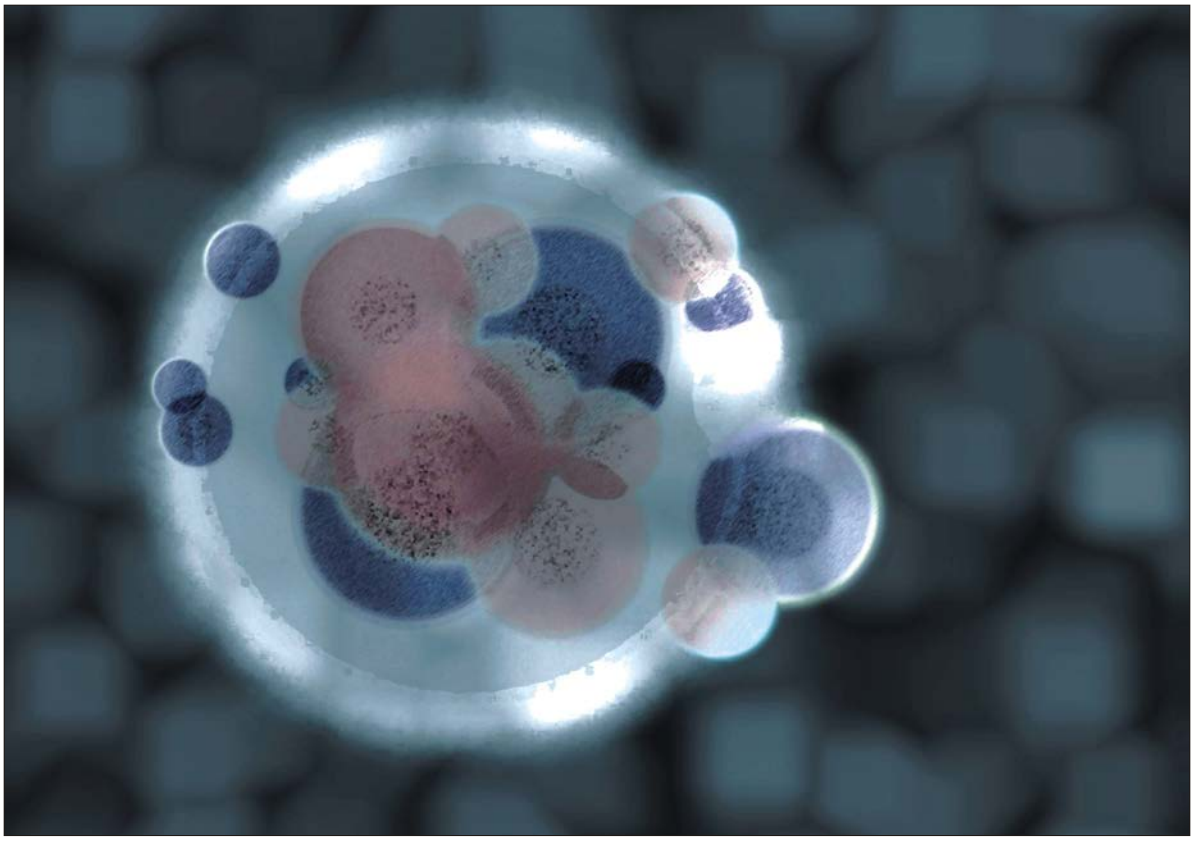

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Phylogenetic Relationships of Wildlife Order Carnivora in Thailand Inferred from the Internal Transcribed Spacer Region

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Abstract: The genetic relationship of 20 Carnivora species in Thailand was determined based on sequence analysis of the Internal Transcribed Spacer (ITS) region of ribosomal DNA. Aligned sequences of the complete ITS region obtained from the 20 taxa and two primate outgroups resulted in 890 characters with 710 variable sites. Genetic distances and a phylogenetic tree were constructed from comparisons of ITS sequences using the Neighborjoining method. The dendogram demonstrates that the 22 taxa can be clearly grouped in six clusters: Mustelidae, Ursidae, Canidae, Felidae, Viveridae and Hylobatidae. Of these clusters, the 20 Canivora species are clustered together and the superfamilies Caniformia and Feliformia can be separated apart, whereas the outgroup Hylobatidae is segregated from the carnivora. In superfamilies Caniformia, the families Mustelidae, Ursidae and Canidae are clustered together. In superfamilies Feliformia, the family Felidae is clustered with Viverridae. The phylogenetic tree of Viverridae species does not completely match the classification based on morphological characters. The Paradoxurinae, *Arctictis binturong* was grouped into the Viverrinae while the other Paradoxurinae, *Paradoxurus hermaphroditus*, *Paguma larvata* and *Arctogalidia trivirgata* are clustered together.

Key words: Phylogenetic, carnivora, ITS, caniformia, feliformia, nrRNA

INTRODUCTION

In Thailand, the mammalian order Carnivora includes 6 families and has been classically divided into two monophyletic superfamilies, Carniformia and Feliformia (Eisenberg, 1989; Wozencraft, 1989; Wyss and Flynn, 1993). Caniformia has usually been organized into the families Canidae, Ursidae and Mustelidae while Feliformia has been partitioned into the families Viveridae, Felidae and Herpestidae (Eisenberg, 1989; Flynn and Nedbal, 1998). Despite numerous efforts, however, evolutionary relationships within and among the diverse families of living Carnivora species remain controversial. Habitat loss has occurred violently throughout Southeast Asia over the past 20 years and some of these carnivore species are vulnerable to population pressures and habitat fragmentation. Forest destruction has negatively affected wild animals such as carnivores. It reduces habitat for wild animals and causes population fragmentation due to the loss of genetic heterogeneity and thus they become vulnerable to environmental change and risk extinction. Molecular genetic data for Carnivora in Thailand, such as phylogenetic relationships, is directly needed for conservation.

During the past decades, the phylogenetic relationships among the Carnivora have been addressed by several studies that employed both morphological and molecular techniques. Early efforts included comparative karyology (Wurster-Hill and Centerwall, 1982; Modi and O'Brien, 1988), cross-species chromosome painting (Tian *et al*., 2004), albumin immunological distance (Collier and O'Brien, 1985), comparative morphology (Salles, 1992), allozyme electrophoresis (O'Brien *et al*., 1987; Pecon-Slattery *et al*., 1994) and two-dimensional protein electrophoresis (Pecon-Slattery *et al*., 1994). More recently, efforts to resolve phylogenetic relationships have focused on the nuclear DNA sequence (Flynn *et al*., 2005; Sato *et al*., 2006; Zhang *et al*.,

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2006) and mitochondrial genome (Lopez *et al*., 1994; Janczewski *et al*., 1995; Johnson *et al*., 1996; Uphyrkina *et al*., 2002; Johnson *et al*., 2004; Flynn *et al*., 2005; Koepfli *et al*., 2006). In more recent studies, sequences from two other nuclear genes, Interphotoreceptor Retinoid-Binding Protein gene (IRBP) and recombination-activating gene 1 (RAG 1), have also been applied in phylogenetic analyses (Sato *et al*., 2003, 2004; Yoder *et al*., 2003; Yu *et al*., 2004). However, few analyses have included information from Internal Transcribed Spacer (ITS) sequence data.

The Internal Transcribed Spacer (ITS) of nuclear ribosomal DNA (rDNA) is one of the most extensively sequenced molecular markers (Alvarez and Wendel, 2003). The two internal transcribed spacers, ITS-1 and ITS-2, are located between genes encoding the 5.8, 18 and 26 sec nuclear ribosomal RNA (nrRNA) subunits (Baldwin, 1992). The ITS-1 and ITS-2 spacers, in addition to the 5.8 sec nrRNA are referred to as the ITS region (Baldwin, 1992). Individually, ITS-1 and ITS-2 are around 300 basepairs (bp) in length and the 5.8 subunit is almost invariant in length making the ITS region variant in varies species and several factors make the ITS region valuable for use in phylogenetic analyses (Baldwin *et al*., 1995). Moreover, the genetic relationships within wildlife order Carnivora in Thailand have never been addressed using the sequence of ITS region.

In this study, we analyzed the sequence of internal transcribed spacer regions to determine the phylogenetic relationships among some wildlife Carnivora in Thailand.

MATERIALS AND METHODS

Sample collections and DNA extraction: Blood samples of 20 Carnivora species and primate outgroups (*Hylobates lar* and *Hylobates agilis*) were taken from Northern, Northeastern and Southern regions of Thailand in 2003-2004 (Table 1). Genomic DNA was isolated from blood samples using protinase K digestion and treatment with phenol/chloroform (Sambrook *et al*., 1989). The quality and quantity of extracted DNA was checked by 0.8% agarose gel electrophoresis and spectrophotometry.

Table 1: Twenty Carnivora species and two primate outgroups, their systematic classification, Fig. 1 lane number, source of collection and ITS length

Taxon	Lane No.	Source	Length (bp)
Ingroup taxa			
(Order Carnivora)			
Superfamilies Caniformia			
Families Canidae			
Canis aureus (Asiatic Jacckal)	1	Nakhon Ratchasrima Zoo, Northeastern	773
Cuon alpinus (Dhole)	2	Song Khla Zoo, Southern	767
Families Ursidae			
Ursus thibetanus (Black Bear)	3	Nakhon Ratchasrima Zoo, Northeastern	748
Ursus malayanus (Sun Bear)	4	Song Khla Zoo, Southern	736
Families Mustelidae			
Lutrogale perspicillata (Smooth-Coated Otter)	5	Song Khla Zoo, Southern	745
Aonyx cinerea (Small-Clawed Otter)	6	Chiangmai Zoo, Northern	728
Superfamilies Feliformia			
Families Viverridae			
Viverra zibetha (Large Indian Civet)	7	Chiangmai Zoo, Northern	713
Viverricular indica (Small Indian Civet)	8	Nakhon Ratchasrima Zoo, Northeastern	774
Paradoxurus hermaphroditus (Common Palm Civet)	9	Nakhon Ratchasrima Zoo, Northeastern	752
Paguma larvata (Masked Palm Civet)	10	Song Khla Zoo, Southern	757
Arctogalidia trivirgata (Small-Toothed Palm Civet)	11	Nakhon Ratchasrima Zoo, Northeastern	756
Arctictis biuturong (Binturong)	12	Chiangmai Zoo, Northern	718
Families Felidae			
Panthera tigris (Tiger)	13	Nakhon Ratchasrima Zoo, Northeastern	746
Panthera pardus (Leopard)	14	Nakhon Ratchasrima Zoo, Northeastern	733
Neofelis nebulosa (Clouded Leopard)	15	Nakhon Ratchasrima Zoo, Northeastern	730
Prionailurus bengaleusis (Leopard Cat)	16	Song Khla Zoo, Southern	728
Prionailurus viverrinus (Fishing Cat)	17	Nakhon Ratchasrima Zoo, Northeastern	743
Catopuma temminckii (Asian Golden Cat)	18	Song Khla Zoo, Southern	745
Felis chaus (Jungle Cat)	19	Nakhon Ratchasrima Zoo, Northeastern	739
Pardofelis marmorata (Marbled Cat)	20	Song Khla Zoo, Southern	723
Outgroup taxa (Order Primate)			
Families Hylobatidae			
Hylobates lar (White Handed Gibbon)	21	Nakhon Ratchasrima Zoo, Northeastern	720
Hylobates agilis (Black Handed Gibbon)	22	Song Khla Zoo, Southern	699

Polymerase chain reaction amplification and sequencing: The complete ITS region of each species was amplified with primers ITSI (5'-TCGTAACAAGGTTTCCGTAGGT-3') and ITSII (5'-GTAAGTTTCTTCTCCTCCGCT-3') (Tsai *et al*., 2004). The ITS amplification reaction consisted of 20 ng DNA extract, 1.5 mM $MgCl_2$, 1X buffer, 2.0 mM dNTP mix, 0.075 mM ITSI and ITSII primers and 0.5 U AmpliTaq DNA polymerase (Invitrogen, USA) in a final volume of 25 μL. Amplification was completed in a thermalcycler (9700, GeneAmp® PCR system, Applied Biosystem, USA) with the following cycling parameters: 94°C for 5 min, 40 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C and a final extension of 10 min at 72°C. Amplification products of the correct size were verified on 1% agarose gels. The sequencing reactions were performed by using ABI

PRISM™ big dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The ITSI primer was used as sequencing primer for the ITS amplification. Reactions were then electrophoresed on an ABI 377 automatic sequencer (Perkin Elmer, Applied Biosystem, USA).

DNA sequence alignment and phylogenetic analysis: Genetic relationships were determined using the program MEGA version 2.1 (Tamura *et al*., 2007). The genetic distance matrix was calculated by the two-parameter method of Kimura (1980) and then was used to construct a phylogenetic tree using the Neighbor-Joining method (NJ) (Saitou and Nei, 1987) with interior branch tests of 1000 replicates (Sitnikova *et al*., 1995).

RESULTS AND DISCUSSION

The primers (ITSI and ITSII) were used for PCR and sequencing. The PCR product of the 20 Carnivora species and two primate outgroups were analyzed by agarose gel electrophoresis and a single fragment (approximately 750 bp in length) shown (Fig. 1). The nucleotide sequences were further determined. The length of the ITS region in Carnivora species varied from 713 (*Viverra zibetha*) to 775 bp (*Catopuma temminckii*). ITS regions of the outgroups were 699 bp in *Hylobates agilis* and 720 bp in *Hylobates lar* (Table 1). ITS sequences of the 20 Carnivora species and two primate outgroups were aligned and resulted in 890 characters with 710 variable sites (79.78%) as show in Fig. 2.

Genetic distances among the 22 taxa ranged from 0.05 between *Ursus malayanus* and *Ursus thibetanus* to 0.93 between *Hylobates lar* and *Viverricular indica* according to the two-parameter method of Kimura (1980). A genetic distance matrix is shown in Table 2. Among the 20 Carnivora species, the range of genetic distances was from 0.05 between *Ursus malayanus* and *Ursus thibetanus* to 0.89 between *Cuon alpinus* and *Paradoxurus hermaphroditus*.

The dendrogram constructed from comparisons of ITS sequences using the Neighbor-Joining method (NJ) demonstrate that the 22 taxa can be clearly grouped in six clusters: Mustelidae, Ursidae, Canidae, Felidae, Viveridae and Hylobatidae. Of these clusters, the 20 canivora species are clustered together and the superfamilies Caniformia and Feliformia can be separated apart, whereas the outgroup Hylobatidae is segregated from the canivora with 100% bootstrap value (Fig. 3). In superfamilies Caniformia, the families Mustelidae, Ursidae and Canidae are clustered together with 76% bootstrap support. In superfamilies Feliformia, the family Felidae is clustered with Viverridae with 98% bootstrap support. The representative species in families Canidae (*Canis aureus* and *Cuon alpinus*), Ursidae (*Ursus thibetanus* and *Ursus malayanus*) and Mustelidae (*Lutrogale perspicillata* and *Aonyx cinerea*), are clustered together. In family Viverridae, the viverrinae, *Viverra zibetha* and *Viverricular indica* are clustered together attaching with *Arctictis binturong* of Paradoxurinae, while the other Paradoxurinae, *Paradoxurus hermaphroditus*, *Paguma larvata* and *Arctogalidia trivirgata* are clustered together. In family Felidae, the Felinae, *Prionailurus bengalensis*, *Prionailurus viverrinus*, *Felis chaus* and *Catopuma temminckii* are clustered together without *Pardofelis marmorata*. Moreover, *Pardofelis marmorata* produces a sister species to the Pantherinae, *Neofelis nebulosa* and these sister species clustered with *Panthera tigris* and *Panthera pardus*.

The ITS sequence has been widely used for phylogenetic analyses, especially in plants (Tsai *et al*., 2004). In the present study, the genetic relationships among Carnivora species in Thailand have been determined by using the ITS region including ITS1, 5.8 sec ribosomal DNA and the ITS2 region. As shown in Fig. 2, the ITS regions are highly variable (79.78%). The numerous variable sites of ITS region reflect the genetic diversity of Carnivora species. Interspecific variations in ITS regions among Carnivora species were very high as indicated by the range of genetic distances from 0.05 to 0.89.

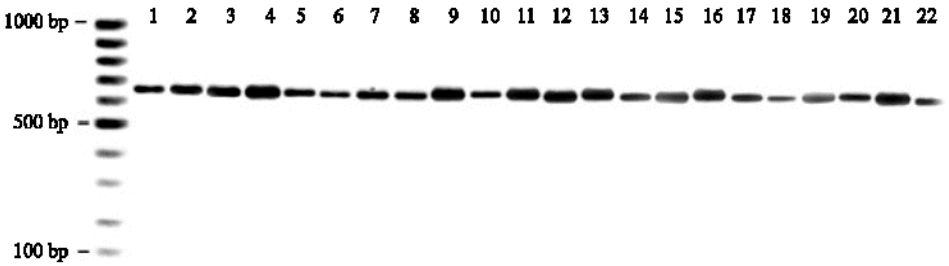

Fig. 1: Gel electrophoresis of the amplified ITS region of 20 Carnivora species and two primates outgroups. Lanes are numbered according to the species list shown in Table 1

Fig. 2: Continued

Fig. 2: Sequence comparison of ITS regions from 20 Carnivora species and two primate outgroups in Thailand. Dots (·) indicate the same nucleotides and dashes (-) are introduced to gap

Table 2: Genetic distances of the ITS sequence among twenty Carnivora species and two primate outgroups according to the two-parameter method of Kimura

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
L. perspicillata	0.00																					
A. cinerea	0.12	0.00																				
U. malayanus	0.41	0.37	0.00																			
U. thibetanus	0.43	0.40	0.05	0.00																		
C. alpinus	0.45	0.44	0.39	0.40	0.00																	
C. aureus	0.35	0.35	0.24	0.25	0.30	0.00																
P. pardus	0.53	0.52	0.55	0.58	0.75	0.53	0.00															
P. tigris	0.59	0.55	0.54	0.57	0.77	0.54	0.12	0.00														
N. nebulosa	0.53	0.50	0.55	0.56	0.70	0.48	0.14	0.18	0.00													
P. marmorata	0.52	0.51	0.59	0.61	0.72	0.55	0.21	0.29	0.17	0.00												
P. bengaleusis	0.65	0.57	0.47	0.49	0.77	0.57	0.38	0.37	0.34	0.31	0.00											
P. viverrinus	0.65	0.56	0.50	0.53	0.77	0.55	0.35	0.37	0.31	0.30	0.10	0.00										
C. temminckii	0.58	0.55	0.54	0.56	0.71	0.52	0.32	0.31	0.31	0.28	0.29	0.29	0.00									
F. chaus	0.60	0.56	0.44	0.47	0.69	0.49	0.33	0.26	0.34	0.33	0.29	0.29	0.22	0.00								
A. trivirgata	0.60	0.59	0.58	0.60	0.81	0.51	0.40	0.43	0.35	0.37	0.37	0.37	0.35	0.40	0.00							
P. larvata	0.58	0.56	0.54	0.55	0.81	0.48	0.36	0.41	0.31	0.36	0.39	0.36	0.35	0.40	0.09	0.00						
P. hermaphroditus	0.64	0.65	0.65	0.68	0.89	0.57	0.44	0.51	0.41	0.41	0.44	0.42	0.40	0.47	0.13	0.13	0.00					
V. indica	0.73	0.69	0.66	0.70	0.85	0.66	0.54	0.57	0.52	0.53	0.53	0.51	0.48	0.53	0.29	0.31	0.31	0.00				
V. zibetha	0.57	0.55	0.53	0.57	0.73	0.46	0.44	0.44	0.40	0.43	0.44	0.41	0.37	0.44	0.15	0.18	0.20	0.18	0.00			
A. binturong	0.56	0.56	0.57	0.59	0.78	0.49	0.40	0.45	0.35	0.36	0.42	0.40	0.36	0.44	0.24	0.24	0.24	0.30	0.20	0.00		
H. lar	0.75	0.75	0.65	0.68	0.77	0.78	0.78	0.86	0.83	0.79	0.71	0.74	0.82	0.73	0.84	0.79	0.84	0.92	0.82	0.83	0.00	
H. agilis	0.74	0.73	0.66	0.67	0.76	0.74	0.78	0.86	0.82	0.81	0.70	0.73	0.77	0.74	0.83	0.78	0.84	0.93	0.80	0.81	0.12	0.00

(1) *L. perspicillata*, (2) *A. cinerea*, (3) *U. malayanus*, (4) *U. thibetanus*, (5) *C. alpinus*, (6) *C. aureus*, (7) *P. pardus*, (8) *P. tigris*, (9) *N. nebulosa*, (10) *P. marmorata*, (11) *P. bengaleusis*, (12) *P. viverrinus*, (13) *C. temminckii*, (14) *F. chaus*, (15) *A. trivirgata*, (16) *P. larvata*, (17) *P. hermaphroditus*, (18) *V. indica*, (19) *V. zibetha*, (20) *A. binturong*, (21) *H. lar* and (22) *H. agilis*

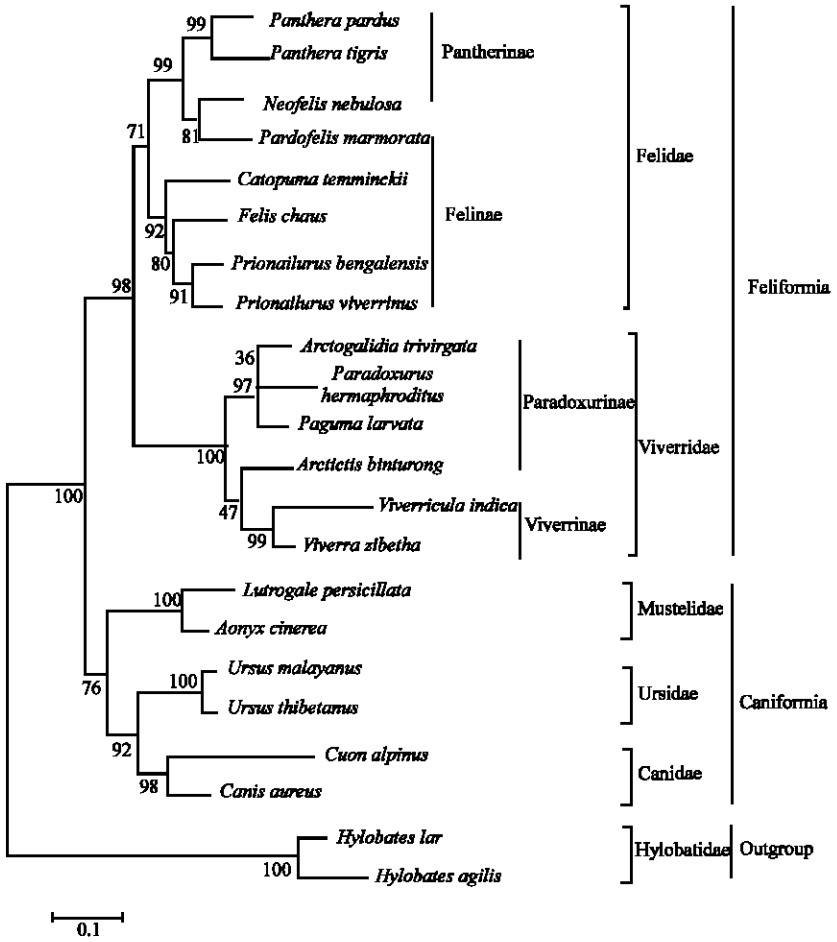

Fig. 3: A dendrogram of the twenty Carnivora species and two primate outgroups constructed from sequences comparisons of the ITS region using the Neighbor-Joining (NJ) method

Based on morphological data, the classification of the mammalian order Carnivora in this study should group the five families namely Canidae, Ursidae, Mustelidae, Viveridae and Felidae (Eisenberg, 1989; Wozencraft, 1989; Wyss and Flynn, 1993). Thus, the phylogenetic tree of Carnivora species deduced from the ITS sequence completely matches the classification based on morphological characters. Furthermore, the two monophyletic superfamilies Carniformia and Feliformia can be separated apart, which supports molecular data previously reported by Yu *et al.* (2004). In superfamilies Caniformia, the family Ursidae was depicted as the closest lineage to the Canidae followed by the Mustelidae. In contrast, the phylogenetic of the Caniformia constructed from the first intron of TTR gene (Flynn and Nedbal, 1998), the combining phylogenetic information (Bininda-Emonds *et al.*, 1999) and the sequence of the TTR gene and the combination of IRBP and TTR gene data sets (Yu *et al.*, 2004) demonstrated that the Ursidae was positioned as the sister group of the Mustelidae, followed by the Canidae. Moreover, the IRBP data placed the Ursidae as a sister group of the Mustelidae and Canidae clade (Yu *et al.*, 2004). Based on the average genetic distances between groups (Table 3), however, the Mustelidae posses equal genetic distance (0.40) to the Ursidae and Canidae. Present results suggest that the superfamilies Feliformia, family Felidae and Viverridae are clustered together and completely separated into two groups. The phylogenetic tree of the Viverridae species deduced from the ITS sequence, however, does not completely match the classification based on morphological characters (Eisenberg, 1989; Flynn and Nedbal, 1998). The Paradoxurinae, *Arctictis binturong* was grouped into the Viverrinae, clustered with 47% bootstrap support, while the other Paradoxurinae, *Paradoxurus hermaphroditus*, *Paguma larvata* and *Arctogalidia trivirgata* are clustered together with 97% bootstrap value. However, the combining phylogenetic information form Bininda-Emonds *et al.* (1999) and the mitochondrial cytochrome b sequence data by Veron and Heard (2000) have reported the close genetic relationships of *Paguma larvata* and *Arctictis binturong*. In order to evaluate and maintain the biological diversity represented in the Viverridae, an improved understanding of the taxonomy and phylogeny of the viverrids is of major importance. Clarification of this phylogeny is also of great significance in understanding carnivore evolution, given that the viverrids retain many of the phylogenetically primitive characteristics of the first Feliformia. To date, the phylogeny of the Viverridae remains controversial (Wyss and Flynn, 1993; Flynn and Nedbal, 1998) and very few Viverrid species have been included In previous molecular phylogenetic studies (Veron and Heard, 2000). However, recently their cytogenetics were studied by Tanomtong *et al.* (2005) with wild animal species of the subfamily Paradoxurinae in Thailand including *Paguma larvata*, *Arctictis binturong*, *Paradoxurus hermaphroditus* and *Arctogalidia trivirgata* and their results showed the number of diploid chromosomes as 44, 42, 42 and 40, respectively. Morover, Tanomtong *et al.* (2006) reported chromosome numbers of 38 for *Viverra zibetha* and 36 for *Viverricular indica*. These show the genetic diversity of the Viveridae species. In this study, the average genetic distances of *Arctictis binturong* to the other Paradoxurinae (0.24) is lower than the Viverrinae (0.25). These indicate the high relationships in the Paradoxurinae, although, *Arctictis binturong* was placed into the Viverrinae group, but is only a bridge for Viverinae and Paradoxurinae.

Table 3: Average genetic distances within and between group of carnivora species and two primate outgroups

Families	Mustelidae	Ursidae	Canidae	Felidae	Viveridae	Outgroup
Mustelidae	0.00					
Ursidae	0.40	0.00				
Canidae	0.40	0.32	0.00			
Felidae	0.56	0.53	0.63	0.00		
Viveridae	0.61	0.60	0.67	0.42	0.00	
Outgroup	0.74	0.67	0.76	0.78	0.84	0.00

In family Felidae, the Felinae, *Prionailurus bengalensis* and *Prionailurus viverrinus* are more closely related to *Felis chaus* than *Catopuma temminckii* with 92% bootstrap support, agreeing with Bininda-Emonds *et al.* (1999), Mattern and McLennan (2000) and Pecon-Slattery *et al.* (2004), while Johnson and O'Brien (1997), Ortolani (1999) and Srisamoot *et al.* (2007) reported that *Prionailurus bengalensis* and *Prionailurus viverrinus* is more closely related to *Catopuma temminckii* than *Felis chaus*. However, based on present study, *Prionailurus bengalensis* and *Prionailurus viverrinus* (Asian leopard cat group) possessed the equal genetic distances (0.29) to those *Catopuma temminckii* and *Felis chaus*.

Astonishingly, the Felinae, *Pardofelis marmorata* was placed as a sister species to the Pantherinae with high bootstrap value (81%). Recently, it has been considered that *Pardofelis marmorata* should be placed in the subfamily Felinae (Bininda-Emonds *et al.*, 1999; Mattern and McLennan, 2000; Pecon-Slattery *et al.*, 2004; Srisamoot *et al.*, 2007). From the cluster analysis of this study, *Pardofelis marmorata* was separated from the subfamily Felinae, which is contrary to morphological classification and the above other studies. However, the separation of Felidae into two groups of big cats (Pantherinae) and small cats (Felinae) is generally

supported by most studies. Moreover, a clear size dichotomy does seem to exist in Felidae and the shape and size of *Pardofelis marmorata* is according to Felinae (Bininda-Emonds *et al*., 2001). Based on our study, we found a close relationship between *Pardofelis marmorata* and *Neofelis nebulosa* with low genetic distance (0.17), according to the phylogenetic tree recently constructed by Bininda-Emonds *et al.* (1999), Ortolani (1999) and Srisamoot *et al.* (2007). Support for monophyletic groups composing the Felidae species is abundant from morphological characters (Neff, 1982; Salles, 1992; Peters and Hast, 1994) as the separation of Felidae into three groups of big cats, medium cats and small cats. Therefore, in order to resolve the genetic relationships between the Carnivora species, especially in Felidae properly, it is necessary to study larger samples, which are most difficult for wildlife studies. Thus, these results suggest that genetic analysis based on the sequence of ITS region has a good capacity for the study of genetic relationships, especially in Carnivora species.

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