Kasetsart J. (Nat. Sci.) 43 : 709 - 719 (2009)

An Analysis of the Phylogenetic Relationship of Thai Cervids Inferred from Nucleotide Sequences of Protein Kinase C Iota (PRKCI) Intron

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ABSTRACT

The phylogenetic relationship of five Thai cervid species (n=21) and four spotted deer (*Axis axis*, n=4), was determined based on nucleotide sequences of the intron region of the protein kinase C iota (PRKCI) gene. Blood samples were collected from seven captive breeding centers in Thailand from which whole genomic DNA was extracted. Intron1 sequences of the PRKCI nuclear gene were amplified by a polymerase chain reaction, using L748 and U26 primers. Approximately 552 base pairs of all amplified fragments were analyzed using the neighbor-joining distance matrix method, and 19 parsimony-informative sites were analyzed using the maximum parsimony approach. Phylogenetic analyses using the subfamily Muntiacinae as outgroups for tree rooting indicated similar topologies for both phylogenetic trees, clearly showing a separation of three distinct genera of Thai cervids: *Muntiacus, Cervus*, and *Axis*. The study also found that a phylogenetic relationship within the genus *Axis* would be monophyletic if both spotted deer and hog deer were included. Hog deer have been conventionally classified in the genus *Cervus* (*Cervus porcinus*), but this finding supports a recommendation to reclassify hog deer in the genus *Axis*.

Key words: Thailand, the family Cervidae, protein kinase C iota (PRKCI) intron, phylogenetic tree, taxonomy

INTRODUCTION

The family Cervidae is one of the most specious families of artiodactyls, with an extensive morphological and ecological divergence (Grubb, 1993). The diversity of their terrestrial habitats is thought to reflect several adaptive radiations and their high level of homoplasy (Groves and Grubb, 1987; Gentry, 1994). Cervids, or what is commonly called "deer," are mostly characterized by antlers with a bony inner core and velvet skin cover. Antlers are presented in males, except in *Rangifer* in which both sexes have antlers, and the single species in the genus *Hydropotes*, which lacks antlers (Lekagul and McNeely, 1977; Nowak, 1999). In the classification of Janis and

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Received date : 20/03/09 Accepted date : 20/05/09

Scott (1987), and Groves and Grubb (1987), Cervidae are classified into six subfamilies: Muntiacinae, Cervinae, Alcinae, Rangiferinae, Odocoileinae and Hydropotinae. The subfamily Muntiacinae contains two Asian genera: *Muntiacus* and *Elaphodus*, and the subfamily Cervinae includes four genera: *Dama*, *Axis*, *Elaphurus* and *Cervus*.

According to the traditional classification of Lekagul and McNeely (1977), which is based on characteristics of antlers and skulls, there are six species from two subfamilies (Muntiacinae and Cervinae) of Cervidae in Thailand. The subfamily Muntiacinae in Thailand consists of common barking deer (*Muntiacus muntjak*) and Fea's barking deer (*Muntiacus feae*). The subfamily Cervinae consists of sambar deer (*Cervus unicolor*), the extinct Schomburgk's deer (*Cervus shomburgki*), Eld's deer (*Cervus eldii*) and hog deer (*Cervus porcinus*). Lekagul and McNeely's classification is different from many modern authors who have placed hog deer in the genus *Axis*, based on the lack of upper canines, which are variably present in other *Cervus* species and the presence of glands in the feet (Groves and Grubb, 1987; Nowak, 1999). This contradiction is one of many remaining inconsistencies concerning the morphological classifications of intergeneric relationships among the family Cervidae.

For the comparative morphology of extant deer, the discrepancies between phylogenies of Cervidae can be explained by the fact that most previous studies have used different matrices of morphological characteristics (Groves and Grubb, 1987; Meijaard and Groves, 2004). It is very difficult to appraise the reliability of their inferences because of the high level of homoplasy affecting these morphological characteristics. Consequently, the results from the decipherment in the phylogeny of Cervidae have been repeatedly questioned (Janis and Scott, 1987; Gentry, 1994; Hassanin and Douzery, 2003). Several dental and cranial characters, especially those of antlers, were traditionally used to resolve intra-subfamily relationships (Bubenik and Bubenik, 1990). These diagnostic characteristics either could be a phylogenetically informative inheritance from their ancestors, or caused by a convergence due to ecological adaptations, which could represent different ecomorphs (Geist, 1998; Grubb, 1993; Vrba and Schaller, 2000). Therefore, traditional classifications with merely morphological characteristics are still inadequate.

Molecular phylogenetic investigations have greatly helped in taxonomical organization and also to understand better the evolution of morphological characters. Several molecular studies on Cervidae have considerably contributed to resolving the debate on 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; Pitra *et al*., 2004; Gilbert *et al*., 2006). However, these previous studies did not have enough representative taxa of cervid species found in Thailand. Moreover, they did not properly clarify the taxonomy of Thai deer due to the intergeneric relatedness between genera within Cervidae.

Therefore, this study selected the nuclear DNA intron region of the protein kinase C iota gene (PRKCI), a neutral and rapidly evolving gene, as a molecular marker to analyze phylogenetic relationships among Thai deer. This study addressed two important phylogenetic questions by determining: (1) whether within the subfamily Cervinae, the genera *Cervus* and *Axis* are monophyletic; and (2) the location of the best phylogenetic position of hog deer.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from five species of Thai deer: hog deer (*Cervus porcinus* or *Axis porcinus*); sambar deer (*C. unicolor*); both subspecies of Eld's deer (Siamese Eld's deer, *C.*

eldii siamensis, and thamin Eld's deer, *C. eldii thamin*); common barking deer (*Muntiacus muntjak*); and Fea's barking deer (*M. feae*). Moreover, one additional species of *Axis* deer (spotted deer, *Axis axis*) that is not native to Thailand was included in the sample. All deer specimens were sampled from seven zoos and captive breeding centers around Thailand. These centers are operated by the Zoological Park Organization under the Royal Patronage of His Majesty the King and by the Wildlife Propagation Division, Department of National Park, Wildlife and Plants Conservation (DNP). The sources of each sample are shown in Table 1. The collected blood samples in vacutainers were kept in ice while being transported to the laboratory at Kasetsart University. Then, they were kept cool at 4°C in a refrigerator.

Laboratory methodology

Whole genomic DNA from the blood samples was extracted using an Aquapure Genomic Blood Kit (Bio-RAD). The extracted DNA with a 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 by Ropiquet and Hassanin (2005). PCR products of PRKCI intron 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 and 0.75 µl of 5 pmol/µl of each primer. The thermal cycling program consisted of an initial denaturation step at 94°C for 4 minutes, 35 cycles of a denaturation step at 94°C for 20 seconds, an annealing step at 53°C for 30 seconds, with an extension step at 72°C for 1 minute and a final extension step at 72°C for 5 minutes. The PCR products were then purified by hydrolysis of the primers with exonuclease I and removal of phosphoric acid of dNTPs by alkaline

Table 1 Names and number of deer samples used in the study.

Common name	Scientific name	Sample name	Source	Number of sample
Common barking deer	*Muntiacus muntjak*	M2, M3	Pattalung Wildlife Station[a]	2
Fea's barking deer	*Muntiacu feae*	F1, F2	Ton Nga-Chang Wildlife Station[a]	2
Hog deer	*Cervus porcinus* or *Axis porcinus*	P1, P5, P6	Hauy Sai Wildlife Station[a]	6
		P9	Dusit Zoo[b]	
		P11	Korat Zoo[b]	
		P12	Songkhla Zoo[b]	
Sambar deer	*Cervus unicolor*	U2, U3, U4,	Hauy Sai Wildlife Station[a]	5
		U5	Songkhla Zoo[b]	
		U8	Khao Kho Wildlife Station[a]	
Siamese Eld's deer	*Cervus eldii siamensis*	S1	Dusit Zoo[b]	1
Thamin Eld's deer	*Cervus eldii thamin*	T1, T2, T7	Hauy Sai Wildlife Station[a]	5
		T9, T11	Dusit Zoo[b]	
Spotted deer	*Axis axis*	A1, A2	Songkhla Zoo[b]	4
		A3, A4	Korat Zoo[b]	

[a] = Wildlife Propagation Division, DNP
[b] = The Zoological Park Organization under the Royal Patronage of His Majesty the King

phosphatase using ExoSAP-IT® (USB Corporation, USA), with 1 μl of ExoSAP-IT® added in each PCR product tube. The mixture had to be left at room temperature for 30 minutes before being placed into the thermal cycler. The standard conditions used for PCR purification consisted of the first step at 37°C for 20 minutes and the second step at 80°C for 15 minutes. The purified PCR products were sent to the KU-Vector Custom DNA Synthesis Service, Kasetsart University, for DNA sequencing.

Data analysis

All sequence outputs were compared with the GenBank database by the BLASTn program (http://blast.ncbi.nlm.nih.gov/blast.cgi) to ensure correct sequences. Each base characteristic for all sequences was edited by Chromas version 2.33. The edited sequences were assembled for each complete sequence using the CAP3 program (http://phil.univ-lyon1.fr/cap3.php). All complete sequences were aligned together using the ClustalX version 1.82 software (Thompson *et al.*, 1997), followed by visual inspection. The excess nucleotides from each target sequence were removed and all gaps were treated as the fifth characteristic state in the phylogenetic analysis.

In the phylogenetic analyses, the members of the subfamily Muntiacinae, *Muntiacus mumtjak* and *M. feae*, were used as outgroups. The phylogenetic trees were constructed based on the 552 aligned base sites of the amplified PRKCI gene intron sequences. Two types of phylogenetic analyses were carried out to investigate genetic relationships: (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 1000 replicates were performed to obtain estimates of support for each node of the NJ tree. For the MP analysis, all uninformative sites were included with informative sites and the informative sites were analyzed using equally weighted characteristics. A branch-and-bound search option was used and the statistical support for recovered nodes was assessed using non-parametric bootstrap analysis with 1000 replicates.

RESULTS

PRKCI intron characteristics

The final alignment of the PRKCI intron sequences of all taxa consisted of 552 characteristics. This alignment was generated by the insertion of a single gap at position (pos.) 8, 104, 105, 476 and 517. Of these 552 characteristics, 526 were constant and 19 were parsimony informative. These PRKCI intron sequences were AT-rich, with an AT component of approximately 70%. The mean base composition and base sequence amount of each cervid species are shown in Table 2.

Table 2 Mean base composition and base sequence amounts of PRKCI intron of cervid species examined.

Scientific 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 the two *Muntiacus* outgroup taxa, 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 were found after a comparison was made with the ingroups.

Among the ingroup taxa, there was 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 one insertion of C in pos. 8 of the spotted deer and hog deer sequences. When only the hog deer was compared 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, a deletion of A in pos. 476 in four of the five sambar specimens was found.

Monophyly of the subfamily Cervinae

Phylogenetic relationships among Thai deer were investigated by two types of phylogenetic trees: (1) a neighbor-joining (NJ) tree (shown in Figure 1); and (2) a maximum parsimony (MP) tree (Figure 2). For the MP tree, a branch-and-bound tree search revealed the three most parsimonious trees and the semi-strict

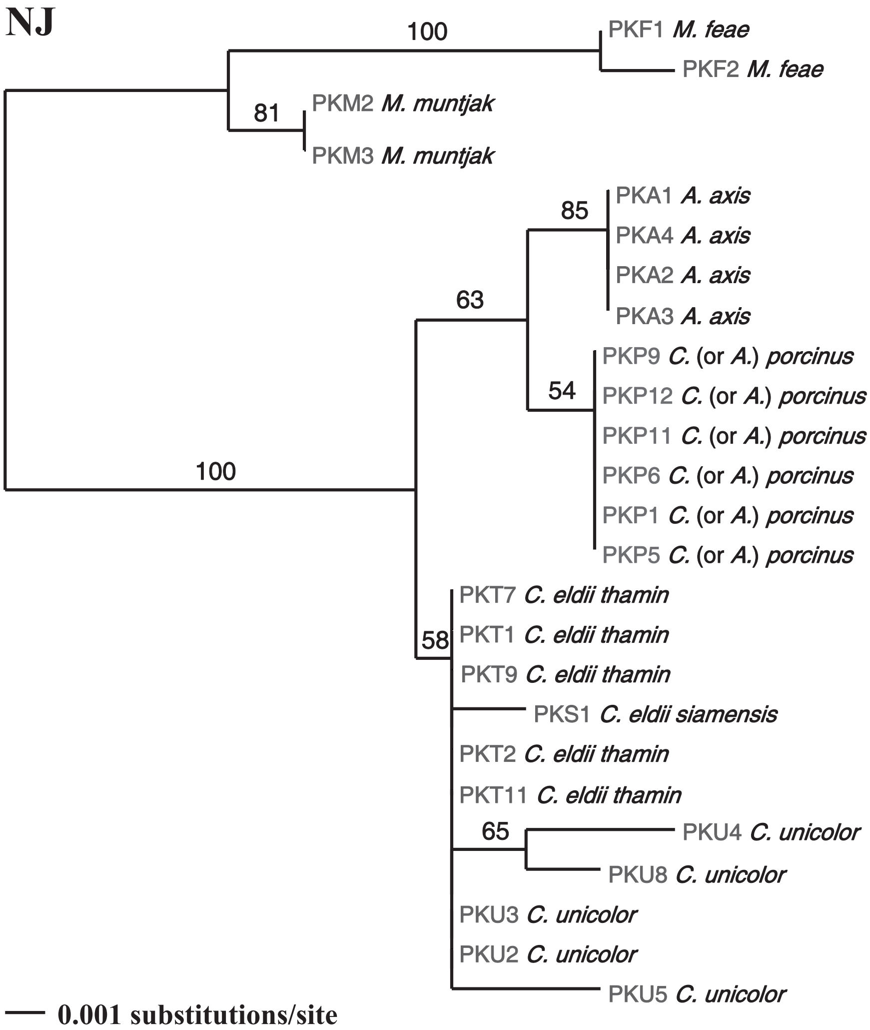

Figure 1 Neighbor-joining tree from PRKCI intron sequences of 25 deer taxa. Numbers above branches indicate % bootstrap support (only >50%) generated from 1000 replications.

consensus tree (Figure 2). From both phylogenetic trees of the deer PRKCI intron sequences, all species of the subfamily Cervinae formed a strongly supported monophyletic group (with 100% statistical bootstrap support on both NJ and MP).

Among the four samples of *Axis*, the study found that they were also grouped monophyletically with moderate statistical bootstrap support (63% on the NJ tree and 67% on the MP tree). In the case of the genus *Cervus*, except for *Cervus porcinus*, the other *Cervus* taxa formed a group with weak support of only 58% and <50% bootstrap values on the NJ tree and the MP tree, respectively. Moreover, this grouping indicated that the PRKCI gene intron may not give enough resolution, as it could not distinguish between two species (*C. eldii* and *C. unicolor*) of the genus *Cervus*.

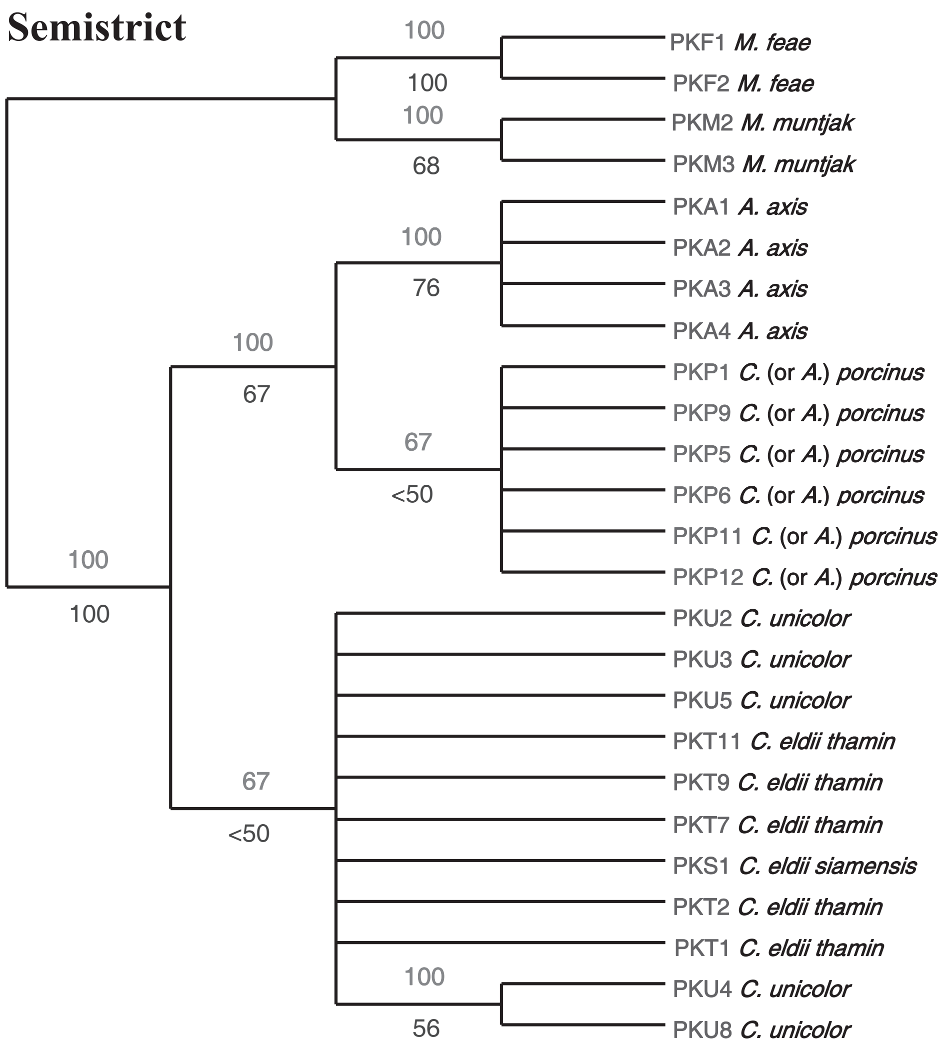

Figure 2 Semi-strict consensus tree of the most three parsimonious trees from PRKCI intron sequences of 25 deer taxa. Numbers above branches show % semi-strict congruent proportion among the MP trees. Numbers below branches indicate % bootstrap support (only >50%) based on 1000 replications.

Phylogenetic position of hog deer

Both NJ and MP phylogenetic analyses yielded a similar topology of the trees in which all six hog deer were clustered, sisterly in the same clade of spotted deer (*Axis axis*) with moderate bootstrap support (63% on the NJ tree and 67% on the MP tree). All four spotted deer taxa were grouped as one species with strong bootstrap support (85% on the NJ tree and 76% on the MP tree) and all hog deer (by now the would-be-named *Axis porcinus*) taxa were grouped as a single species with moderate bootstrap support (54% on the NJ tree).

To distinguish the genus *Axis* from the genus *Cervus* (not including *Axis porcinus*), 17 base changes in the PRKCI intron sequences were needed. These 17 synapomorphic nucleotide characteristics were counted from eight base changes of the genus *Axis*, which could group spotted deer and hog deer together, and nine base synapomorphic characteristics between *C. eldii* and *C. unicolor*, as shown in Figure 3.

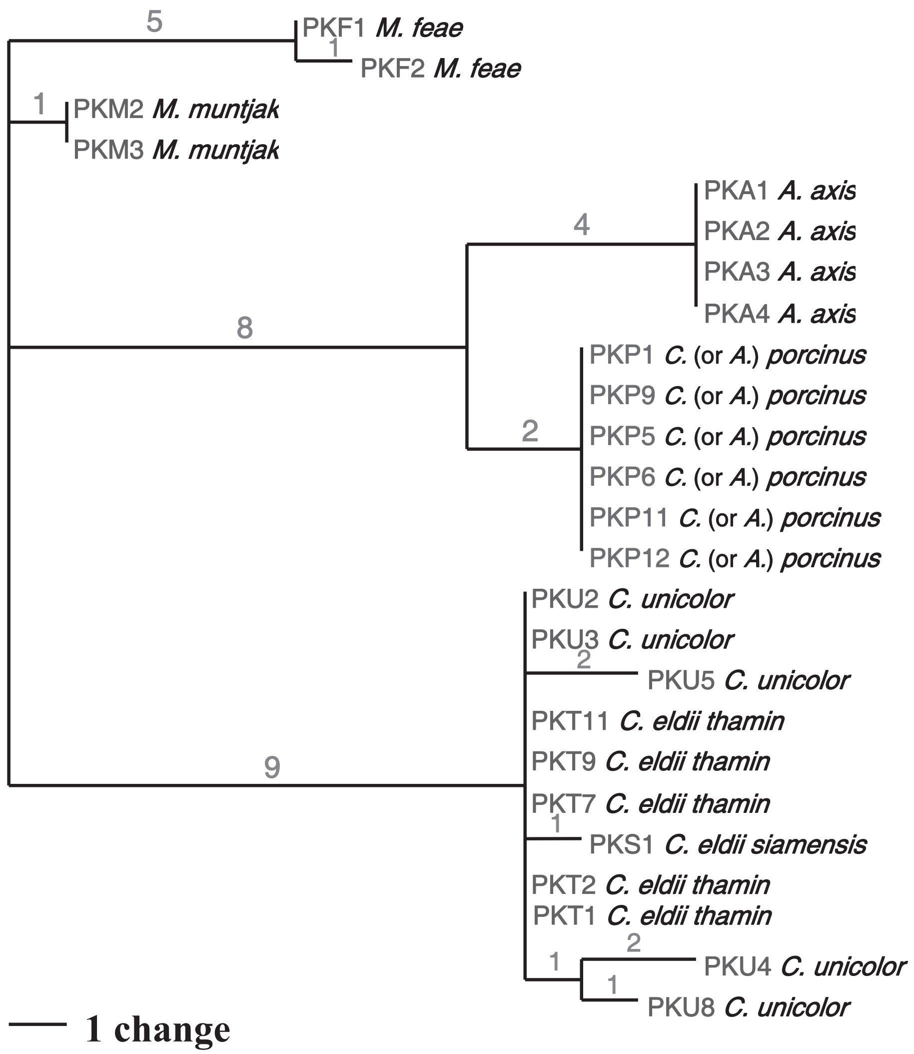

Figure 3 One of the most three parsimonious trees from PRKCI intron sequences of 25 deer taxa. Numbers above branches show the number of base changes.

DISCUSSION

Phylogeny and taxonomy of Thai deer

The analyses showed that there was a monophyly of the subfamily Cervinae (*Cervus* and *Axis*) with strong statistical support. This result was in agreement with previous molecular phylogenetic studies based on the cytochrome *b*, the control region (D-loop region) and nuclear DNA intron (Douzery and Randi, 1997; Randi *et al*., 2001; Pitra *et al*., 2004; and Gilbert *et al*., 2006).

For the genus *Axis*, the study found that it formed a monophyletic clade with moderate support. This result supported a previous study of Bonnet (2001) which analyzed the D-loop region and that 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. In addition, the study of Ouithavon (2009), in which all phylogenetic analyses were based on the cytochrome *b*, the control region, the PRKCI intron region and combined data sequences, yielded the concordant results of the genus *Axis* monophyletic clade. However, the finding in the current study was in contrast with the former study of Pitra *et al*. (2004). They found that *Axis* was paraphyletic, having *A. porcinus* joined with *C. timorensis* and *A. axis* related to *C. duvauceli*. The work of Pitra *et al*. (2004) published the first molecular phylogeny of old-world deer with the comparison of cytochrome *b* sequences for an almost-complete set of the subfamily Cervinae. Nevertheless, their study was performed using only one sequence of *A. porcinus* produced by Ludt *et al*. (2004) in accession No. AY035874. Gilbert *et al*. (2006) suggested that this *A. porcinus* sequence may have been obtained from a tissue sample of a misidentified specimen. The paraphyletic grouping of *Axis* suggested by Pitra *et al*. (2004) is also considered questionable by the authors of the current study. Consequently, the findings of the current study are proposed as confirmation of a monophyly of the subfamily Cervinae and the genus *Axis* of deer in Thailand.

In the case of the genus *Cervus*, all of the previous studies uniformly agreed that *Cervus* is polyphyly, either based on cytochrome *b*, control region, nuclear DNA intron, or combined data (Randi *et al*., 2001; Liu *et al*., 2003; Pitra *et al*., 2004; Gilbert *et al*., 2006). However, the results of the current study 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. 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 analysis.

New classification of Thai deer

Current traditional, morphological taxonomy of deer in Thailand splits the family Cervidae on the basis of antlers and craniometrical characteristics. These criteria separate Thai deer into only two genera: *Muntiacus* and *Cervus* (Lekagul and McNeely, 1977). However, the taxonomic value of antlers has been repeatedly questioned because some morphological characteristics seem to evolve with high homoplasy in ungulates (Scott and Janis, 1987). In this study, the results based on nucleotide sequence comparison of the nuclear DNA intron of Thai deer showed that Thai cervids should be divided into the genera: *Muntiacus* and *Cervus*, and additionally *Axis*, since hog deer were found positioned along side spotted deer within the monophyletically inferred genus *Axis*. This study suggests that the scientific name of hog deer should be renamed to *Axis porcinus* and the new systematic classification of Thai deer is proposed 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 *Axis*

- *Axis porcinus* (Hog deer)

Ecological comparison between spotted deer, hog deer and sambar deer

The distributions of spotted deer (*Axis axis*) and hog deer (renamed as *A. porcinus*) are commonly found overlapped in Nepal, Sri Lanka and India. Another area of distribution of *A. axis* includes Bangladesh, while *A. porcinus* has 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 for living in grasslands or open forests, which is still an indication of their close evolutionary relatedness.

In contrast, the comparison between hog deer and sambar deer (*Cervus unicolor*), revealed that there are similarities in antlers and body shapes, but still differences in body sizes. The taxonomic differentiation occurs in the craniometrical characteristic, as sambar deer possess a larger skull (Lekagul and McNeely, 1977). Both species are found in Nepal, Sri Lanka and India, however sambar deer are widely distributed into China, Indochina, the Malay Peninsula and on the islands of Indonesia. Although some areas of their distribution are similar, there are differences in their living habitats, as sambar deer prefer penetrating into heavier jungle than hog deer (Nowak, 1999). From this fact, this study hypothesized 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.

CONCLUSION

In conclusion, the current study found that hog deer (traditionally named *Cervus porsinus*) and spotted deer (*Axis axis*) were grouped together as a monophyly. Therefore, it is proposed that the taxonomic position of hog deer should be changed and be placed into the genus *Axis*, and that the scientific nomenclature for hog deer be changed to *Axis porcinus* from *Cervus porcinus* as it has been known in the formal classification of Thai deer. Consequently, this study concluded that there are three genera of cervids in Thailand: *Muntiacus*, *Cervus* and *Axis*. Based on the knowledge of the authors of the current study, the results reported in this paper are the first from Thai samples that show a new classification of deer in Thailand. It is suggested that the PRKCI intron region as a tool for deer classification has sufficient resolution to distinguish between genera and to characterize each species in the genus *Axis* (such as spotted deer (*A. axis*) and hog deer (*A. porcinus*)), but not enough for the genus *Cervus*.

ACKNOWLEDGEMENTS

This study was supported by the Thesis and Dissertation Support Fund, the Graduate School, Kasetsart University. The authors would like to thank the veterinarian team from the Zoological Park Organization and the officers of the Wildlife Breeding Station in the National Park, Wildlife and Plants Conservation Department (DNP) for their assistance in collecting samples. The authors express their gratitude to the KU-

Vector Custom DNA Synthesis Service, Kasetsart University for help with DNA sequencing work. Editorial assistance, comments and suggestions by anonymous reviewers are also acknowledged.

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