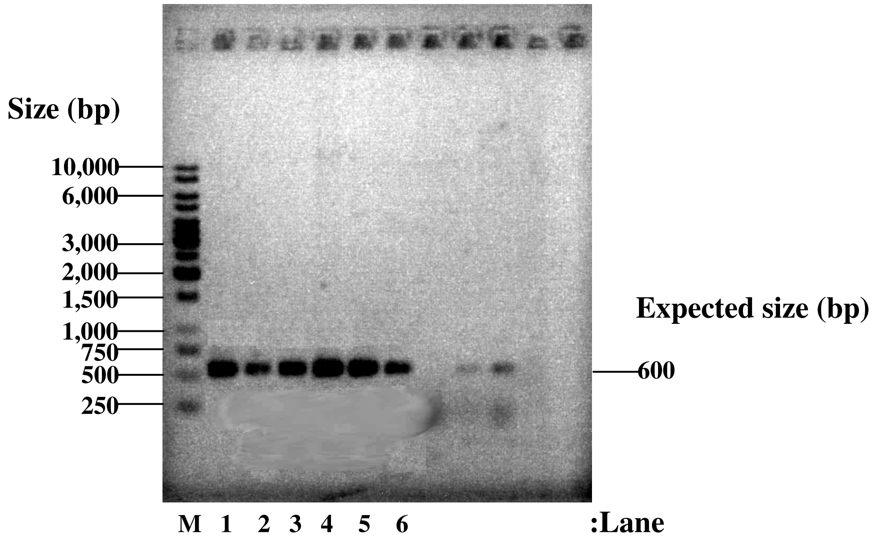


Figure 32 PCR products of Cervidae's intron1 of protein kinase C, *iota* gene (PRKCI). Lane M indicated the size standard marker. Lane 1= *Muntiacus muntjak*, lane 2 = *Cervus eldii thamin*, lane 3 = *Cervus eldii siamensis*, lane 4-5 = *C. unicolor* and lane 6 = *Cervus* or *Axis porcinus*.

C. PRKCI intron1 characteristics

The final alignment of PRKCI intron1 sequences of all taxa consisted of 552 characters. This alignment was generated by the insertion of single gap at position (pos.) 8, 104, 105, 476 and 517. Of these 552 characters, 523 were constant and 19 were parsimony informative. These PRKCI intron sequences were AT-rich, with approximately 70 % AT component. The base composition and base sequence amount (not included sequence samples from GenBank) were calculated by averaging base composition and base sequence amount values from individuals of each species in as shown in Table 11.

Table 10 Cervid samples for PRKCI intron phylogenetic analysis used in this study.

Common name	Zoological name	Sample name /Accession no.	Source	No. of sample
Common barking deer	*Muntiacus muntjak*	PKM2, PKM3	Pattalung Wildlife Station[a]	2
Fea's barking deer	*Muntiacu feae*	PKF1, PKF2	Ton Nga-Chang Wildlife Station[a]	2
Hog deer	*Cervus* or *Axis porcinus*	PKP1, PKP5, PKP6 PKP9 PKP11 PKP12	Hauy Sai Wildlife Station[a] Dusit Zoo[b] Korat Zoo[b] Songkhla Zoo[b]	6
Sambar deer	*Cervus unicolor equinus*	PKU2, PKU3, PKU4 PKU5 PKU8	Hauy Sai Wildlife Station[a] Songkhla Zoo[b] Khao Kho Wildlife Station[a]	5
Siamese Eld's deer	*Cervus eldii siamensis*	PKS1	Dusit Zoo[b]	1
Thamin Eld's deer	*Cervus eldii thamin*	PKT1, PKT2, PKT7 PKT9, PKT11	Hauy Sai Wildlife Station[a] Dusit Zoo[b]	5
Rusa deer	*Cervus timorensis*	DQ379333	GenBank	1
Swamp deer	*Cervus duvaucelii*	DQ379331	GenBank	1

Table 10 (Continued)

Common name	Zoological name	Sample name /Accession no.	Source	No. of sample
Sika deer	*Cervus nippon*	DQ379332	GenBank	1
Thorold’s deer	*Cervus albirostris*	DQ379330	GenBank	1
Spotted deer	*Axis axis*	PKA1, PKA2	Songkhla Zoo[b]	5
		PKA3, PKA4	Korat Zoo[b]	
		DQ379329	GenBank	
Total				30

[a] Wildlife Propagation Division, DNP

[b] The Zoological Park Organization under the Royal Patronage of His Majesty the King

Table 11 Percentage of average base composition and base sequence amounts of PRKCI intron of each cervid species examined by PAUP* version 4.010b.

Zoological name	**Base composition (%)**				**Amount**
	A	**C**	**G**	**T**	
M. mumtjak	31.81	12.03	17.50	38.65	548.50
M. feae	31.79	12.02	18.12	38.07	549.00
C. (or *A.*) *porcinus*	33.82	11.57	16.67	38.96	551.83
C. unicolor	32.86	11.31	16.79	39.04	550.20
C. eldii siamensis	33.21	11.25	16.52	39.02	551.00
C. eldii thamin	33.03	11.25	16.70	39.02	551.00
A. axis	32.80	11.55	16.49	39.15	551.75
All taxa	**32.76**	**11.57**	**16.97**	**38.84**	**550.47**

Among two *Munticus* outgroup taxa, there are six transitions (C→T in pos. 51, 261 and 433; G→A in pos. 456, 500 and 505), two transversions (C→A in pos. 439 and 535) and one deletion of TA in pos. 104-105 found after compared with the ingroups.

Among the ingroup taxa, there are one transversion (G→T in pos. 165) between the pair of sambar deer and Eld's deer and that of spotted deer and hog deer, and also one insertion of C in pos. 8 of spotted deer and hog deer sequences. If compare only hog deer with the others, one transition (A→G in pos. 169) was observed while a comparison of spotted deer with the others revealed one transversion (A→T in pos. 271). For sambar deer, deletion of A in pos. 476 of four in five sambar specimens was found.

D. Phylogenetic analysis

In the phylogenetic analyses, four taxa of the subfamily Muntiacinae; *Muntiacus muntjak* and *M. feae* and were used as outgroups. The phylogenetic trees were executed as 2 types, which were (1) neighbor-joining (NJ) analysis and (2) maximum parsimony (MP) analysis using PAUP* version 4.010b (Swofford, 2002). For the NJ tree, non-parametric bootstrap analyzes with 1,000 replicates were performed to obtain estimates of support for each node of the NJ tree as shown in Figure 33. For the MP tree, the informative sites were analyzed using equally weighted characters and were searched by heuristic option with a stepwise starting tree, a random stepwise addition of 1,000 replicates, tree-bisection-reconnection (TBR), and steepest descent branch-swapping algorithm. Gaps were treated as missing data. With this heuristic search option, only one most parsimonious tree was generated as shown in Figure 34. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

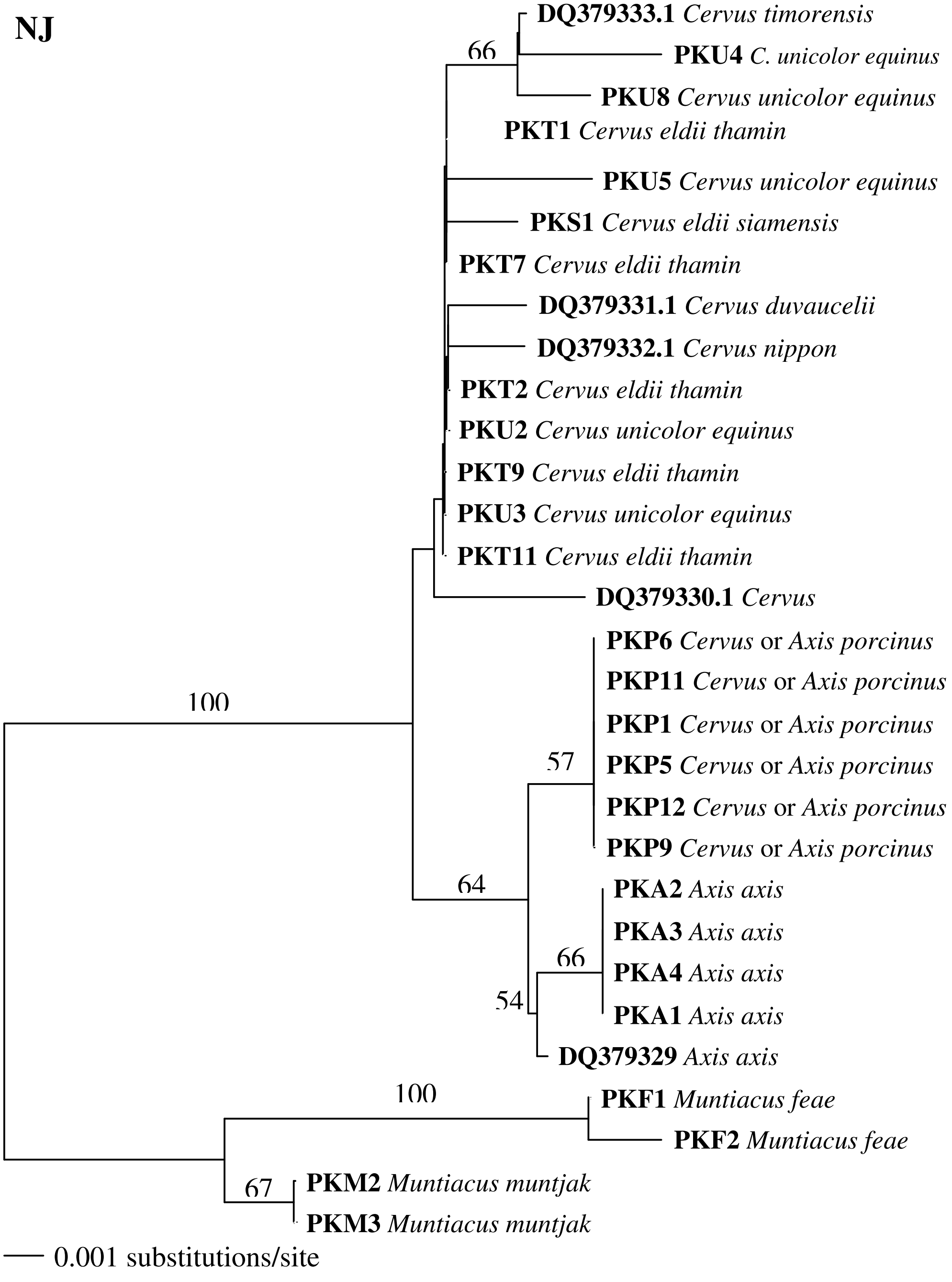


Figure 33 Neighbor-joining tree from PRKCI intron sequences of 30 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) generated from 1,000 replications.

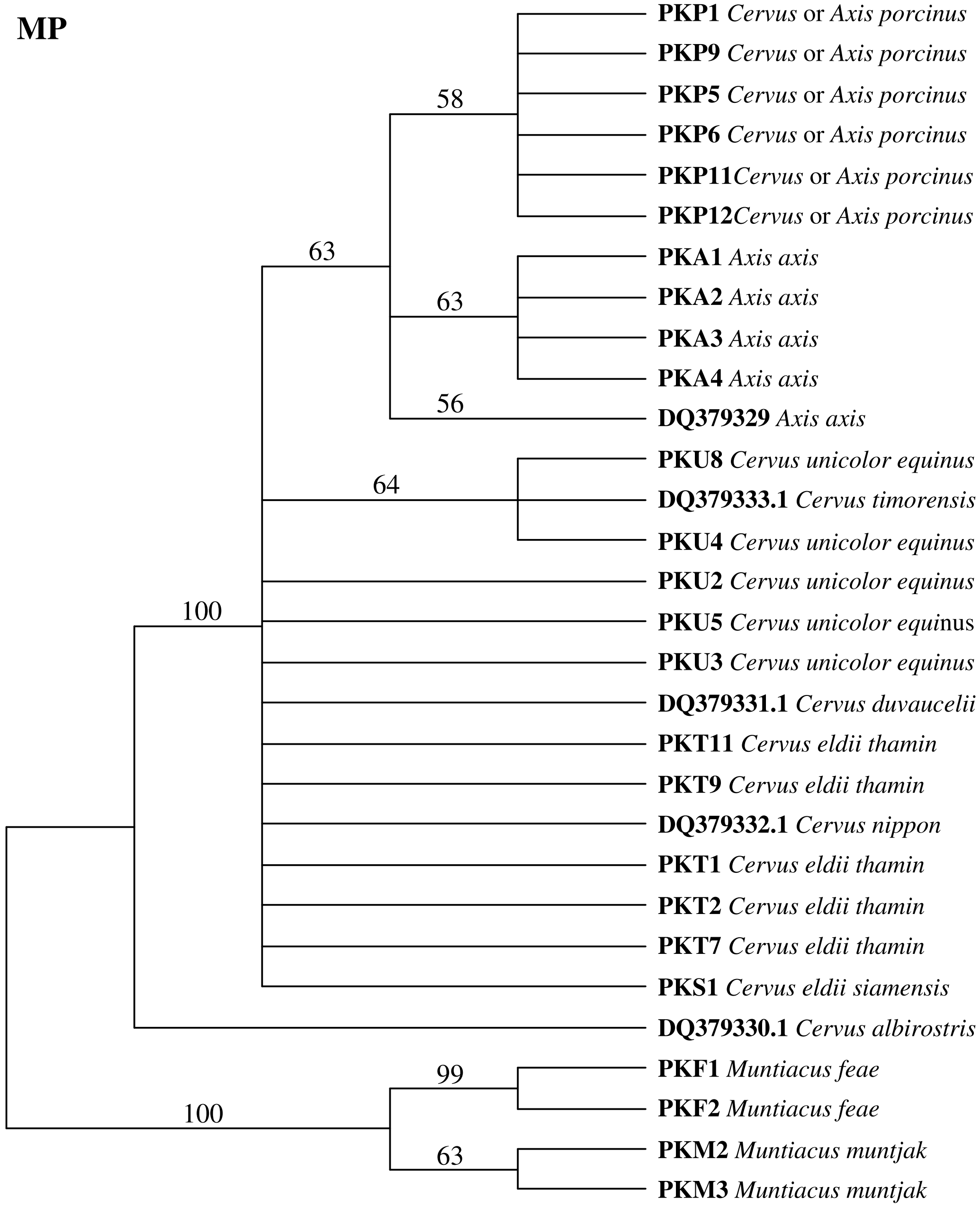


Figure 34 The consensus parsimonious tree from PRKCI intron sequences of 30 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) based on 1,000 replications.

E. Phylogenetic relationship result

Among hog deer (*Cervus* or *Axis porcinus*) and spotted deer (*Axis axis*) in which assumption as the genus *Axis* clade, it was found that they were grouped monophyletically with moderate statistical bootstrap support (64 % on NJ tree and 63 % on MP tree). In the case of the genus *Cervus*, except hog deer, they were formed a group with very weak support of < 50 % bootstrap values on NJ tree and polytomy branches have been occurred among them on MP tree. Moreover, this topology indicated that PRKCI gene intron1 may not give enough resolution as it could not distinguish the species among the genus (*Cervus unicolor*, *C. timorensis*, *C. duvaucelii*, *C. eldii thamin*, C. *eldii siamensis*, *C. nippon*, *C. albirostris*) apart.

To distinguish the genus or the species by focus on base changes, the parsimonious analysis yielded tree which was not clear separation among different genus and also among different species. The genus *Axis* was joined in the same group of the genus *Cervus* (except Thorold's deer; *Cervus albirostris*), in which a lot of polytomy branches were occurred among them. This tree presented 18 different bases between Thorold's deer and the clade of the others *Cervus* and the genus *Axis*. These 18 synapomorphic nucleotide characters were counted from nine base changes of Thorold's deer and nine base synapomorphic characters among the others *Cervus* and the genus *Axis*.

To consider only hog deer and spotted deer, these two species could be clearly separated into different species, although the number of base changes was not much found. There are 2 base changes between them, in which only one base change of hog deer were counted and only one base change of spotted deer were counted. The parsimonious tree presented the number of base changes are shown in Figure 35.

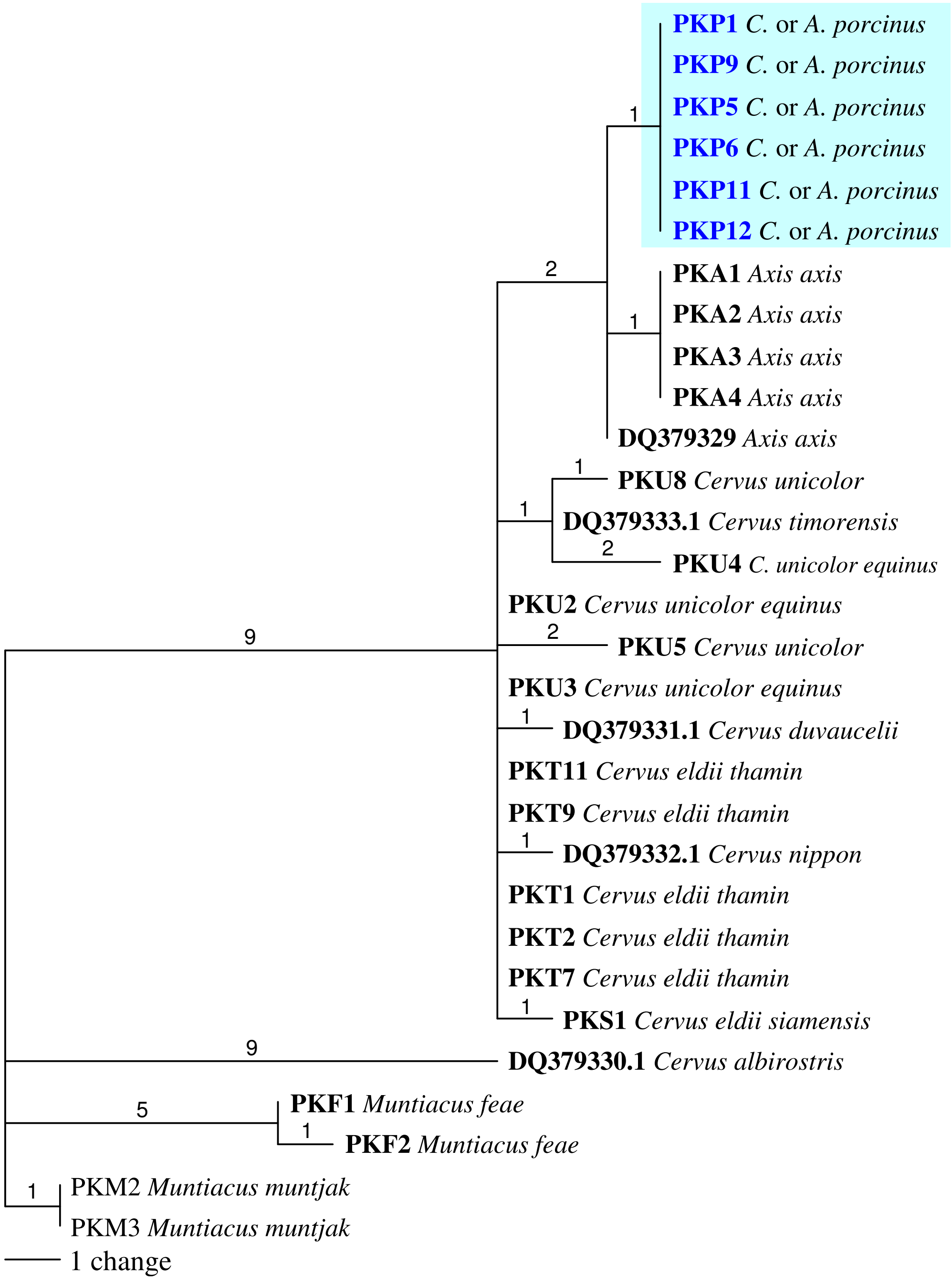


Figure 35 The most parsimonious tree from PRKCI intron sequences of 30 deer taxa. Numbers above branches show numbers of base changes.

5. Phylogenetic Relationship Analysis Inferred from Mitochondrial DNA Sequences

A. Cytochrome *b* gene and control region assembly

After DNA sequences assembly in each mitochondrial DNA target (cytochrome *b* gene and control region portion) were finished. Each sequences portion from the same sample was assembled together using CAP3 program (http://pbil.univ-lyon1.fr/cap3.php). All sequences were aligned together using the software ClustalX version 1.82 (Thomson *et al.*, 1977) along with complete mitochondrion DNA genome sequences obtained from GenBank, followed by visual inspection to ensure correct sequences. The excessive nucleotides from each target sequence have to be removed. Finally, each one of assemblage sequence still remains approximately 2,600 bp long which mainly includes complete sequences of cytochrome *b* and control region, additionally with several of tRNA genes.

Each mitochondrial DNA sequences contained eight portion of partial NADH dehydrogenase subunit 6 (ND6), complete sequence of tRNA^{Glu} gene, complete sequence of cytochrome *b* gene, complete sequence of tRNA^{Thr} gene, complete sequence of tRNA^{Pro} gene, complete sequence of control region, complete sequence of tRNA^{Phe} gene and partial sequence of 12S ribosomal RNA gene, respectively. The example of mitochondrial DNA component and each portion position which was obtained from this study as shown in Figure 36.

>MTA1_*Axis_axis*

```
CAAAAATAATACTTAAGATAAATACAATGTATGTTATCATTATTCTCACATGGAATCTAACC
ATGACTAATGATATGAAAAACCATCGTTGTCATTCAACTACAAGAACACTAATGATCAACAT
CCGAAAAACTCACCCATTAATAAAAATTGTAAACAACGCATTCATTGACCTCCCAACCCCAT
CAAATATTTCATCCTGATGGAACTTCGGCTCTTTGCTAGGAGTCTGCTTAATTCTACAAATT
CTCACGGGCTTATTCTTAGCAATACACTATACATCTGATACAATAACAGCATTCTCCTCTGT
CACTCATATCTGTCGAGACGTCAACTACGGCTGAATTATTCGATATATGCACGCAAATGGAG
CATCAATATTTTTTATTTGCTTATTTTTACATGTAGGACGAGGTCTGTATTACGGATCATAT
```

Figure 36 The mitochondrial DNA sequence of spotted deer; *Axis axis* (MTA1) as the example shows the sequence component and their positions on sequence.

ACCTTTTTAGAAACATGAAACATTGGAGTAATCCTCCTATTTACAGTTATAGCCACAGCATT
TGTGGGATACGTCCTACCATGAGGACAGATATCATTCTGAGGAGCAACAGTTATTACCAATC
TCCTCTCAGCAATCCCTTACATTGGTACAAATCTAGTCGAATGAATTTGAGGAGGCTTTTCA
GTAGATAAAGCAACCCTAACCCGATTTTTCGCTTTCCACTTTATTCTCCCATTTATCATTGC
AGCACTTGCTATAGTTCACCTACTCTTCCTTCACGAAACAGGATCCAATAACCCAACAGGAA
TTCCATCAGATGCAGATAAAATTCCCTTCCACCCTTACTATACCATTAAAGATACTCTAGGT
ATTATTCTTCTAATTATATTCCTAATACTACTAGTGCTATTTGCACCAGATATGCTTGGAGA
CCCAGACAACTATACCCCAGCAAACCCACTCAATACACCTCCCCATATTAAACCCGAATGGT
ATTTCCTATTTGCATACGCAATCCTACGATCAATTCCCAATAAACTAGGAGGAGTTTTAGCC
CTAGCTTCATCCATCCTGATCCTAATTCTTATACCCCTCCTCCACACATCCAAACAACGCAG
CATAATATTCCGACCATTTAGCCAATGCTTATTTTGAATCTTAGTGGCAGACCTACTAACAC
TTACATGAATCGGAGGACAACCAGTTGAATACCCCTTTATTATTATCGGACAACTAGCATCT
ATTCTATACTTTCTTATTATCCTAATTCTTATACCAATTATCAGCTCAATCGAAAATAACCT
CCTAAAATGAAGATAAGTCTTTGTAGTATACTGAATACACTGGTCTTGTAAATCAGAAAAGG
AGAGTAATTAACCTCCCTAAGACTCAAGGAAGAAGCAATAGCCCCACGTATCAACACCCAAA
GCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTGTTAATATAGTTCCATAAAAATCAAG
AACTTTATTAGTATTAAATTTCCAAAAATTTTAACAATTCAATACAGCTTTCTACTCAACAT
CCAATTTACACCCAACATACCATTTTCTACACAACATGACATGCAATGCATTGCATGCGCTT
ATAGTACATAAAATTAATGTATTAGGACATACTATGTATAATAGTACATTACATTACATTCC
CCATGCTTATAAGCATGTACCCCTCATCATTTATAGTACATAGTACATAGTGTTGTTCATCG
TACATAGCACATTGAGTCAAATCAATCCTTGTCAACATGCGTATCCCGTCCCCTAGATCACG
AGCTTAATTACCATGCCGCGTGAAACCAACAACCCGCTAGGCAGGGATCCCTCTTCTCGCTC
CGGGCCCATAAACTGTGGGGGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTT
CAGGGCCATCTCACCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATGGACTA
ATGACTAATCAGCCCATGCTCACACATAACTGGGGGGTCATACATTTGGTATTTTTAATTTT
TGGGGGATGCTTGGACTCAGCATTGGCCGTCTGAGGCCCCGACCCGGAGCATAAATTGTAGC
TGGACTTAACTGCATCTTGAGCATCCCCATAATGGTAGGCGCGGGGCATGGCAGTCAATGGT
CCCAGGACATAGTTATTATTTCAGGGGTCAACCCTTTAATTTTTTTTCCCCCCCCCTCATAT
TTTCCCCCTTATATAGTTACCACCATTTTTAACACGCTTTCCCCTAGATATTATTTTAAATT
TATCACATTTCCAATACTCAAATGGCACTCCATAAGGAGGTAAGTATATAAGCGCCTATTTT
TTCCTAATTACGCACAGTTAATGTAGCTTAAACAATAAAGCAAGGCACTGAAAATGCCTAGA
TGAGTATATTAACTCCATAAACATATAGGTTTGGTCCCAGCCTTCCTATTAGCCCGTAATAG
ACTTACACAGGGAAGCA

🟩 = partial sequence of NADH dehydrogenase subunit 6 (ND6) gene
🟧 = complete sequence of tRNA^{Glu} gene
🟦 = complete sequence of cytochrome *b* gene
🟫 = complete sequence of tRNA^{Thr} gene
🟥 = complete sequence of tRNA^{Pro} gene
🟩 = complete sequence of control region
🟪 = complete sequence of tRNA^{Phe} gene
🟪 = partial sequence of 12S ribosomal RNA gene

Figure 36 (Continued)

B. Samples for phylogenetic analysis

Eleven sample sequences from the assembled mitochondrial DNA sequences obtained from this study (Appendix D), including hog deer (*Cervus* or *Axis porcinus*), sambar deer (*Cervus unicolor equinus*), thamin Eld's deer (*C. eldii thamin*), common barking deer (*Muntiacus muntjak*), Fea's barking deer (*M. feae*) and one cervid species which not native to Thailand; spotted deer (*Axis axis*) were aligned using ClustalX version 1.82 (Thompson *et al.* 1997) along with five cervid sequences obtained from GenBank. Before launching to PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis, multiple sequences alignment on ClustalX was saved as nexus file (*.nxs). Sample names and accession number of all sequences for phylogenetic analysis as shown in Table 12.

C. Mitochondrial DNA characteristics

The final alignment of cervid mitochondrial DNA of all taxa consisted of 2,599 characters, in which scattered from 2,434 up to 2,559 bp long. Of these 2,599 characters, 1,979 were constant and 439 were parsimony informative. The average base composition and base sequence amount of each cervid sample as shown in Table 13.

D. Phylogenetic analysis

The phylogenetic trees were executed as 2 types, which were (1) neighbor-joining (NJ) analysis and (2) maximum parsimony (MP) analysis using PAUP* version 4.010b (Swofford, 2002). For the NJ tree, non-parametric bootstrap analyzes with 1,000 replicates were performed to obtain estimates of support for each node of the NJ tree as shown in Figure 37. For the MP tree, the informative sites were analyzed using equally weighted characters and were searched by branch-and-bound search option. Gaps were treated as missing data. With this branch-and-bound search option, three most parsimonious trees were found, and the semi-strict consensus of the most three parsimonious trees is shown in Figure 38. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

Table 12 Cervid samples for mitochondrial DNA phylogenetic analysis used in this study.

Common name	Zoological name	Sample name /Accession no.	Source	No. of sample
Common barking deer	*Muntiacus muntjak*	MTM2, MTM3	Pattalung Wildlife Station[a]	3
		NC_004563	GenBank	
Fea's barking deer	*Muntiacus feae*	MTF1	Ton Nga-Chang Wildlife Station[a]	1
Reeves's barking deer	*Muntiacus reevesi micrurus*	NC_008491	GenBank	1
Hog deer	*Cervus* or *Axis porcinus*	MTP5, MTP6	Hauy Sai Wildlife Station[a]	4
		MTP9	Dusit Zoo[b]	
		MTP12	Songkhla Zoo[b]	
Sambar deer	*Cervus unicolor equinus*	MTU4	Hauy Sai Wildlife Station[a]	2
	Cervus unicolor swinhoei			
		EF035448	GenBank	
Thamin Eld's deer	*Cervus eldii thamin*	MTT1	Hauy Sai Wildlife Station[a]	2
		MTT9	Dusit Zoo[b]	
Sika deer	*Cervus nippon taiouanus*	EF058308	GenBank	1

Table 12 (Continued)

Common name	**Zoological name**	**Sample name /Accession no.**	**Source**	**No. of sample**
Red deer or wapiti	*Cervus elaphus*	NC_007704	GenBank	1
Spotted deer	*Axis axis*	MTA1	Songkhla Zoo[b]	1
Total				16

[a] Wildlife Propagation Division, DNP

[b] The Zoological Park Organization under the Royal Patronage of His Majesty the King

Table 13 Percentage of average base composition of mitochondrial DNA of each cervid sample examined by PAUP* version 4.010b.

Zoological name	Sample name /Accession No.	Base composition (%)				Amount (bp)
		A	C	G	T	
Muntiacus muntjak	MTM2	31.66	25.02	13.86	29.45	2,438
	MTM3	31.56	24.96	13.89	29.59	2,440
	NC_004563	31.46	25.31	14.07	29.16	2,438
Muntiacus feae	MTF1	31.70	25.25	13.59	29.46	2,451
Muntiacus reevesi micrurus	NC_008491	31.62	25.05	14.00	29.32	2,435
Cervus or *Axis porcinus*	MTP5	31.83	24.47	13.29	30.41	2,460
	MTP6	31.76	24.52	13.34	30.38	2,459
	MTP9	31.81	24.53	13.30	30.35	2,458
	MTP12	31.83	24.50	13.31	30.36	2,457
Cervus unicolor equinus	MTU4	32.06	24.67	14.03	29.24	2,558
Cervus unicolor swinhoei	EF035448	32.00	24.15	14.22	29.62	2,559
Cervus eldii thamin	MTT1	31.42	25.39	13.95	29.24	2,438
	MTT9	31.49	25.42	13.94	29.15	2,439

Table 13 (Continued)

Zoological name	Sample name /Accession No.	Base composition (%)				Amount (bp)
		A	C	G	T	
Cervus nippon taiouanus	EF058308	31.55	24.70	14.26	29.48	2,510
Cervus elaphus	NC_007704	31.35	24.94	14.21	29.50	2,434
Axis axis	MTA1	31.33	24.15	14.13	30.39	2,435
Mean		31.65	24.81	13.84	29.69	2,463.06

E. Phylogenetic relationship result

All phylogenetic analyses yielded the similar topology of tree. It found that hog deer (*Cervus* or *Axis porcinus*) were grouped monophyletically in the same clade of spotted deer (*Axis axis*) (assumption as genus *Axis* clade) with absolutely strong statistical bootstrap support (100 % on NJ tree and 100 % on MP tree). These phylogenetic tree results were powerful declaration that hog deer should be placed in the genus *Axis*, not genus *Cervus*. For the genus *Cervus*, it was also formed as clearly monophyletic clade with very strong statistical bootstrap support (99 % on NJ tree and 97 % on MP tree). Among Cervus clade, thamin Eld's deer (*Cervus eldii thamin*) were found as former sister lineage of all *Cervus* taxa, while sambar deer (*Cervus unicolor*) was the latter of clade.

To distinguish the genus *Axis* clade from the genus *Cervus* clade (not include *Cervus* or *Axis porcinus*), 189 base change were needed. These 189 synapomorphic nucleotide characters were counted from 72 base synapomorphic characters of the genus *Axis* clade, which could group spotted deer and hog deer together, and 117 base synapomorphic characters among genus *Cervus* species. For hog deer if compared with spotted deer, there are 235 base changes between them, which were counted from 80 base synapomorphic characters of hog deer and 155 base synapomorphic characters of spotted deer. Among the genus *Cervus*, Eld's deer were found as former sister lineage of the others *Cervus*, with 122 base changes among them.

For Fea's barking deer (*Muntiacus feae*); one member of outgroups as the important endangered species of Thailand, it could be noticed that they were former sister lineage of the others *Muntiacus*. There are 125 base changes among them, with 76 base synapomorphic characters of Fea's barking deer and 49 base synapomorphic characters between the others *Muntiacus* species. The parsimonious tree that shows numbers of base changes is shown in Figure 39.

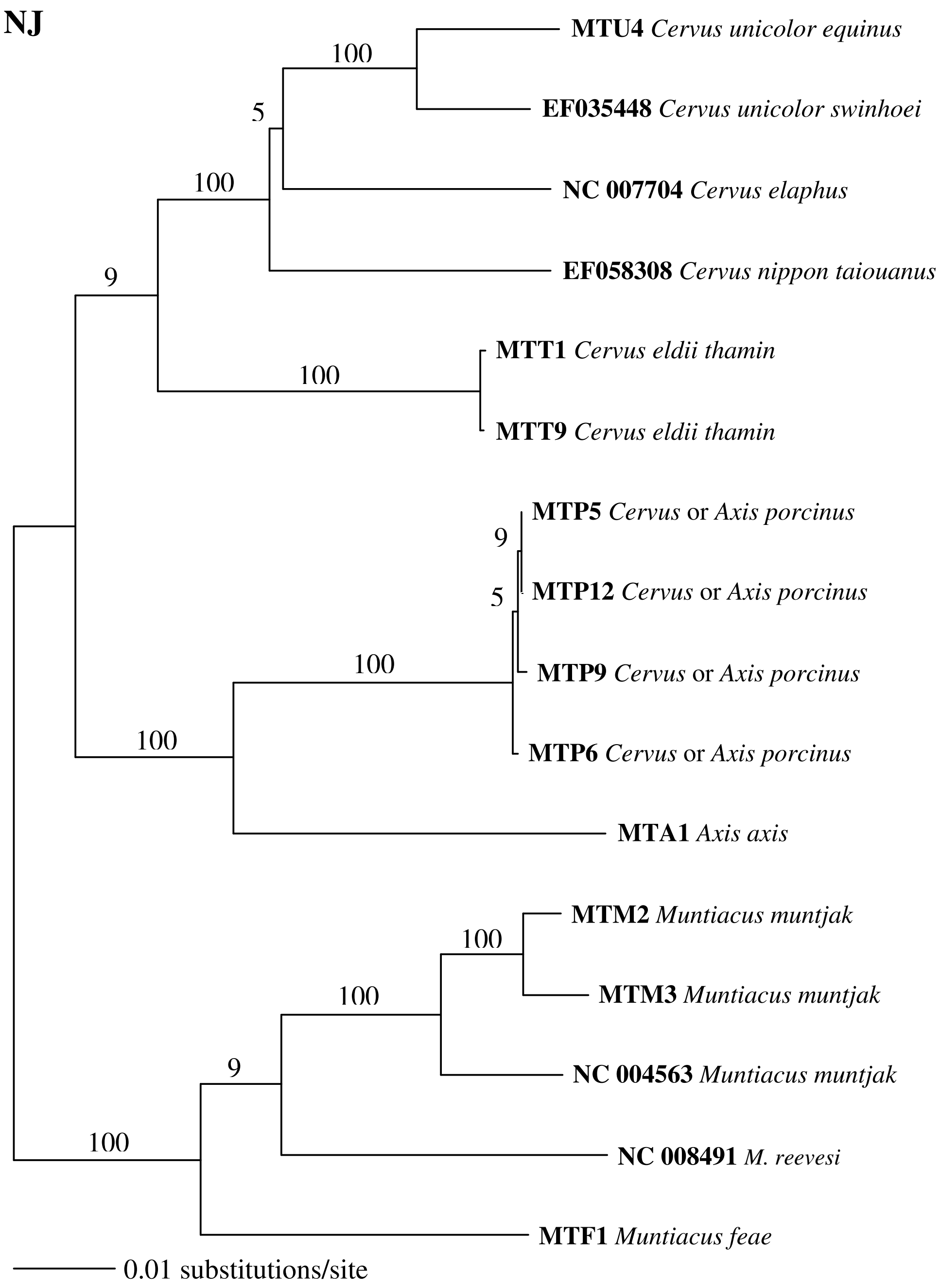


Figure 37 Neighbor-joining tree from cervid mitochondrial DNA sequences of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) generated from 1,000 replications.

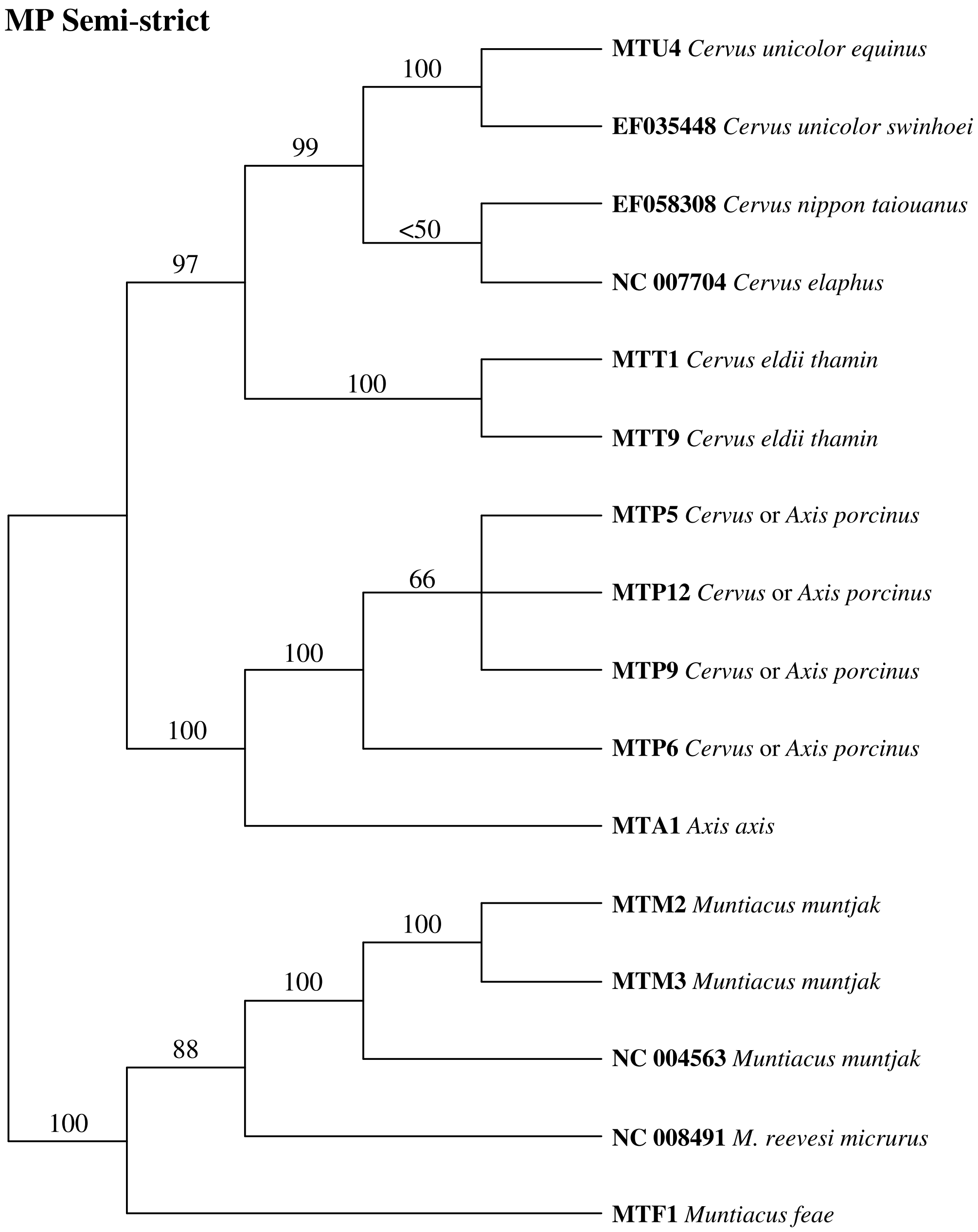


Figure 38 Semi-strict consensus tree of the most three parsimonious trees from cervid mitochondrial DNA sequences of 16 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) based on 1,000 replications.

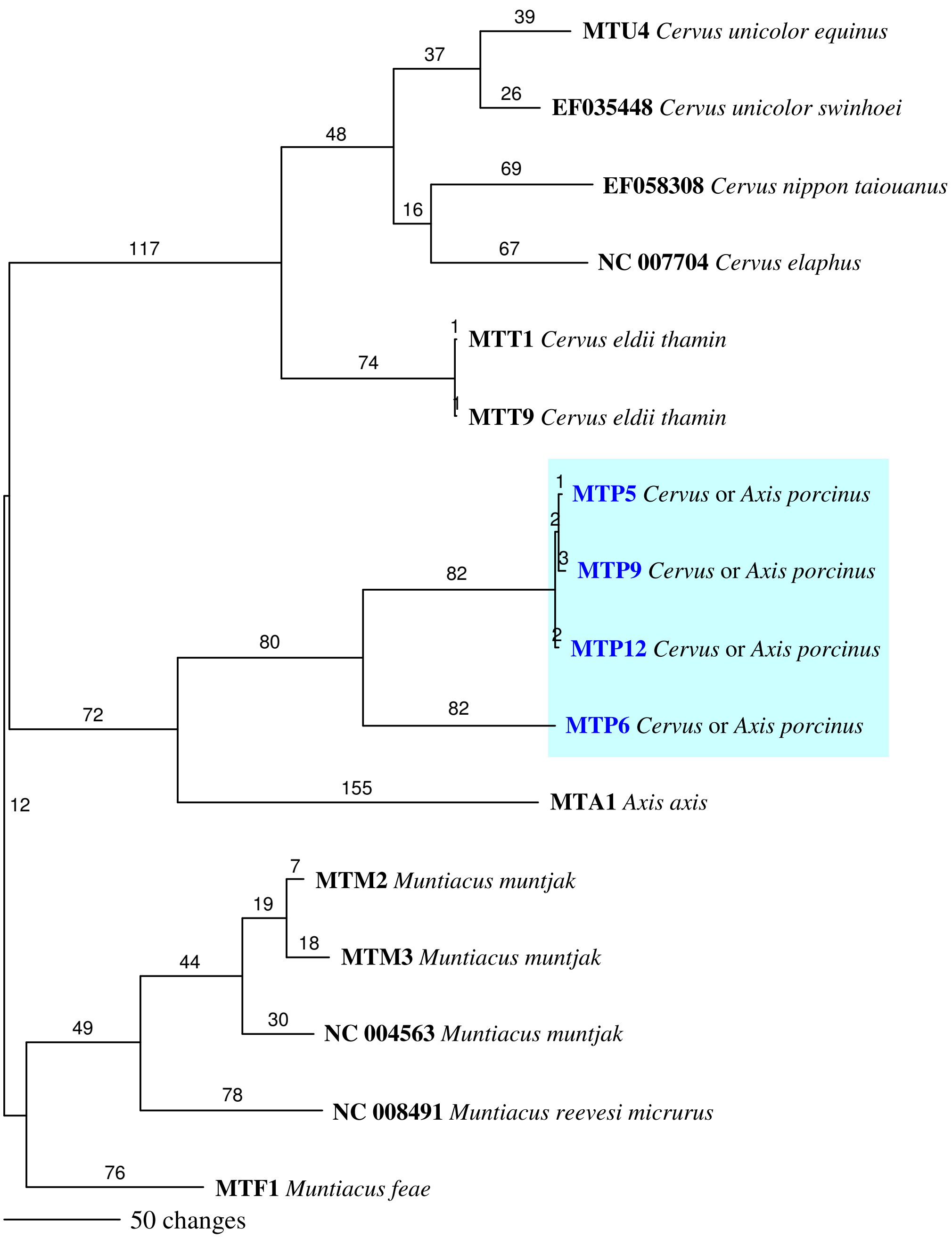


Figure 39 One of the most three parsimonious trees from cervid mitochondrial DNA sequences of 16 deer taxa. Numbers above branches show numbers of base changes.

6. Phylogenetic relationship analysis inferred from mitochondrial DNA and nuclear DNA of PRKCI intron sequences

A. Combined data set of mitochondrial DNA and nuclear DNA of PRKCI intron

After DNA sequences assembly in each sample of mitochondrial DNA (cytochrome *b* gene and control region portion) were finished. Each mitochondrial DNA sequences and each PRKCI intron1 nuclear DNA from the same sample were combined together as each one of data set. All sequences were aligned together using the software ClustalX version 1.82 (Thomson *et al.*, 1977), followed by visual inspection to ensure correct sequences. Finally, each one of combined sequence contained approximately 3,150 bp long. The example of combined sequence in each sample was obtained from this study as shown in Figure 40.

>ALA1_*Axis_axis*

```
CAAAAATAATACTTAAGATAAATACAATGTATGTTATCATTATTCTCACATGGAATCTAACC
ATGACTAATGATATGAAAAACCATCGTTGTCATTCAACTACAAGAACACTAATGATCAACAT
CCGAAAAACTCACCCATTAATAAAAATTGTAAACAACGCATTCATTGACCTCCCAACCCCAT
CAAATATTTCATCCTGATGGAACTTCGGCTCTTTGCTAGGAGTCTGCTTAATTCTACAAATT
CTCACGGGCTTATTCTTAGCAATACACTATACATCTGATACAATAACAGCATTCTCCTCTGT
CACTCATATCTGTCGAGACGTCAACTACGGCTGAATTATTCGATATATGCACGCAAATGGAG
CATCAATATTTTTTATTTGCTTATTTTTACATGTAGGACGAGGTCTGTATTACGGATCATAT
ACCTTTTTAGAAACATGAAACATTGGAGTAATCCTCCTATTTACAGTTATAGCCACAGCATT
TGTGGGATACGTCCTACCATGAGGACAGATATCATTCTGAGGAGCAACAGTTATTACCAATC
TCCTCTCAGCAATCCCTTACATTGGTACAAATCTAGTCGAATGAATTTGAGGAGGCTTTTCA
GTAGATAAAGCAACCCTAACCCGATTTTTCGCTTTCCACTTTATTCTCCCATTTATCATTGC
AGCACTTGCTATAGTTCACCTACTCTTCCTTCACGAAACAGGATCCAATAACCCAACAGGAA
TTCCATCAGATGCAGATAAAATTCCCTTCCACCCTTACTATACCATTAAAGATACTCTAGGT
ATTATTCTTCTAATTATATTCCTAATACTACTAGTGCTATTTGCACCAGATATGCTTGGAGA
CCCAGACAACTATACCCCAGCAAACCCACTCAATACACCTCCCCATATTAAACCCGAATGGT
ATTTCCTATTTGCATACGCAATCCTACGATCAATTCCCAATAAACTAGGAGGAGTTTTAGCC
CTAGCTTCATCCATCCTGATCCTAATTCTTATACCCCTCCTCCACACATCCAAACAACGCAG
CATAATATTCCGACCATTTAGCCAATGCTTATTTTGAATCTTAGTGGCAGACCTACTAACAC
TTACATGAATCGGAGGACAACCAGTTGAATACCCCTTTATTATTATCGGACAACTAGCATCT
ATTCTATACTTTCTTATTATCCTAATTCTTATACCAATTATCAGCTCAATCGAAAATAACCT
CCTAAAATGAAGATAAGTCTTTGTAGTATACTGAATACACTGGTCTTGTAAATCAGAAAAGG
AGAGTAATTAACCTCCCTAAGACTCAAGGAAGAAGCAATAGCCCCACGTATCAACACCCAAA
```

Figure 40 DNA sequence of spotted deer; *Axis axis* (ALA1) as the example shows the combined sequence in each sample of the data set.

```
GCTGAAGTTCTATTTAAACTATTCCCTGATGCCTTTGTTAATATAGTTCCATAAAAATCAAG
AACTTTATTAGTATTAAATTTCCAAAAATTTTAACAATTCAATACAGCTTTCTACTCAACAT
CCAATTTACACCCAACATACCATTTTCTACACAACATGACATGCAATGCATTGCATGCGCTT
ATAGTACATAAAATTAATGTATTAGGACATACTATGTATAATAGTACATTACATTACATTCC
CCATGCTTATAAGCATGTACCCCTCATCATTTATAGTACATAGTACATAGTGTTGTTCATCG
TACATAGCACATTGAGTCAAATCAATCCTTGTCAACATGCGTATCCCGTCCCCTAGATCACG
AGCTTAATTACCATGCCGCGTGAAACCAACAACCCGCTAGGCAGGGATCCCTCTTCTCGCTC
CGGGCCCATAAACTGTGGGGGTAGCTATTTAATGAATTTTATCAGACATCTGGTTCTTTCTT
CAGGGCCATCTCACCTAAAATCGCCCACTCTTTCCTCTTAAATAAGACATCTCGATGGACTA
ATGACTAATCAGCCCATGCTCACACATAACTGGGGGTCATACATTTGGTATTTTTAATTTT
TGGGGGATGCTTGGACTCAGCATTGGCCGTCTGAGGCCCCGACCCGGAGCATAAATTGTAGC
TGGACTTAACTGCATCTTGAGCATCCCCATAATGGTAGGCGCGGGGCATGGCAGTCAATGGT
CCCAGGACATAGTTATTATTTCAGGGGTCAACCCTTTAATTTTTTTCCCCCCCCCTCATAT
TTTCCCCCTTATATAGTTACCACCATTTTTAACACGCTTTCCCCTAGATATTATTTTAAATT
TATCACATTTCCAATACTCAAATGGCACTCCATAAGGAGGTAAGTATATAAGCGCCTATTTT
TTCCTAATTACGCACAGTTAATGTAGCTTAAACAATAAAGCAAGGCACTGAAAATGCCTAGA
TGAGTATATTAACTCCATAAACATATAGGTTTGGTCCCAGCCTTCCTATTAGCCCGTAATAG
ACTTACACAGGGAAGCATTTATGCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTA
TTTATGCAATGAAAGTTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATATGA
TGTTTTATTACCTCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAAT
GTTTGTGCCTTATTGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATA
AGGTATATTTAAAGTTGTCTGATGATTATTAGATTTTTTAAAAATAAACATCAAACCATATT
ATTATCAAGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGA
TTTGCTGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTG
ATGTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGGAT
ATTGACTGGG
```

🟦 = Sequence of mitochondrial DNA

🟥 = Sequence of PRKCI intron nuclear DNA

Figure 40 (Continued)

B. Samples for phylogenetic analysis

This analysis part was conducted only the samples which were obtained from this study, because of in GenBank, there was no sequence samples from PRKCI gene intron1 which were the same sample of mitochondrial DNA sequence samples. Therefore, only eleven sample sequences from combined sequences of mitochondrial DNA and nuclear DNA of PRKCI intron obtained from this study were analyzed, including hog deer (*Cervus* or *Axis porcinus*), sambar deer (*Cervus unicolor equinus*), thamin Eld's deer (*C. eldii thamin*), common barking deer (*Muntiacus muntjak*), Fea's barking deer (*M. feae*) and one cervid species which not native to Thailand;

spotted deer (*Axis axis*). Eleven of them were aligned using ClustalX version 1.82 (Thompson *et al*. 1997). Before launching to PAUP* version 4.010b (Swofford, 2002) for phylogenetic analysis, multiple sequences alignment on ClustalX was saved as nexus file (*.nxs). Sample names and accession number of all sequences for phylogenetic analysis as shown in Table 14.

C. Combined sequences of mitochondrial DNA and nuclear DNA of PRKCI intron characteristics

The final alignment of cervid mitochondrial DNA of all taxa consisted of 3,152 characters, in which scattered from 2,434 up to 2,559 bp long. Of these 3,152 characters, 2,634 were constant and 392 were parsimony informative. The average base composition and base sequence amount of each cervid sample as shown in Table 15.

D. Phylogenetic analysis

The phylogenetic trees were executed as 2 types, which were (1) neighbor-joining (NJ) analysis and (2) maximum parsimony (MP) analysis using PAUP* version 4.010b (Swofford, 2002). For the NJ tree, non-parametric bootstrap analyzes with 1,000 replicates were performed to obtain estimates of support for each node of the NJ tree as shown in Figure 41. For the MP tree, the informative sites were analyzed using equally weighted characters and were searched by branch-and-bound search option. Gaps were treated as missing data. With this branch-and-bound search option, three most parsimonious trees were found, and the semi-strict consensus of the most three parsimonious trees is shown in Figure 42. Finally, the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1,000 replicates.

Table 14 Cervid samples for mitochondrial DNA and nuclear DNA of PRKCI intron phylogenetic analysis used in this study.

Common name	Zoological name	Sample name	Source	No. of sample
Common barking deer	*Muntiacus muntjak*	ALM2, ALM3	Pattalung Wildlife Station[a]	2
Fea's barking deer	*Muntiacus feae*	ALF1	Ton Nga-Chang Wildlife Station[a]	1
Hog deer	*Cervus* or *Axis porcinus*	ALP5, ALTP6	Hauy Sai Wildlife Station[a]	4
		ALP9	Dusit Zoo[b]	
		ALP12	Songkhla Zoo[b]	
Sambar deer	*Cervus unicolor equinus*	ALU4	Hauy Sai Wildlife Station[a]	1
Thamin Eld's deer	*Cervus eldii thamin*	ALT1	Hauy Sai Wildlife Station[a]	2
		ALT9	Dusit Zoo[b]	
Spotted deer	*Axis axis*	ALA1	Songkhla Zoo[b]	1
Total				11

[a] Wildlife Propagation Division, DNP

[b] The Zoological Park Organization under the Royal Patronage of His Majesty the King

Table 15 Percentage of average base composition of mitochondrial DNA and nuclear DNA of PRKCI intron of each cervid sample examined by PAUP* version 4.010b.

Common name (Zoological name)	**Sample name**	**Base composition (%)**				**Amount (bp)**
		A	**C**	**G**	**T**	
Common barking deer (*Muntiacus muntjak*)	ALM2	31.68	22.64	14.53	31.14	2,986
	ALM3	31.62	22.58	14.55	31.25	2,989
Fea's barking deer (*Muntiacus feae*)	ALF1	31.73	22.83	14.40	31.03	3,000
Hog deer (*Cervus* or *Axis porcinus*)	ALP5	32.00	22.11	13.91	31.97	3,012
	ALP6	31.95	22.15	13.95	31.95	3,011
	ALP9	32.00	22.13	13.92	31.94	3,009
	ALP12	32.00	22.13	13.92	31.94	3,009
Sambar deer (*Cervus unicolor equinus*)	ALU4	32.21	22.33	14.51	30.95	3,108
Thamin Eld's deer (*Cervus eldii thamin*)	ALT1	31.72	22.78	14.45	31.05	2,989
	ALT9	31.77	22.81	14.45	30.97	2,990
Spotted deer (*Axis axis*)	ALA1	31.61	21.80	14.57	32.02	2,986
Mean		31.85	22.39	14.29	31.47	3,008.09

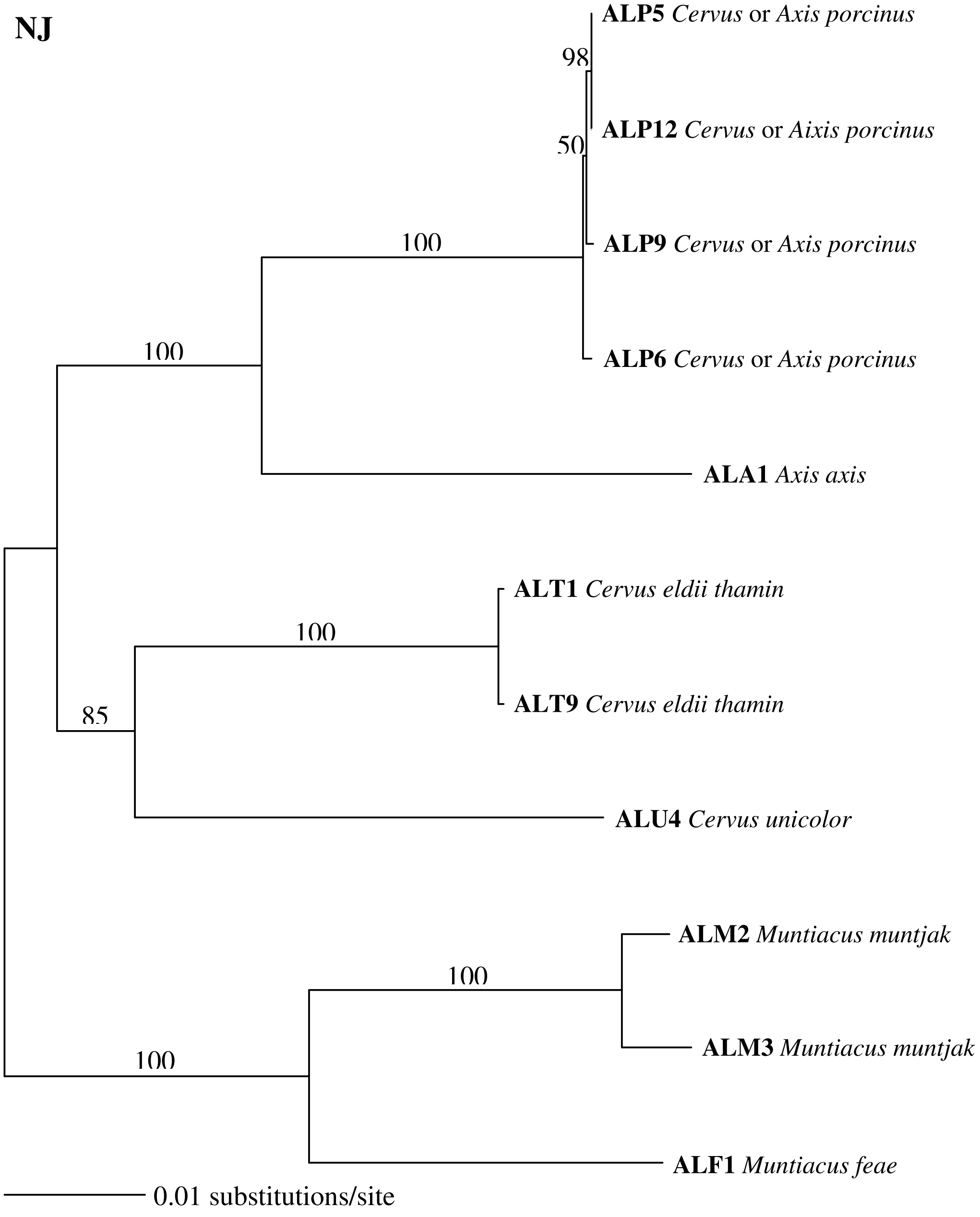


Figure 41 Neighbor-joining tree from cervid mitochondrial DNA sequences and PRKCI intron nuclear DNA sequences of 11 deer taxa. Numbers above branches indicate % bootstrap support (only > 50%) generated from 1,000 replications.

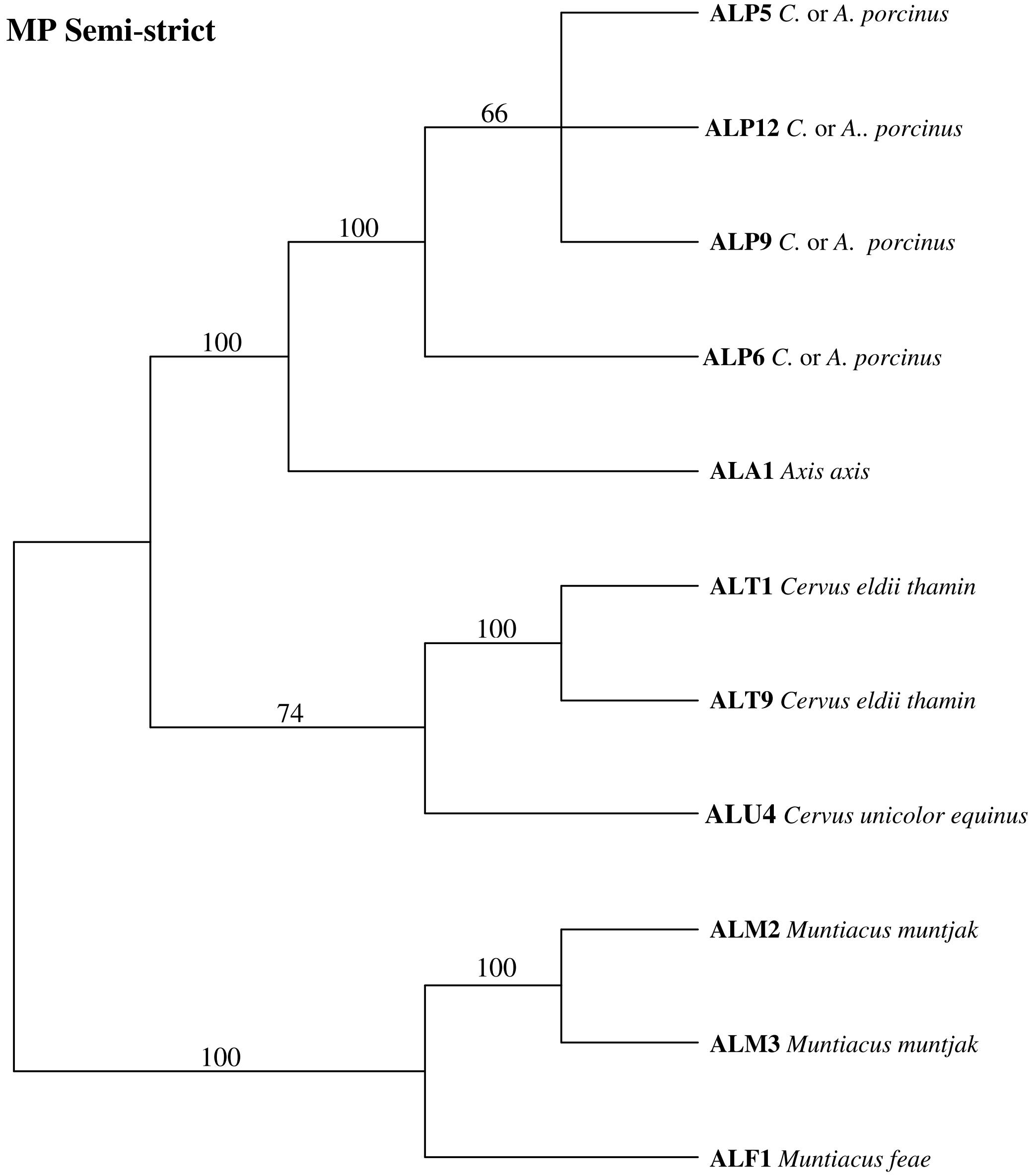


Figure 42 Semi-strict consensus tree of the most three parsimonious trees from cervid mitochondrial DNA and PRKCI intron nuclear DNA sequences of 11 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) based on 1,000 replications.

E. Phylogenetic relationship result

All phylogenetic analyses yielded the similar topology of tree. It found that hog deer (*Cervus* or *Axis porcinus*) were grouped monophyletically in the same clade of spotted deer (*Axis axis*) (assumption as genus *Axis* clade) with absolutely strong statistical bootstrap support (100 % on NJ tree and 100 % on MP tree). These phylogenetic tree results were powerful declaration that hog deer should be placed in the genus *Axis*, not genus *Cervus*. For the genus *Cervus*, it was also formed as the same clade with strong statistical bootstrap support (85 % on NJ tree and 74 % on MP tree).

To distinguish the genus *Axis* clade from the genus *Cervus* clade (not include *Cervus* or *Axis porcinus*), 198 base change were needed. These 198 synapomorphic nucleotide characters were counted from 79 base synapomorphic characters of the genus *Axis* clade, which could group spotted deer and hog deer together, and 119 base synapomorphic characters between sambar deer (*Cervus unicolor equinus*) and thamin Eld's deer (*C. eldii thamin*). For hog deer if compared with spotted deer, there are 259 base changes between them, which were counted from 90 base synapomorphic characters of hog deer and 169 base synapomorphic characters of spotted deer. Among the genus *Cervus* with only two species of sambar deer and thamin Eld's deer, 178 base changes were needed to distinguish them. These 178 synapomorphic nucleotide characters were counted from 91 base synapomorphic characters of sambar deer and 87 base synapomorphic characters of thamin Eld's deer.

For Fea's barking deer (*Muntiacus feae*); one member of outgroups as the important endangered species of Thailand, it could be noticed that they were former sister lineage of common barking deer (*Muntiacus muntjak*). Among the *genus Muntiacus*, there are 145 base changes to distinguish the species of them, in which were counted from 69 base synapomorphic characters of Fea's barking deer and 76 base synapomorphic characters of common barking deer. The parsimonious tree that shows numbers of base changes is shown in Figure 43.

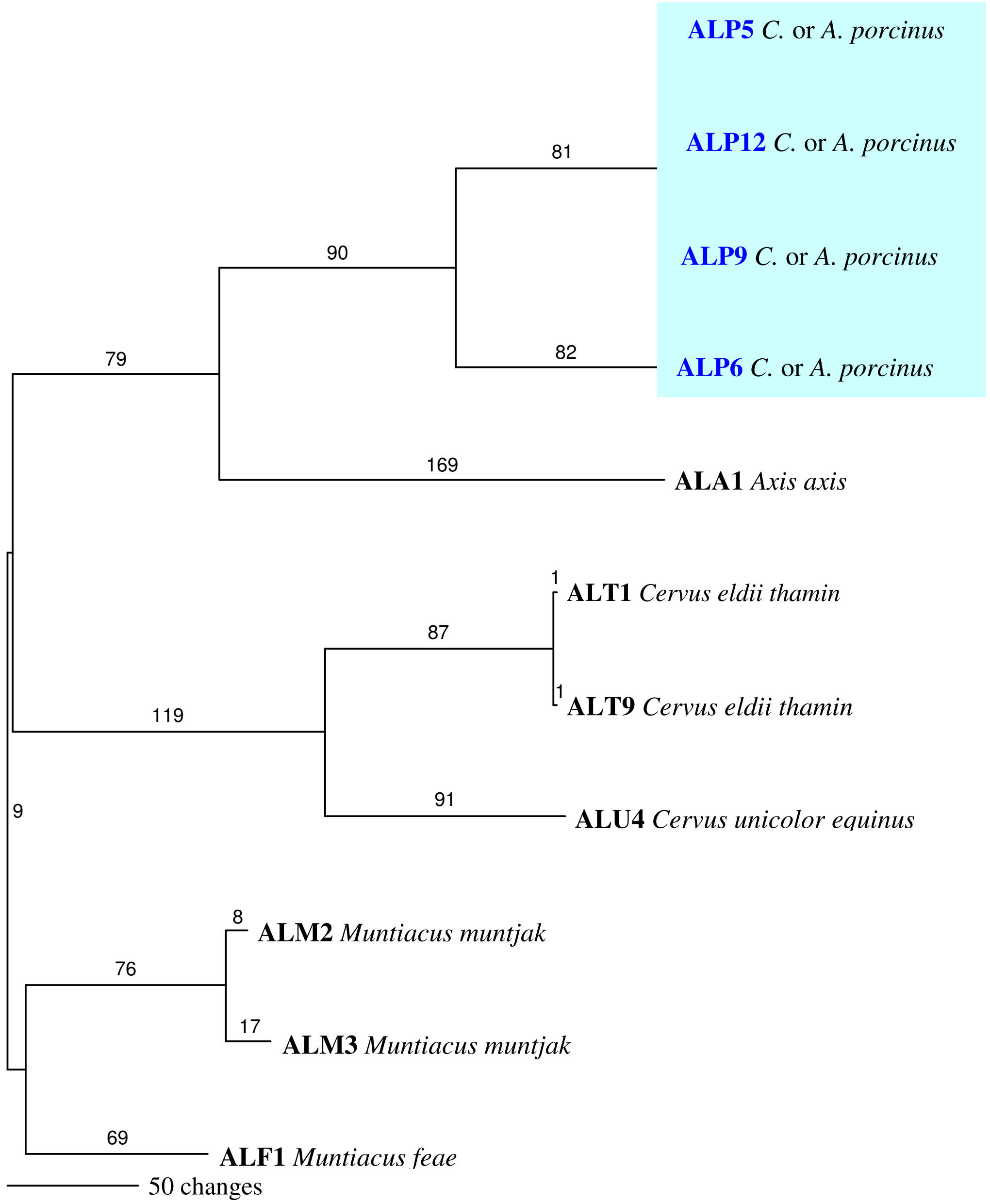


Figure 43 One of the most three parsimonious trees from cervid mitochondrial DNA and PRKCI intron nuclear DNA sequences of 11 deer taxa. Numbers above branches show numbers of base changes.

Discussion

1. Phylogeny and Taxonomy of Thai Deer Inferred from Each Molecular Marker

A. Cytochrome *b* gene with supplement of partial NADH dehydrogenase subunit 6 gene, complete $tRNA^{Glu}$ gene and complete $tRNA^{Thr}$ gene

This study showed that there is a monophyly of the subfamily Cervinae (*Cervus* and *Axis*) with strongly statistic support, whereas a polyphyly of the genus Cervus was found. This result is in agreement with previous molecular phylogenetic studies based on cytochrome *b* (Randi *et al*., 1998; Pitra *et al*., 2004; and Gilbert *et al*., 2006). This analyzed is different from the others molecular marker by including of Schomburgk's deer and swamp deer into the data set. Consequently, the derived trees have been shown more informative classification; with clearly divide Thai Cervine into three main clades of *Cervus*, *Rucervus* and *Axis* which was corresponded to the new revised classification of Gilbert *et al*. (2006). They classified the subfamily Cervinae into four genera; consisted of *Cervus*, *Rucervus*, *Axis* and *Dama* in which the last one is not native to Thailand. The classification of Gilbert *et al*. (2006) was differed from Groves and Grubb (1987) and Grubb (1993) who divided subfamily Cervinae into four genera; consisted of *Cervus*, *Elaphurus*, *Axis* and *Dama*, in which genus *Cervus* was divided into four subgenera; consists of *Rusa*, *Rucervus*, *Cervus* and *Przewalskium*. This former classification classified Schomburgk's deer and Eld's deer into subgenus *Rucervus* and classified sambar deer into subgenus *Rusa*.

From the derived tree topology, it showed that there is polyphyly of the genus *Cervus*, because of Eld's deer (or *Rucervus eldii* in the former classification) was allied with the genus Cervus clade, was not joined as the sister lineage of Schomburgk's deer (or *Rucervus schomburgki* in the former classification), whereas Schomburgk's deer and swamp deer (or *Rucervus duvaucelii* in the former classification) were grouped nearly with the genus *Axis* clade, depart from the genus *Cervus* clade with strong bootstrap support (88 % on NJ tree and 83 % on MP tree) and wide range of base changes between them. This study confirms a polyphyly of

Cervus as previously found by Pitra *et al.* (2004) and Gilbert *et al.* (2006). Therefore, the results from this study suggest that Schomburgk's deer (*Cervus schomburgki*) should be placed in the genus *Rucervus,* as defined by Hodgson (1838) and Eld's deer (*Cervus eldii*) should be placed in the genus *Cervus*, not was *Rucervus* as defined by Groves and Grubb (1987) and Grubb (1993).

For the genus *Axis* (includes spotted deer and hog deer), it was found that it formed a monophyletic clade with very strong support (99 % on NJ tree and 86 % on MP tree). This result supports a previous study of Gilbert *et al.* (2006) which found that *Axis* was a strongly supported monophyly by three independent markers of cytochrome *b* and two nuclear DNA introns. However, this finding is in contrast with the former study of Pitra *et al.* (2004). They found that *Axis* was paraphyly, having *A. porcinus* joined with *C. timorensis* and *A. axis* related to *C. duvaucelii*. The work of Pitra *et al.* (2004) published the first molecular phylogeny of old world deer with the comparison of cytochrome *b* sequences of an almost complete set of the subfamily Cervinae. Nevertheless, their study was performed by using only one sequence of *A. porcinus* produced by Ludt *et al.* (2004) in accession No. AY035874. Gilbert *et al.* (2006) has suggested that this *A. porcinus* sequence may obtain from a tissue sample of a misidentified specimen. This study confirms a monophyly of *Axis* as previously found by Gilbert *et al.* (2006). Therefore, the results from this study suggest that hog deer (*Cervus* or *Axis porcinus*) should be placed in the genus *Axis,* as defined by Groves and Grubb (1987) and Grubb (1993).

B. Control region (CR) with supplement of complete tRNA^{Phe} gene and partial 12 ribosomal RNA gene

Because Fea's barking deer (*Muntiacus feae*) could not be joined as outgroups with the others *Muntiacus* species, therefore two kinds of data sets were made as (1) the data set of was not included Fea's barking deer and (2) the data set which was included Fea's barking deer, but not set it as outgroups.

In the data set which not included Fea's barking deer, the phylogenetic trees show a monophyly of the subfamily Cervinae (*Cervus* and *Axis*) with strongly statistic support. This result is in agreement with previous studies based on control region (D-loop) sequences of Douzery and Randi (1997), Bonnet (2001), Randi *et al.* (2001). Actually, CR contains sequences which are both of central conserved portion (CCR) and highly divergent peripheral portion (5'-left and 3'-right). The more slowly evolving CCR has been used to reconstruct phylogenies of mammal and placental orders (Saccone *et al.*, 1991), whereas the peripheral region which were more high rate of nucleotide change have been widely used in population and evolutionary genetics of mammals (Loftus *et al.*, 1994). Nevertheless, the complete sequences of CR were obtained from this study, but not all portions of sequences could be aligned for data analysis. Almost 5'-left peripheral portion were cut off and CCR and 3'-right peripheral portion were still remained. The derived trees based on this portion were similar topology with the derived from cytochrome *b*, in which found that there is a monophyly of the genus *Axis* clade that spotted deer and hog deer were grouped together.

For the data set which included Fea's barking deer, but not set it as outgroups, the strange and incredible result was happened. The derived tree shows Fea's barking deer (*Muntiacus feae*) was joined close to the genus *Cervus* clade. This occurrence could be explained that Fea's barking deer have similar sequences, particularly in domain III (3'-right peripheral domain). It was found that Fea's barking deer have a pattern and a number of VNTRs copies in the CSB-2+3 region as same as a pattern of *Cervus* species with equally copied. This unexpected evidence of VNTRs copied from this study is the first one incorporating from Thai cervid samples, especially, this is first complete cytochrome *b* and control region sequences which obtained from Fea's barking deer (*Muntiacus feae*), thamin Eld's deer (*Cervus eldii thamin*), hog deer (*Cervus* or *Axis porcinus*) and spotted deer (*Axis axis*).

C. Intron1 of PRKCI gene

This study showed that there is a monophyly of the subfamily Cervinae (*Cervus* and *Axis*) with strongly statistic support. This result is in agreement with previous molecular phylogenetic studies based on mitochondrial DNA and nuclear DNA intron (Gilbert *et al*., 2006).

In the case of the genus *Cervus*, all of the previous studies totally agreed that *Cervus* is polyphyly, either based on cytochrome *b*, control region, nuclear DNA intron, or combine data (Randi *et al*., 2001; Liu *et al*., 2003; Pitra *et al*., 2004; and Gilbert *et al*., 2006). However, our current result based on PRKCI intron sequences may not help finalizing the phylogenetic relationships among *Cervus* because the PRKCI gene intron could not give enough resolution to appropriately distinguish each *Cervus* species within the genus (< 50 % bootstrap values on NJ tree and polytomy branches have been occurred among them on MP tree). To get a better resolution of phylogenetic relationships among *Cervus*, more appropriate genes or sequence portions should be added as molecular phylogenetic markers for data analyzing.

For the genus *Axis*, we found that it formed a monophyletic clade with moderate support (64 % on NJ tree and 63 % on MP tree). Although the number of base changes was not much found based on this molecular marker, this was enough to distinguish spotted deer and hog deer into two difference species in the same genus. This result supports a previous study of Gilbert *et al*. (2006) which found that *Axis* was a strongly supported monophyly by three independent markers of cytochrome *b* and two nuclear DNA introns.

D. Mitochondrial DNA (cytochrome *b* gene and control region)

This phylogenetic analysis was conducted on the assemblage of the complete cytochrome *b* gene and the complete control region mitochondrial DNA sequences with supplement of their adjacent gene elements. The derived trees show the monophyletic clade of subfamily Cervinae, as same as the monophyletic clade of the genus *Axis* with very strong statistic supported. Without the sample of Thai *Rucervus* deer such as Schomburgk's deer (*Cervus schomburgki*) based on this portion, the genus *Cervus* become strong monophyly forming, with Eld's deer was the former sister lineage of the others *Cervus* species. For the position of hog deer, Eld’s deer and sambar deer, the topology of trees is congruously with the previous studies based on mitochondrial DNA and nuclear DNA intron of Gilbert *et al*. (2006).

When the sequences of the complete cytochrome *b* gene and the complete control region were assembled, the sequences in the data set could be multiple aligned together in the ClustalX program neatly and easily. There is no problem concern with alignment in the samples of sambar deer and Fea’s barking deer just like was found in alignment based on control region solely. Their VNTRs copies could be arranged as neaten blocks. For Fea’s barking deer as the one of outgroups, it was found placing as the former sister lineage of the others *Muntiacus* species, with strongly bootstrap supported (96 % on NJ tree and 88 % on MP tree). For hog deer, it was reliably grouped in the genus Axis with absolutely bootstrap supported (100 % on NJ tree and 100 % on MP tree). To the best of knowledge, this is the first one incorporating all extant Thai cervid, with the longest sequences of mitochondrial DNA.

E. Mitochondrial DNA (cytochrome *b* gene and control region) and nuclear DNA of PRKCI intron1

This phylogenetic analysis was combined the sequences of the cytochrome *b* and control region mitochondrial DNA and nuclear DNA of PRKCI intron1 together. The eleven sequences in data set were obtained from only this study. The derived trees show the monophyletic clade of subfamily Cervinae, and the monophyletic clade of the genus *Axis* with very strong statistic supported just the same as the tree topology which inferred from mitochondrial DNA. This result is in agreement with the previous studies based on mitochondrial DNA and nuclear DNA intron of Gilbert *et al*. (2006).

For the position of hog deer, the derived tree inferred from this combined data shows the reliable confirming that it was placed in the genus Axis with absolutely bootstrap supported (100 % on NJ tree and 100 % on MP tree). To the best of knowledge, this is the first one incorporating all extant Thai cervid, with the longest combined sequences of mitochondrial DNA and nuclear DNA.

2. Comparison of Molecular Marker Capability for Cervid Taxonomic Classification

To compare capability and efficiency of each molecular marker that was chosen to analyze the phylogenetic data in this study, the comparison of the parsimonious informative sites among each molecular marker as shown in Table 16 and the value of % bootstrap support on trees were accepted for the consideration content as shown in Table 17.

All of three molecular markers (cytochrome *b* gene, control region and PRKCI intron1) which were selected in this study are neutral marker, with high polymorphism level. The cytochrome *b* gene has been used in numerous studies of phylogenetic relationships among mammals because its tempo and mode of evolution is well understood. Its high level of variability makes it most generally use for the comparison of species in the same genus or family (Jonhs and Avise, 1998). Similar to the cytochrome *b*, the whole control region has been commonly used to reconstruct the phylogenies of placental orders (Saccone *et al*., 1991). Because of their high mutation rate in the peripheral regions, control region sequences are also widely used in population and evolutionary study in mammals (Loftus *et al*., 1994). From these three molecular markers, PRKCI intron1 is slower evolving marker (Gilbert *et al.* (2006).

From the comparison data in table 16, all markers (except control region) show absolute efficiency for cervid classification in the subfamily level with 100 % on all trees. To consider each gene or portion of marker solely, the cytochrome *b* gene is most effective classification in every taxonomic level, whereas control region could not be good resolution for all level of subfamily, generic and species classification. Depending on the number of VNTRs copies in control region, they maybe the cause of bias effect in which could not distinguish Fea’s barking deer from the species in the genus *Cervus*. For PRKCI intron1, this marker, with small informative characters, is still fair resolution for distinguish the species in the genus *Axis*, but could not give enough resolution for the species in the genus *Cervus*.

For mitochondrial DNA, the assemblage between the complete cytochrome *b* gene and the complete control region, this assemblage marker could provide better signal than alone marker of cytochrome *b*. The values of bootstrap support are increasing up to 100 % or almost in every taxonomic level, especially in genus *Axis* which their bootstrap support are not absolutely 100 % and *Muntiacus muntjak* which are only moderately bootstrap support in cytochrome *b* gene. For combined data of mitochondrial DNA and PRKCI intron, this combined marker is not much different from assemblage mtDNA marker, maybe because of small informative characters of PRKCI intron1 could not equally weight with the longer sequences of mtDNA. Moreover, the value of % bootstrap support in the genus *Cervus* is decreasing if compare to the mtDNA marker, probably is the effect of PRKCI intron1 which could not distinguish the species in the genus *Cervus*.

Therefore, this study suggest that the assemblage of complete cytochrome *b* and complete control region mitochondrial DNA is the best molecular marker for classification in all taxonomic level. If the laboratory procedure has been limited by the budget, alone molecular marker of complete cytochrome *b* gene could be most useful, with enough signals and capability for classification in every taxonomic level.

Table 16 The constant and the parsimony informative character of the sequences from each molecular marker after aligned by ClustalX version 1.82.

Molecular marker	**Number of sequences character**		
	All	Constant	Parsimonious informative site
Cytochrome *b*	1,328	985	290
Control region	923	731	149
PRKCI intron1	552	523	19
Mitochondrial DNA (*Cyt. b* + CR)	2,599	1,979	439
Combined data (*Cyt. b* + CR + PRKCI intron1)	3,152	2,634	392

Table 17 Percentage of bootstrap support on NJ and MP trees of each molecular marker using for cervid taxonomic classification. (UK = Unknown; because of only one taxon on the tree, NO = can not be classified)

Taxonomy Classification by Phylogenetic Approach		**Molecular Marker**									
		Cytochrome *b*		**CR**		**mtDNA**		**PRKCI intron**		**mtDNA & PRKCI intron**	
		NJ	**MP**	**NJ**	**MP**	**NJ**	**MP**	**NJ**	**MP**	**NJ**	**MP**
Subfamily level	Cervinae	100	100	NO	NO	100	100	100	100	100	100
Generic level	*Muntiacus*	100	100	NO	NO	100	100	100	100	100	100
	Cervus	100	99	<50	NO	99	97	<50	NO	85	74
	Axis	99	86	98	97	100	100	64	63	100	100
Species level	*Muntiacus muntjak*	64	55	100	100	100	100	67	63	100	100
	M. feae	100	100	NO	NO	UK	UK	100	99	UK	UK
	Cervus unicolor	100	100	56	NO	100	100	NO	NO	UK	UK
	C. eldii	100	100	100	100	100	100	NO	NO	100	100
	C. or *Axis porcinus*	100	100	100	100	100	100	57	58	100	100
	A. axis	100	100	UK	UK	UK	UK	54	56	UK	UK

3. Occurrences of Multiple Repeats (VNTRs) in the mtDNA Control Region of Cervidae

Although the phylogenetic trees inferred from control region sequences in this study presented as not good resolution for cervid taxonomy classification, it does not mean it is not appropriate tool for species classification at finer scale. Because of the 5'-left and 3'-right peripheral domain of control region can have highly variable numbers of tandem repeats (VNTRs) in mammals, this is the useful tool for classification at the finer geographical scale, especially for better understanding in local wildlife population dynamics on spatial genetic patterns (Avise, 1994; Rhodes *et al.*, 1996), whereas cytochrome *b* gene is useful tool for well understood of species evolution at macrogeographic scale (Avise, 2000). There are many previous studies revealed the occurrences of VNTRs in mammals control region, such as in bats (Wilkinson and Chapman, 1991), in cats (Lopez *et al*., 1996) and in sheep (Wood and Phua, 1996) For Cervidae, the previous study of Douzery and Randi (1997) presented only sika deer (*Cervus nippon*) and species of *Mazama* and *Odocoileus* have two copies sequences in the RS2 region of 5'-left peripheral domain, whereas Purdue *et al*. (2000) reported white-tailed deer (*Odocoileus virginianus*) have 2-4 copies sequences in the same region of 5'-left peripheral domain. The numbers of these copies are different in difference white-tailed deer population. They suggest that two copies of the repeat in RS2 had an ancient origin, while three or four copies of the repeat in RS2 region were occurring at the present time (Purdue *et al*., 2000). The RS2 region has repeated copies of TAS and D-loop termination sequences. This is likely a general character of VNTRs at these regions of the mammalian mtDNA (Stewart and Baker, 1994).

In this study, three copies of tandem repeats (VNTRs) were found in the RS2 region of 5'-left peripheral domain of Thai sambar deer (*Cervus unicolor equinus*), while the others of Thai cervid have no any tandem repeats in this region. These copies were tandemly duplicated from TAS-4 and TAS-3 region. TAS region (TAS-1, 2, 3 and 4) is the termination-associated sequences on the 5'-left domain which is the sites of the nacent H-strand syntheses ending (Clayton, 1991). The presence of tandem repeats of these functionally important TAS sequences could enable the cell to use alternative pathways for regulation of mtDNA replication and transcription and can provide the adventages to mtDNA molecules with multiple copies of replication signals (Kuzminov, 1996)

In the 3'-peripheral domain, multiple copies of VNTRs of CSB-2+3 sequences were found in this study. CSBs are conserved sequence blocks which is the sites of the L-strand transcription is cleaved by the RNase mitochondrial RNA processing (Clayton, 1991). For CSB-2 and CSB-3, they are apparently fused as CSB-2+3 regions among ruminant artiodactyls (Clayton, 1992; Ghivizzani *et al.*, 1993). To the new of knowledge, this is the first evidence yielded from Thailand cervid samples that showed multiple repeats in CSB-2+3 regions. From this new knowledge, Thai cervid species could be indicated by the number of copies in this region. Only hog deer (*Cervus* or *Axis porcinus*) have two copies of CSB-2+3-like, spotted deer (*Axis axis*), which not native to Thailand have only one copies, the remaining of Thai cervid species (except common barking deer) have only one copies, whereas common barking deer (*Muntiacus muntjak*) have no any copies. To compare the species in the genus *Muntiacus*, unexpected results was observed in this study, in which Fea's barking deer (*Muntiacus feae*) have one copies of CSB-2+3-like, whereas common barking deer (*Muntiacus muntjak*) have none. This study suggest that VNTRs of CSB-2+3-like in the 3'-peripheral domain could be distinguished hog deer from spotted deer, as same as could be characterized Fea's barking deer from common barking deer. The numbers of these VNTRs in the cervid control region mtDNA which were obtained from this study as shown in Table 18.

Table 18 Number of copies and basepair amount of tandem repeats (VNTRs) in the cervid control region mtDNA.

Cervid species	**VNTRs**			
	5' left peripheral domain (TAS-4 and 3-like)		**3' right peripheral domain (CSB-2+3-like)**	
	no. of copies	**bp**	**no. of copies**	**bp**
Muntiacus reeversi	-	-	-	-
Muntiacus muntjak	-	-	-	-
Muntiacus feae	-	-	1	35
Cervus unicolor equinus	3	120	1	35
Cervus unicolor swinhoei	3	120	1	35
Cervus elaphus	-	-	1	33
Cervus nippon taiouanus	2	78	1	32
Cervus eldii thamin	-	-	1	34
Cervus or *Axis porcinus*	-	-	2	52-54
Axis axis	-	-	1	33

4. Phylogenetic Position of Hog Deer

All of NJ and MP phylogenetic analyses inferred from each molecular marker, consisted of cytochrome *b* gene, control region, mitochondrial DNA and PRKCI intron1, including combined data of all molecular marker using in this study yielded congruent topology of the trees in which all hog deer were clustered, sisterly in the same clade of spotted deer (*Axis axis*) with moderately up to absolutely bootstrap supported (as the description in discussion 1). Therefore, hog deer by now would be named *Axis porcinus*, should not be named *Cervus porcinus* according to the traditional classification of Lekagul and McNeely (1977). The position of hog deer among the others Thai cervid and spotted deer or its closely relative, which not native to Thailand is presented in Figure 44.

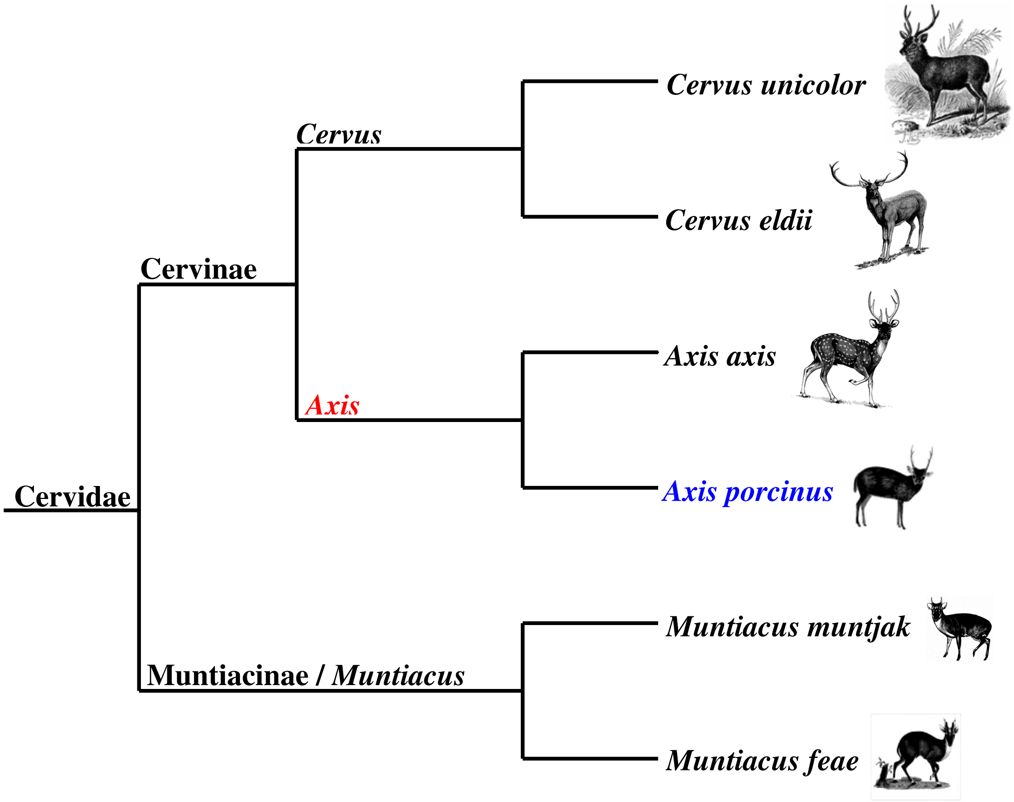


Figure 44 The tree model of Cervidae in Thailand display the phylogenetic position of hog deer (by now would be named *Axis porcinus*)

5. The Evolution of Cervinae: the Exposure of Thai Deer History

The subfamily Cervinae is one of the most specious group of artiodactyls, with extensive of morphological and ecological divergence (Grubb, 1993). There is widespread homoplasy among them caused by climatically similar conditions (Gentry, 1994). The first old world deer (subfamily Cervinae) originated in the Middle to Late Tertiary and radiated repeatedly from tropical into cold climates during the Pleistocene. The cervine deer that enter temperate zone have developed large and complex display organs, typically based on the head-pole and rump-pole strategy (Geist, 1998). From nowadays of molecular phylogenetic knowledge which advanced understanding of the Cervinae evolution, many conspicuous external features such as antler complexity, mane and rump-patch development turned out to be not related to phylogeny, but were bind to climatic related and ecological lifestyle factor (Groves, 2005). This hidden knot effected to Thai deer taxonomy classification also. Because of the traditional classifications with merely morphological characters were still inadequate and probably lead to expose in illusive classification.

The good point knowledge gained from phylogenetic approach in this study suggests that Thai cervine deer should be revised in a new taxonomy classification. Subfamily Cervinae in Thailand should be divided into three genera of *Cervus*, *Rucervus* and *Axis*, in which the extinct Schomburgk's deer should be placed in the genus *Rucervus* and hog deer should be placed in the genus *Axis*. These three genera are quite different in ecological lifestyle as the description below:

Genus *Cervus*: These deer are specialized for solitary life in thickets and have been adapted to quite dense habitats of forest with some open grass and scrub vegetation. They are rather browsing species (Geist, 1998).

Genus *Rucervus*: These species are deer of open woodland and riparian habitats, with the tall grass of flood plains. They are specialized graminivores with uniquely folded cheek teeth (Grubb, 1990)

Genus *Axis*: These species are deer of grassland and open forest habitats. Their typical feature is dwelling in such open terrain in large herds, and seems to be adapted to regularly burn grassy areas. They also occur in forest edge areas, where they grazed and feed on fallen fruit (Grubb, 1990; Geist, 1998).

To better understand Thai cervine history through their evolutionary time, the chronogram of Cervinae including of all Thai cervine species were created by adaptation from Pitra *et al*. (2004) fossil-constrained phylogram in the same time scale for the discussion in this part as shown in Figure 45.

Cervine deer such as *Axis* and *Cervus* are diverged rapidly in the Late Miocene to Early Pliocene. At this time, the climate was changed from the generally humid and warm of the Miocene to drier and cooler conditions in the Pliocene. On the Indian Peninsula and Indochina land were gradually changed from evergreen tropical rainforest and were replaced by deciduous forests. For the Early Pliocene about 5 myr ago, a vegetation mosaic of grasslands and forest was probably present in the Himalayan foothills (Morley, 2000). After 5 myr, grasses were dominant vegetation, possible interspread with riparian habitats. Such conditions would be initial evolution time of deer of grasslands and open forest types such as the *Axis* species as same as the initial establishment time of deer of riparian habitats such as the *Rucervus* species (Quade *et al*, 1989). The Early Pliocene cooling event was followed by a Middle Pliocene warmer period (about 3.5-3.0 myr ago). Evergreen forest was expanded into southern china, whereas Indochina was covered by rainforest, possible with patches of deciduous forest in the area of nowadays Burma (Dowsett *et al*., 1994). Such conditions could have led to isolation of grassland-dependent species and riparian-dependent species in areas where such vegetation remained. This is maybe the reason of small riparian area was remained as the habitats of Thai extinct Schomburgk's deer. The Middle Pliocene warm period was followed by a severe cooling period at the Pliocene-Pleistocene transition (about 2.4-1.8 myr ago). Indochina and southern China were still covered by humid rainforest, but this zone was pushed into the south during the Pleistocene until no rainforest remained in China (Liu and Ding, 1984). At the end of the Pliocene, most of China was still covered by tropical, subtropical and warm temperate forests; presumably the first *Rusa* species (Genus *Cervus*) have been adapted to quite dense habitats of forest with some open grass and scrub vegetation (Jablonksi and Whitfort, 1999). There is fossil evidence of the first *Cervus* or *Rusa unicolor* which was appeared in Middle Pleistocene (Dong, 1993). Because of the tropical forest zone moved to the south and almost disappeared in China, it was led to the disappearance of *Rusa* deer from most of China, while possibly being a factor in initial evolution of modern *Cervus* such as *Cervus elaphus* and *Cervus nippon* in China (Zong, 1987).

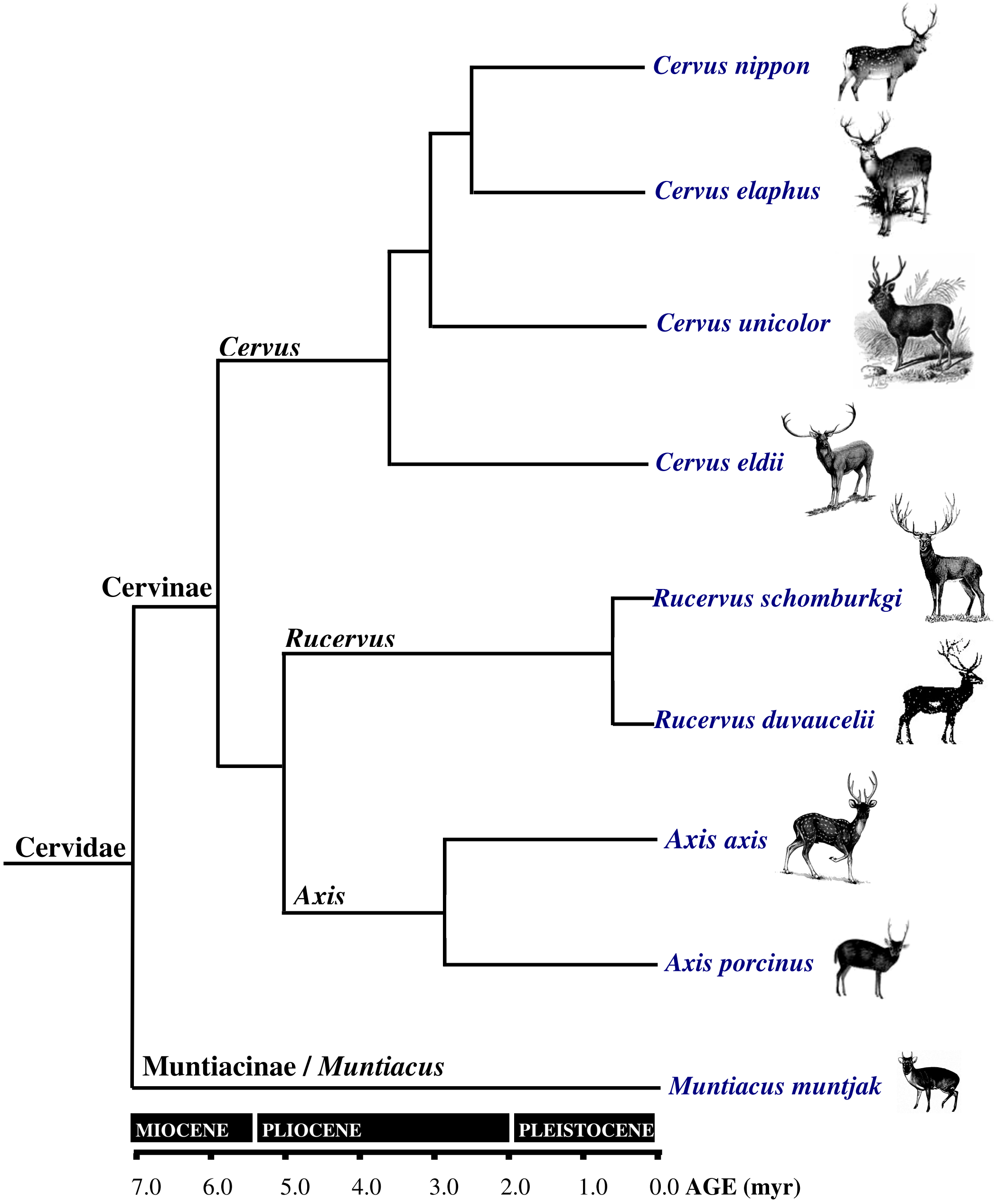


Figure 45 The chronogram of Cervinae including of all Thai cervine species which is adapted from fossil-constrained phylogram of Pitra *et al.* (2004) and Gilbert *et al.* (2006) in the same evolutionary time scale.

6. New Classification of Thai Deer

Current traditional, morphological taxonomy of deer in Thailand splits the family Cervidae on the basis of antlers and craniometrical character. This criterion separates Thai deer to only two genera: *Muntiacus* and *Cervus* (Lekagul and McNeely, 1977). However, the taxonomic value of antlers has been repeatedly questioned because some morphological characters seem to evolve with high homoplasy in ungulates (Scott and Janis, 1987). In this study, the integrated results based on nucleotide sequence comparison of mitochondrial DNA and nuclear DNA intron of Thai deer showed that Thai cervid should be divided into four genera: *Muntiacus*, *Cervus* and additionally *Rucervus* and *Axis* since the extinct Schomburgk's deer were found positioning nearby the genus *Axis* clade, with clearly separated from the genus *Cervus* clade inferred genus *Rucervus* and for hog deer which were found positioning along side spotted deer within the monophyletically inferred genus *Axis*. We suggested that the zoological name of Schomburgk's deer should be renamed to "*Rucervus schomburgki*" and hog deer should be renamed to "*Axis porcinus*". The new systematic classification of Thai deer is proposed as below:

- Family Cervidae
  - Subfamily Muntiacinae
    - Genus *Muntiacus*
      - *Muntiacus muntjak* (Common barking deer)
      - *Muntiacus feae* (Fea's barking deer)
  - Subfamily Cervinae
    - Genus *Cervus*
      - *Cervus unicolor* (Sambar deer)
      - *Cervus eldii* (Eld's deer)
        - ▪ *Cervus eldii thamin* (Thamin Eld's deer)
        - ▪ *Cervus eldii siamensis* (Siamese Eld's deer)
    - Genus *Rucervus*
      - *Rucervus schomburgki* (Schomburgk's deer)
    - Genus *Axis*
      - *Axis porcinus* (Hog deer)

7. A Key to the Thai Cervidae

A key to the Thai Cervidae has been revised, with adaptation from Lekagul and McNeely (1977), Groves and Grubb (1987), WWW Thailand Programme Office (2000) and proposed in this study as below:

A. ▪ Antler two tines .... Muntiacinae (B)
▪ Antler at least three tines .... Cervinae (C)
B. ▪ Reddish or yellowish brown body hair, skull shallow double lachrymal orifice .... *Muntiacus muntjak*
▪ Dark brown body hair, black dorsal surface of tail, skull deep double lachrymal orifice .... *M. feae*
C. ▪ Antler three tines, with beam unforked .... (D)
▪ Antler more than three tines, normally branches trez tine, shoulder height greater than 720 mm .... (E)
D. ▪ Small body size, with 650-720 mm shoulder height, reduced lachrymal pits .... *Axis porcinus*
▪ Large body size, with greater than 720 mm shoulder height, deep lachrymal pits .... *Cervus unicolor*
E. ▪ Deep lachrymal pits, antler rugose, lyrate, very obtuse brow-tine and supernumerary snags, with beam unforked .... (F)
▪ Shallow lachrymal pits, antler smooth, with beam forked .... (G)
F. ▪ Terminal of trez tine branch into very short tines like palmate, reddish brown body hair .... *C. eldii siamensis*
▪ Terminal of trez tine branch lesser palmate or none, yellowish brown body hair .... *C. e. thamin*
G. ▪ Trez tine branch into numerous tines like candelabra .... *Rucervus schomburgki*

8. Ecological Comparison between Spotted Deer, Hog deer and Sambar deer

Distributions of spotted deer (*Axis axis*) and hog deer (renamed as *Axis porcinus*), are commonly found overlapped in Nepal and India. Another area of distribution of *A. axis* includes Bangladesh while *Axis porcinus* dispersed into Indochina, Pakistan and China where ecological and vegetative types are diverse. These two species are quite different in size and coat color (Nowak, 1999). Moreover, they display divergent behaviors during sexual arousal. It could be hypothesized that these differences may be a result of their adaptive radiation in different environments. However, both deer have a similar preference of living in grasslands or open forests, which is still an indication of their closely evolutionary relatedness.

In contrast, comparison between hog deer and sambar deer, sambar deer (*Cervus unicolor*), revealed that there are similarities in antlers and body shapes, but still different in body sizes. The taxonomic differentiation is the craniometrical characteristic, in which sambar deer possess a larger skull (Lekagul and McNeely, 1977). Both species are found in Nepal and India, but sambar deer are widely distributed into China, Indochina, Malay Peninsula and islands of Indonesia. Although some areas of their distribution are similar, there are differences in their living habitats, in which sambar deer prefer penetrating into heavier jungles than hog deer (Nowak, 1999). This study hypothesized from this fact that the two species seem to have high level of homoplasy due to their convergent evolution, in which a trait similarity has occurred independently from their ancestors.

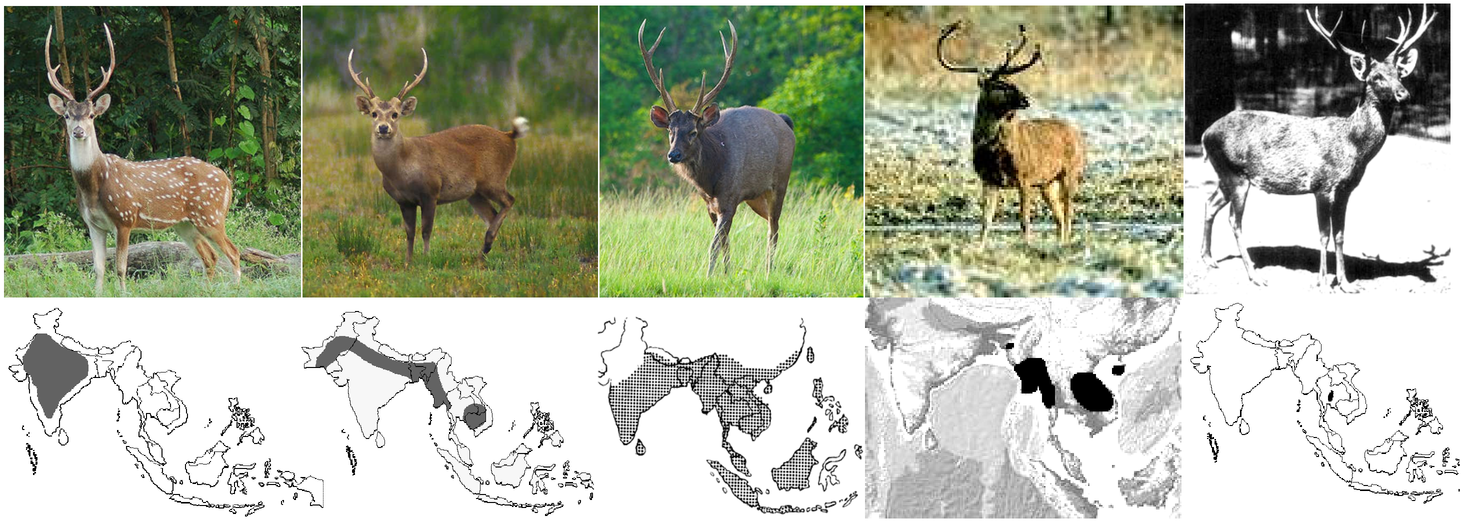
9. Ecological Comparison between Eld's Deer, Schomburgk's Deer and Sambar deer

These three species are quite different in morphological character and rather different in ecological habitats also. The extinct Schomburgk's deer was found in riparian and swampy areas, Eld's deer are found most in dry and dipterocarp forest, with and open understorey (Salter and Sayer, 1986), whereas sambar deer are found in more dense forests.

In the classification of Groves and Grubb (1987) and Grubb (1993), Schomburgk’s deer and Eld’s deer were classified in the genus *Rucervus* that were characterized by the basis of the branching of the trez tine, which is unique among the Cervinae (Pocock, 1943). This study is not in agreement with this classification, since the topology of all trees presented Eld’s deer were still grouped in the genus *Cervus* clade, not in the genus *Rucervus* clade as Schomburgk’s deer was. Therefore, this study suggest that these two species should be placed in the different genus, in which Schomburgk’s deer are in genus *Rucervus* and Eld’s deer are in genus *Cervus*. This classification corresponded to their quite different ecology habitats. Exception of ecology habitats, such classification is also corresponded to some morphological character, in which found that Eld’s deer differ from Schomburgk’s deer in having a deep lachrymal pit, like sambar deer (Groves and Grubb, 1987).

To compare between Eld’s deer and sambar deer, these two species are sympatric species, commonly found overlapped in Burma, Thailand and Cambodia. Although some areas of their distribution are similar, there are rather differences in their living habitats, in which sambar deer prefer dense forests, whereas Eld’s deer prefer dry deciduous dipterocarp forests. These two species are quite different in antlers shape, although they were classified into the same genus clade. It could be hypothesized that these differences may be a result of their adaptive radiation in different environment of living habitats.

The geographic range and some scenery, which probably could be implied their ecological habitats, comparing among spotted deer, hog deer, sambar deer, Eld’s deer and Schomburgk’s deer is shown in Figure 46.



Spotted deer (***Axis axis***)

Hog deer (***Axis porcinus***)

Sambar deer (***Cervus unicolor***)

Eld's deer (***Cervus eldii***)

Schomburgk's deer (***Rucervus schomburgki***)

Figure 46 Geographic range comparison among spotted deer, hog deer, sambar deer, Eld's deer and Schomburgk's deer.

Source: Modified from Lekagul and McNeely (1977), Whitehead (1993) and Nowak (1999)

CONCLUSION AND RECOMMENDATION

Conclusion

1. This study found that hog deer (traditionally named *Cervus porsinus*) and spotted deer (*Axis axis*) were grouped together a monophyly. Therefore, this study suggest a changing the taxonomic position of hog deer to be placed into the genus *Axis* and confirm a scientific nomenclature of hog deer as "*Axis porcinus*", not *Cervus porcinus* as in the formal classification of Thai deer. This study also confirms that Schomburgk's deer (traditionally named *Cervus schomburgki*) and swamp deer (*Cervus duvaucelii*) were grouped together as sister lineage nearby the genus *Axis* clade, with clearly separate from the genus *Cervus* clade. Therefore, this study suggest the splitting Schomburgk's deer into different genus depart from the genus *Cervus* by changing the taxonomic position to the genus *Rucervus* and confirm a scientific nomenclature of Schomburgk's deer as "*Rucervus schomburgki*", not *Cervus schomburgki* as in the formal classification. Consequently, it can be concluded that there are 3 genera of subfamily Cervinae in Thailand; *Cervus*, *Rucervus* and *Axis*. From our knowledge gained, this is the first document of results yielded from Thai samples that showed a new classification of deer in Thailand.

2. To compare capability and efficiency of each molecular marker that was chosen to analyze the phylogenetic data in this study, it was found that the cytochrome *b* gene is the most effective classification in every taxonomic level, PRKCI intron1, is still fair resolution for distinguish the species in the genus *Axis*, but could not give enough resolution for the species in the genus *Cervus*, whereas control region could not be good resolution for all level. For mitochondrial DNA, the assemblage molecular marker between the complete cytochrome *b* gene and the complete control region, it could provide better signal than alone marker of cytochrome *b*. The values of bootstrap support are increasing up to 100 % or almost in every taxonomic level. Therefore, this study suggest that the assemblage of complete cytochrome *b* and complete control region mitochondrial DNA is the best molecular marker for classification in all taxonomic level. If the laboratory procedure

has been limited by the budget, the complete cytochrome *b* gene marker alone could be most useful, with enough signals and capability for classification in every taxonomic level.

3. In this study, three copies of tandem repeats (VNTRs) were found in the RS2 region of 5'-left peripheral domain of Thai sambar deer (*Cervus unicolor equinus*), while the others of Thai cervid have no any tandem repeats in this region. These copies were tandemly duplicated from TAS-4 and TAS-3 region. In the 3'-peripheral domain, multiple copies of VNTRs of CSB-2+3 sequences were found. This is the first evidence obtained from Thailand cervid samples that showed multiple repeats in CSB-2+3 regions. It was found that Thai cervid species could be indicated by the number of copies in this region. Only hog deer (*Axis porcinus*) have two copies of CSB-2+3-like, whereas spotted deer (*Axis axis*), which not native to Thailand and the remaining of Thai cervid species (except common barking deer) have only one copy. To compare species in the genus *Muntiacus*, unexpected results were observed, in which Fea's barking deer (*Muntiacus feae*) have one copy of CSB-2+3-like, whereas common barking deer (*Muntiacus muntjak*) have none. This study suggests that VNTRs of CSB-2+3-like in the 3'-peripheral domain could distinguish hog deer from spotted deer, as same as could characterize Fea's barking deer from common barking deer.

Recommendation

1. Thailand have own genetic material of the extinct Schomburgk's deer, in which their ancient carcass such as antlers and bones are still maintained in Thailand's museum. In this study, only one sample of complete cytochrome *b* gene was obtained from GenBank. To better confirm for their genus *Rucervus*, their cytochrome *b* gene and control region sequences should be amplified with the same primers used in this study.

2. Nuclear DNA of PRKCI intron1 rather gives small informative characters for phylogenetic analysis. Its short sequences are probably weightless when it was

combined to longer mtDNA sequences unequally. Therefore, the others nuclear DNA that could give larger size of informative characters should be selected in the next study.

3. Refer to the new systematic classification of Thai deer proposed in this study, especially hog deer and Schomburgk's deer in the new zoological names as "*Axis porcinus*" and "*Rucervus schomburgki*", respectively. This new classification should be responded in the national level by changing the previous zoological names which have still used in Thai Wildlife Protection Act of 1992 or the others Act involved in wildlife conservation in Thailand into these new zoological names.

4. Nowadays, the studies of Thai wildlife using molecular genetic approaches are still limited and inadequate. The knowledge gained from these aspects will be important and useful for wildlife taxonomy, including the implication for wildlife conservation and management in Thailand. Therefore, molecular genetic studies should be expanded to the others wildlife species, particularly in any endangered species and confiscated animal species such as bovines, tiger, pangolin, langur, gibbon, civet, marten etc.

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APPENDICES

Appendix A

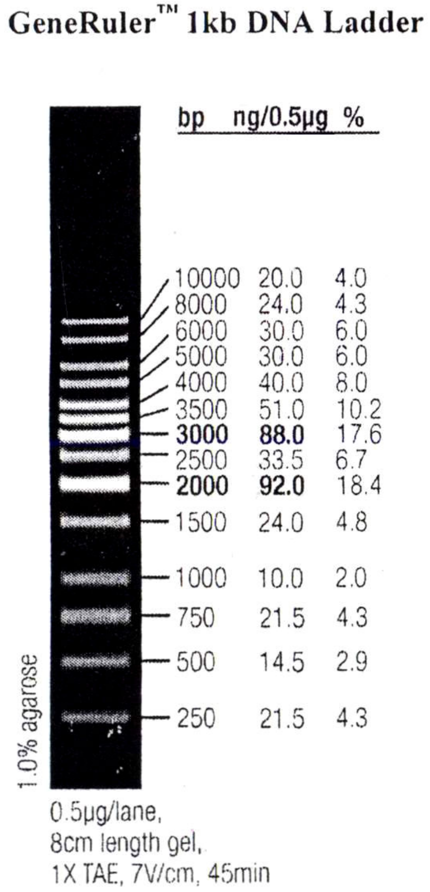
Samples collections

Appendix Figure A1 Cervine blood samples from Hauysai wildlife propagation station, Phetchaburi.

Appendix Figure A2 Blood samples were kept in vacutainers plus EDTA

Appendix B

Standard DNA marker



GeneRuler™ 1kb DNA Ladder

bp	ng/0.5µg	%
10000	20.0	4.0
8000	24.0	4.3
6000	30.0	6.0
5000	30.0	6.0
4000	40.0	8.0
3500	51.0	10.2
3000	**88.0**	17.6
2500	33.5	6.7
2000	**92.0**	18.4
1500	24.0	4.8
1000	10.0	2.0
750	21.5	4.3
500	14.5	2.9
250	21.5	4.3

0.5µg/lane,
8cm length gel,
1X TAE, 7V/cm, 45min

Appendix Figure B3 Gene Ruler 1 kb DNA Ladder (Fermentas)

Appendix C

Cervidae PRKCI intron1 sequences in FASTA format

>PKA1_*A*_*axis*
TTTATGCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTATGCAATGAAAG
TTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATATGATGTTTTATTACCT
CTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAATGTTTGTGCCTT
ATTGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATATT
TAAAGTTGTCTGATGATTATTAGATTTTTTAAAAATAAACATCAAACCATATTATTATCA
AGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTGC
TGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGATG
TTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTGT
CACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGGA
TATTGACTGGG

>PKA2_*A*_*axis*
TTTATGCCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTATGCAATGAAA
GTTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATATGATGTTTTATTACC
TCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAATGTTTGTGCCT
TATTGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATAT
TTAAAGTTGTCTGATGATTATTAGATTTTTTAAAAATAAACATCAAACCATATTATTATC
AAGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTG
CTGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGAT
GTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGG
ATATTGACTGGG

>PKA3_*A*_*axis*
TTTATGCCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTATGCAATGAAA
GTTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATATGATGTTTTATTACC
TCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAATGTTTGTGCCT
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TTAAAGTTGTCTGATGATTATTAGATTTTTTAAAAATAAACATCAAACCATATTATTATC
AAGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTG
CTGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGAT
GTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGG
ATATTGACTGGG

>PKA4_*A*_*axis*
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GTTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATATGATGTTTTATTACC
TCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAATGTTTGTGCCT
TATTGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATAT
TTAAAGTTGTCTGATGATTATTAGATTTTTTAAAAATAAACATCAAACCATATTATTATC
AAGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTG
CTGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGAT
GTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGG
ATATTGACTGGG

>PKF1_*M*_*feae*
TTTATGCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTACGCAATGAAAG
TTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATGATGTTTTATTACCTCT

AAAGTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATGTCAGTGTTTGTGCCTTAT
TGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATATTTA
AAGTTGTCTGATGATTACTAGATTTTTAAAAAATAAACATCAAACCACATTATTATGAAG
GGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTGCTG
GGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAGTGCTTTTGATGTT
TAGCCTAGTCAGTCACTAATTGCATTTATGGTGATGGAATTACTAAGAACAAATTTGTCA
TAGTTTTGATACTGTCGTTCTGGTTTGACAAAAATTTCTTTCATATTTTAACATAGGATA
TTGACTGGG

>PKF2_*M_feae*
TTTATGCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTACGCAATGAAAG
TTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATGATGTTTTATTACCTCT
AAAGTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATGTCAGTGTTTGTGCCTTAT
TGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATATTTA
AAGTTGTCTGATGATTACTAGATTTTTAAAAAATAAACATCAAACCACATTATTATGAAG
GGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTGCTG
GGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAGTGCTTTTGATGTT
TAGCCTAGTCAGTCACTAATTGCATTTATGGTGATGGAATTACTAAGGACAAATTTGTCA
TAGTTTTGATACTGTCGTTCTGGTTTGACAAAAATTTCTTTCATATTTTAACATAGGATA
TTGACTGGG

>PKM2_*M_muntjak*
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AAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATGTCAGTGTTTGTGCCTTAT
TGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATATTTA
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GGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTGCTG
GGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGATGTT
TAGCCTAGTCAGTCACTAATTGCATTTATGGTGATGGAATTACTAAGAACAAATTTGTCA
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TGACTGGG

>PKM3_*M_muntjak*
TTTATGCTAAAGTACTGTTGGTGCGATTGAAAAAAACAGATCGTATTTACGCAATGAAAG
TTGTGAAAAAAGAGCTTGTCAATGATGATGAGGTAAGCACAATGATGTTTTATTACCTCT
AAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATGTCAGTGTTTGTGCCTTAT
TGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATATTTA
AAGTTGTTTGATGATTACTAGATTTTTAAAAAATAAACATCAAACCATATTATTATCAAG
GGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTGCTG
GGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGATGTT
TAGCCTAGTCAGTCACTAATTGCATTTATGGTGATGGAATTACTAAGAACAAATTTGTCA
TAGTTTTGATACTGTCGTTCTGGTTTGACATAAATTTCTTTCATATTTTAACATAGGATA
TTGACTGGG

>PKP1_*A_porcinus*
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TCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAGTGTTTGTGCCT
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TTAAAGTTGTCTGATGATTATTAGATTTTTAAAAAATAAACATCAAACCATATTATTATC
AAGGGAAACTACTTGAGCTTTGTAAAAGTTTGCTAGACTCTAATAATATTGACAGATTTG
CTGGGTAGTAGTTTCTCTTTGTATATAAATTATTATTTCATGATTATCAATGCTTTTGAT

GTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGG
ATATTGACTGGG

>PKP5_*A_porcinus*
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TCTAAACTATTACATTGGCTTAAGTGTATGATATCAGAAAAAATTTCAGTGTTTGTGCCT
TATTGTTTCTGTCTGCTTTTCTAAATACATGCTTCAGACTGCTTAATATATAAGGTATAT
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GTTTAGCCTAGTTAGTCAATAATTGCATTTATGGTAATGGAATTACTAAGAACAAATTTG
TCACAGTTTTGATACTGTCATTCTAGTTTGACATAAATTTCTTTCATATTTTAAAATAGG
ATATTGACTGGG

>PKP6_*A_porcinus*
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Appendix D

Cervidae mtDNA sequences in FASTA format

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CTTACACATGCAAGCA
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Appendix E

ATBC Asia-Pacific Chapter annual meeting 2009

The Association
for Tropical
Biology and
Conservation

12th January 2009

Kanita Ouitavon
Wildlife Research Division, Wildlife Conservation Office
National Park, Wildlife and Plants Conservation Department
Phaholyothin Rd, Jatujak, Bangkok
Thailand

Dear Kanita Ouitavon,

Subject: Invitation to present a **paper** at the ATBC Asia-Pacific chapter conference in Chiang Mai, Thailand 12-15th February 2009

The Asia-Pacific chapter of the Association for Tropical Biology & Conservation would like to invite you to present a paper entitled

Genetic relationship analysis of Thai cervids using nucleotide sequences of protein kinase C iota (PRKC/) intron"

at the above conference. The theme of the conference is "Assessing and restoring biodiversity in a human dominated landscape".

Thank you and I look forward to seeing you in Chiang Mai,

Yours sincerely,

Tommaso Savini
Chair, Organizing Committee for Chaing Mai 2009

SYMPOSIUM J

Sunday 15 February 2009

Molecular approaches to the study of Asian biodiversity

Chair:
David Lohman
National University of Singapore, Singapore

GENETIC RELATIONSHIPS ANALYSIS OF THAI CERVIDS USING NUCLEOTIDE SEQUENCES OF PROTEIN KINASE C IOTA (PRKC*I*) INTRON

Kanita Ouithavon[1] ,Naris Bhumpakphan[2], Jessada Denduangboripant[3] ,Boripat Siriaroonrat[4] and Savitr Trakulnaleamsai[5]

[1] Department of Bioscience, Faculty of Science, Kasetsart University, Bangkok, Thailand.
[2] Department of Forest Biology, Faculty of Forestry, Kasetsart University. Bangkok, Thailand.
[3] Department of Biology, Faculty of Science, Chulalongkorn University. Bangkok, Thailand.
[4] The Zoological Park Organization under the Royal Patronage of His Majesty the King. Bangkok, Thailand.
[5] Department of Microbiology, Faculty of Science, Kasetsart University. Bangkok, Thailand.

The phylogenetic pattern from 21 taxa of Thai cervid species; sambar deer, thamin Eld's deer, siamensis Eld's deer, hog deer, common barking deer and Fea's barking deer and 4 taxa of spotted deer were determined based on nuclear intron of Protein Kinase C, iota. Blood samples of these deer species were collected from captivity for whole genomic DNA extraction. Intron1 of complete protein kinase C iota gene (PRKCI) (approximately 552 bp) was amplified by polymerase chain reaction (PCR) method using known primers of L748 and U26. Using unrooted phylogenies derived from distinct theoretical approaches with 19 parsimony-informative characters sites, strong support was achieved for monophyly of the genus *Axis* with muntjacs as sister group as well as for the divergence of three distinct genera of Thai cervids: *Muntiacus, Cervus* and *Axis*. The latter clade comprises spotted deer (*Axis axis*) and hog deer (*Cervus porcinus*) which has previously been regarded as the genus *Cervus*. This result suggests a strong support for certain position taxonomy of hog deer, which is not nested within the *Cervus* clade, but it should be placed within the *Axis* clade.

CIRRICULUM VITAE

NAME : Ms. Kanita Ouithavon

BIRTH DATE : August 12, 1970

BIRTH PLACE : Bangkok, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
1993	Kasetsart Univ.	B.Sc. (Forestry)
2000	Kasetsart Univ.	M.S. (Forest Biology)

POSITION : Scientist
WORK PLACE : Wildlife Research Division, National Park, Wildlife and Plants Conservation Department (DNP). Bangkok, Thailand.

PUBLICATION:

Ouithavon, K., N. Bhumpakphan, J. Denduangboripant, B. Siriaroonrat and S. Trakulnaleamsai. 2009. An analysis of the phylogenetic relationship of Thai Cervids inferred from nucleotide sequences of protein kinase C iota (PRKCI) intron. **Kasetsart Journal.** (In press).

CONFERENCE (ORAL PRESENTATION):

Ouithavon, K., N. Bhumpakphan, J. Denduangboripant, B. Siriaroonrat and S. Trakulnaleamsai. 2009. **Genetic relationships analysis ofThai cervids using nucleotide sequences of protein kinase C,iota (PRKCI) intron.** ATBC Asia-Pacific Chapter annual meeting “Assessing and Restoring Biodiversity in a Human Dominated Landscape” Imperial Maeping Hotel, Chiang Mai, Thailand. 12-15 February 2009. (Appendix E)