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New Insights into the Evolution of Intronic Sequences of the β-fibrinogen Gene and Their Application in Reconstructing Mustelid Phylogeny

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Mustelidae is the largest and most diverse family in the order Carnivora. The phylogenetic relationships among the subfamilies have especially long been a focus of study. Herein we are among the first to employ two new introns (4 and 7) of the nuclear β-fibrinogen gene to clarify these enigmatic problems. In addition, two previously available nuclear (IRBP exon 1 and TTR intron 1) and one mt (ND2) data sets were also combined and analyzed simultaneously with the newly obtained sequence data in this study. Detailed characterizations of the two intronic regions not only reveal the remarkable occurrences of short interspersed element (SINE) insertion events, providing a new example supporting the attractive hypothesis that attrition of an earlier retroposition may offer a proper environment for successive retropositions by forming a "dimer-like" structure, but also demonstrate their utility in the resolution of mustelid phylogeny. All of our analyses confirm the assemblage of Mustelinae, Lutrinae, and Melinae with confidence; moreover, two clades within Mustelinae were clearly recognized, i.e., genera *Mustela* and *Martes*. Notably, genus *Martes* of Mustelinae was found to branch off first, followed by Melinae and then a clade containing Lutrinae and genus *Mustela* of Mustelinae, indicating paraphyly of Mustelinae. In addition, Mephitinae diverges before the other mustelids and the monophyletic Procyonidae in all cases, supporting its elevation to a separate family. Additional independent genetic markers are still in need to resolve the trichotomy among Mephitinae and the other two carnivoran clades, Ailuridae and Procyonidae/non-mephitine Mustelidae.

Key words: β-fibrinogen, intron, short interspersed element, SINE, Mustelidae, Mephitinae, phylogenetic analysis

INTRODUCTION

The family Mustelidae, the most heterogeneous and morphologically diverse family in the mammalian order Carnivora, includes 66 species and is traditionally grouped into five subfamilies: Mustelinae, Lutrinae, Melinae, Taxidiinae, and Mephitinae (Wozencraft, 1989; Yonezawa et al., 2007). Phylogenetic relationships among these subfamilies of living mustelids have been hotly disputed in recent studies and are not yet well established (Bininda-Emonds et al., 1999; Sato et al., 2003, 2004, 2006; Koepfli and Wayne, 2003; Marmi et al., 2004; Flynn et al., 2005; Yonezawa et al., 2007). The main problem is that Mustelidae represents a typical example of rapid evolutionary radiation and recent speciation events (Koepfli and Wayne, 1998; Marmi et al., 2004), possibly dating back to Oligocene (Wolsan, 1993, 1999; 2005). It even remains ambiguous whether mephitines belongs in Mustelidae, and what their systematic placements are in Order Carnivora, if they are not mustelids. The unresolved trichotomy between Mephitinae and the other two carnivoran clades, Ailuridae and Procyonidae/non-mephitine Mustelidae, has been an evolutionary puzzle over the past few decades (Vrana et al., 1994; Ledje and Arnason, 1996a, b; Dragoo and Honeycutt, 1997; Flynn and Nedbal, 1998; Wolsan,1999; Flynn et al., 2000, 2005; Sato et al., 2004; Delisle and Strobeck, 2005; Fulton and Strobeck, 2006; Yonezawa et al., 2007).

Here, we are the first to employ introns 4 and 7 of the

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fibrinogen gene (FGB) in a phylogenetic study of the Mustelidae. Intron 7 of β-fibrinogen has been used at different taxonomic levels for birds (Prychitko and Moore, 1997), reptiles (Creer et al., 2003), and carnivorans (Yu and Zhang, 2005a, 2006), while β-fibrinogen intron 4 has recently been explored in avian and higher-level carnivoran phylogenies (Barker, 2004; Yu and Zhang, 2006). Interestingly, in the present study, besides providing new insight into the relationships among mustelid subfamilies and the phylogenetic position of Mephitinae relative to the other carnivorans, analyses of these two intronic sequences also reveal the striking occurrences of short interspersed elements (SINEs) insertion events. SINEs are repetitive retroposons less than 500 bp in length that are propagated in eukaryotic genomes by retroposition via an RNA intermediate (Rogers, 1985; Okada, 1991). It has been believed that the enormous dispersion of SINEs may have had a significant impact not only on genomic diversity, but also on regulation of gene expression and function (Deininger, 1989; Maraia, 1995; Shedlock and Okada, 2000).

A comprehensive data set combining the two intronic sequences with previously published nuclear and mitochondrial (mt) data was used here, in both separate and concatenated analyses, with a view to (1) examine the mode of evolution and phylogenetic information in the SINE elements isolated from the two new intronic sequences, (2) evaluate phylogenetic relationships among mustelid subfamilies and investigate the true closest relatives of Mephitinae in Order Carnivora, (3) explore the utility of β-fibrinogen introns 4 and 7 in the resolution of mustelid phylogeny.

MATERIALS AND METHODS

DNA samples and PCR amplification

Ten species representing four subfamilies of Mustelidae (Mustelinae, Lutrinae, Melinae, and Mephitinae) were examined in this study (Table 1). In addition, considering that growing genetic

Table 1. Samples and sequences used in this study

Taxon					Gene				
Family	Subfamily	Scientific name	Common name	Sample source	Accession No. (References) FGB7	FGB4	IRBP	TTR	ND2
Canidae		Canis lupus	Wolf	Yunnan Province, China	AY726652 (Yu & Zhang, 2005b)	AY882026 (Yu & Zhang, 2006)	AY525044 (Yu & Zhang, 2004b)	AF039732 (Flynn & Nedbal, 1998)	AY170044 (Yoder et al., 2003)
		Canis familiaris	Domestic dog	–	Genome Database	Genome Database	Genome Database	Genome Database	Genome Database
Procyonidae		Procyon lotor	Raccoon	San Diego Zoo, USA	AY726653 (Yu & Zhang, 2005b)	AY882028 (Yu & Zhang, 2006)	AY525029 (Yu & Zhang, 2004b)	AF039736 (Flynn & Nedbal, 1998)	AY170046 (Yoder et al., 2003)
		Potos flavus	Kinkajou	San Diego Zoo, USA	AY882046 (Yu & Zhang, 2006)	AY882029 (Yu & Zhang, 2006)	AY525030 (Yu & Zhang, 2004b)	AF039737 (Flynn & Nedbal, 1998)	AY882059 (Yu & Zhang, 2006)
		Nasua narica	White-nosed coati	San Diego Zoo, USA	AY882047 (Yu & Zhang, 2006)	AY882030 (Yu & Zhang, 2006)	AY525031 (Yu & Zhang, 2004b)	AY525054 (Yu & Zhang, 2004b)	AY882060 (Yu & Zhang, 2006)
Mustelidae	Mustelinae	Martes flavigula	Yellow-throated Marten	Kunming Zoo, China	AY726654 (Yu & Zhang, 2005b)	AY882031 (Yu & Zhang, 2006)	AY525048 (Yu & Zhang, 2004b)	AY525050 (Yu & Zhang, 2004b)	AY882061 (Yu & Zhang, 2006)
		Martes zibellina	Sable	Haerbin Zoo, China	AY882048 (Yu & Zhang, 2006)	AY882032 (Yu & Zhang, 2006)	AY525047 (Yu & Zhang, 2004b)	AY525051 (Yu & Zhang, 2004b)	AY882062 (Yu & Zhang, 2006)
		Martes foina	Stone Marten	Kunming Zoo, China	EU021485 (this study)	EU021492 (this study)	AB082965 (Sato et al., 2003)	EU021499 (this study)	EU021504 (this study)
		Mustela kathiah	Yellow-bellied weasel	Yunnan Province, China	AY882049 (Yu & Zhang, 2006)	AY882033 (Yu & Zhang, 2006)	AY525046 (Yu & Zhang, 2004b)	EU021500 (this study)	AY882063 (Yu & Zhang, 2006)
		Mustela nivalis	Least weasel	CGRB*, South Korea	EU021487 (this study)	EU021494 (this study)	AB082973 (Sato et al., 2003)	EU021501 (this study)	AY750629 (Flynn et al. 2005)
		Mustela sibirica	Siberian weasel	CGRB*, South Korea	EU021486 (this study)	EU021493 (this study)	AB082976 (Sato et al., 2003)	AY750594 (Flynn et al., 2005)	AY750641 (Flynn et al. 2005)
	Lutrinae	Lutra lutra	Eurasian otter	CGRB*, South Korea	EU021489 (this study)	EU021496 (this study)	dq205884[#] (Fulton & Strobeck, 2006)	EU021503 (this study)	AY598557[#] (Delisle & Strobeck, 2005)
	Melinae	Meles meles	Eurasian badger	CGRB*, South Korea	EU021488 (this study)	EU021495 (this study)	AB082980 (Sato et al., 2003)	EU021502 (this study)	EU021505 (this study)
		Arctonyx collaris	Hog badger	Yunnan Province, China	AY882050 (Yu & Zhang, 2006)	AY882034 (Yu & Zhang, 2006)	AY525049 (Yu & Zhang, 2004b)	AY525053 (Yu & Zhang, 2004b)	AY882064 (Yu & Zhang, 2006)
	Mephitinae	*Mephitis mephitis*	Striped skunk	San Diego Zoo, USA**	EU021490 (this study)	EU021497 (this study)	AB109331 (Sato et al., 2004)	AF306948 (Flynn et al., 2000)	AY598535 (Delisle & Strobeck, 2005)
Ursidae		*Ursus arctos*	Brown bear	Heilongjiang Province, China	AY726655 (Yu & Zhang, 2006)	AY882035 (Yu & Zhang, 2006)	AY303842 (Yu et al., 2004a)	AF039741 (Flynn & Nedbal, 1998)	AF303110(Delisle & Strobeck, 2002)
		Ursus thibetanus	Asiatic black bear	Yunnan Province, China	AY882051(Yu & Zhang, 2006)	AY882036 (Yu & Zhang, 2006)	AY303841 (Yu et al., 2004a)	AY303847 (Yu et al., 2004a)	AY882065 (Yu & Zhang, 2006)
		Tremarctos ornatus	Spectacled bear	San Diego Zoo, USA	AY882052 (Yu & Zhang, 2006)	AY882037 (Yu & Zhang, 2006)	AY303840 (Yu et al., 2004a)	AF039740 (Flynn & Nedbal, 1998)	AY170045 (Yoder et al., 2003)
		Ailuropoda melanoleuca	Giant panda	Sichuan Province, China	AY882053 (Yu & Zhang, 2006)	AY882038 (Yu & Zhang, 2006)	AY303836 (Yu et al., 2004a)	AF039738 (Flynn & Nedbal, 1998)	AY882066 (Yu & Zhang, 2006)
Ailuridae		*Ailurus fulgens*	Red panda	Yunnan Province, China	AY882054 (Yu & Zhang, 2006)	AY882039 (Yu & Zhang, 2006)	AY525045 (Yu et al., 2004b)	AF039739 (Flynn & Nedbal, 1998)	AY882067 (Yu & Zhang, 2006)
Otariidae		*Zalophus californianus*	Sea lion	San Diego Zoo, USA	AY882055 (Yu & Zhang, 2006)	AY882040 (Yu & Zhang, 2006)	AY525043 (Yu et al., 2004b)	AF039745 (Flynn & Nedbal, 1998)	AY750639 (Flynn et al., 2005)
Phocidae		*Phoca vitulina*	Harbor seal	China	AY882057 (Yu & Zhang, 2006)	AY882042 (Yu & Zhang, 2006)	AB188518 (Sato et al. ,2006)	AY750599 (Flynn et al., 2005)	AY750640 (Flynn et al., 1998)
Odobenidae		*Odobenus rosmarus*	Walrus	San Diego Zoo, USA*	EU021491 (this study)	EU021498 (this study)	DQ205892 (Fulton & Strobeck, 2006)	AF039743 (Flynn & Nedbal, 1998)	AY750637 (Flynn et al., 2005)

* Conservation Genome Resource Bank for Korean Wildlife, Seoul National University. Data were exchanged after sequencing was conducted in each laboratory.

** Data were exchanged after sequencing was conducted in each laboratory.

[#] Sequence from *Lontra canadensis*, instead of that from *Lutra lutra*, was obtained for the concatenated analysis.

evidence supports the exclusion of subfamily Mephitinae from Mustelidae and places it within suborder Caniformia of Carnivora as a separate unresolved clade, we also included 13 non-mustelid caniform carnivorans in our analyses to determine the phylogenetic position of Mephitinae (Table 1). Here, all seven currently recognized extant families of Caniformia, including Canidae, Ursidae, Procyonidae, Mustelidae, Phocidae, Odobenidae, and Otariidae (Eisenberg, 1989; Wozencraft, 1989; Wyss and Flynn, 1993), were represented. We also retrieved published sequences from GenBank for two nuclear genes, part of exon 1 from the gene for interphotoreceptor retinoid binding protein (IRBP) and intron 1 from the transthyretin (TTR) gene, and one mitochondrial protein-coding gene, NADH dehydrogenase subunit 2 (ND2), for these carnivore species (Flynn and Nedbal, 1998; Flynn et al., 2005; Sato et al., 2003; Yu et al., 2004a, b; Yu and Zhang, 2005b, 2006). In addition, we newly sequenced some sequences of these genes from the mustelid species included in this study (Table 1).

For each sample, total genomic DNA was isolated from blood or frozen tissues using standard proteinase K, phenol/chloroform extraction (Sambrook et al., 1989). All target gene segments were amplified by PCR using previously reported primers: FGB-FelF/FGB-FelR and FGB7-CarF/FGB7-CarR for β-fibrinogen intron 7, FGB4-CarF and FGB4-CarR for β-fibrinogen intron 4, ND2-FelF/ND2-FelR and ND2-CarF/ND2-CarR for ND2 (Yu and Zhang, 2006), and T635/T1628 for TTR (Flynn and Nedbal, 1998). The optimal conditions adopted for PCR reactions were 95°C for 5 min; 35 cycles of 94°C for 1 min, 50°C (for β-fibrinogen introns 4 and 7) or 56°C (for ND2 and TTR) for 1 min, and 72°C 1 min; and a final extension at 72°C for 10 min.

Sequencing and data analysis

Amplified PCR products were purified and sequenced in both directions with an ABI PRISM 3730 DNA sequencer. Acquired sequences were put into GenBank for BLAST searching (Altschul et al., 1997) and data validity was ensured.

Separate alignments of four nuclear gene segments (β-fibrinogen introns 4 and 7, IRBP, TTR) and one mt gene sequence (ND2) from 23 caniform carnivores were generated by CLUSTAL X (Thompson et al., 1997) and refined by visual inspection. The β-fibrinogen intron 4 and 7 alignments exhibited obvious sequence length variation due to multiple insertions/deletions. The two intron DNA sequences were thus screened for interspersed repeats known to exist in mammalian genomes by using the program CENSOR (Kohany et al., 2006; http://www.girinst.org/censor/index.php). Matching elements were detected and assigned to specific repeat classes. Target-site duplications flanking putative SINEs due to the integration at staggered chromosomal breaks (Weiner et al., 1986) were identified by the software REPFIND (Betley et al., 2002) and by eye. Notably, besides orthologous mammalian-wide interspersed repeats (MIRs) and species-specific CAN SINEs in two canid taxa (*Canis lupus* and *Canis rufus*) detected within β-fibrinogen intron 7 (reported recently by us elsewhere; Yu and Zhang, 2005a, b, 2006), two new CAN SINEs were also observed here in *Lutra lutra* (otter) β-fibrinogen intron 4 and *Mephitis mephitis* (striped skunk) intron 7 (hereafter referred as Ott4-ins and Sku7-ins).

Phylogenetic analyses were conducted by using the maximum parsimony (MP; PAUP*4.0b10 software; Swofford, 2002), maximum likelihood (ML; PAUP*4.0b10 software; Swofford, 2002), and partitioned Bayesian inference (pBI; MrBayes3.0B4 software; Ronquist and Huelsenbeck, 2003) methods for the separate and combined datasets, with all species-specific SINEs removed. In the end, 514 and 618 nucleotide positions in the β-fibrinogen intron 4 and 7 alignment were used in the phylogenetic analyses, respectively (see Supplemental Material). We designated Canidae as the outgroup on the basis of the consensus that Canidae was the earliest diverging lineage within Caniformia (Wyss and Flynn, 1993; Vrana et al., 1994; Flynn and Nedbal, 1998; Yu et al., 2004b, 2006; Delisle and Strobeck, 2005; Flynn et al., 2005; Fulton and Strobeck, 2006). In MP analyses, a branch-and-bound search strategy with furthest sequence addition and MULPARS was employed. For the model-based ML analyses, we conducted hierarchical likelihood-ratio tests (hLRT) to compare the goodness of fit of 56 nucleotide substitution models by using ModelTest version 3.06 (Posada and Crandall, 1998). Once an appropriate model was established, a ML tree was constructed based on this model of sequence evolution. The reliability of phylogenetic relationships was evaluated by bootstrap analyses for MP and ML trees (BS; 1000 replicates for MP and 100 replicates for ML). The pBI analysis allowed a separate general time reversible (GTR)+I+Γ model and set of parameters for each gene partition. Four Metropolis-coupled Markov-chain Monte-Carlo (MCMC) analyses were run for 2 million generations, with trees sampled every 100 generations. Robustness of branches in the pBI analysis was assessed by posterior probability (PP).

In addition, to examine their potential phylogenetic signal, we also subjected the nucleotide sequence data from the orthologous MIRs embedded in β-fibrinogen intron 7 to phylogenetic analysis by using the three tree-building methods as described above.

RESULTS

Novel findings on SINE elements in β-fibrinogen intronic segments

Our previous study of β-fibrinogen intron 7 in 12 mammalian orders detected an orthologous MIR and several species-specific CAN SINE insertions in Carnivora (Yu and Zhang, 2005b). MIRs represent one of the oldest tRNA-derived SINE elements examined to date, as inferred from its integration into the host genomes before the radiation of mammalian orders (Jurka et al., 1995; Smit and Riggs, 1995), whereas species-specific CAN SINE elements were found to have independently inserted into different nucleotide positions in two feline (*Profelis temminckii* and *Otocolobus manul*) and one canine taxa (*Canis lupus*) (Yu and Zhang, 2005a, b). CAN SINE, as a pan-carnivore SINE family (Vassetzky and Kramerov, 2002), was first found in the American mink, then in the dog and harbor seal (Lavrentieva et al., 1989; Minnick et al., 1992; Coltman and Wright, 1994). Particularly interesting in our previous study is the finding that all identified species-specific SINEs show a strong tendency to insert within or in very close proximity to preexisting MIRs for efficient integration, supporting the attractive hypothesis that attrition of an earlier retroposition may provide a proper environment for successive retropositions (Wang et al., 2004; Yu and Zhang, 2005b).

Here, we extended SINE analyses of the same intron in Carnivora by including Odobenidae and additional taxa of Mustelidae. Interestingly, besides the presence of orthologous MIRs, a 420-bp large insertion located extremely close the 5' B box of MIR (at a distance of 37 bp) was observed uniquely in *Mephitis mephitis*, a representative of Mephitinae (alignment positions 528–947). On closer examination, sequence analyses revealed that this large insertion consists of two CAN SINEs (named Sku7-ins-1 and Sku7-ins-2) separated by a 9-bp sequence (ATATATTCT), each comprising 195 bp of the intact sub-subfamily SINEC1D_CF in subfamily SINEC1 of the CAN SINEs (Repbase Update Release 9.11, December 2004) and having been inserted in complementary orientation. As can be seen from Fig. 1A, there were perfect 15-bp target-site duplications, CTGACTGC(T)AT(C)ATTCT, flanking the large insertion and amazingly, the 9-bp separation sequence between the

A Mephitis mephitis (striped skunk) intron 7

GTATGTATGACGTCTTCTGCCCCTTCTTTAAAAGGCACTACAAATGCTGTAATTAGGATCCTTAATGGGAGCTCATATTA

MIR ← 3' variable segments

TTGACAACTATCATTTCTTAGGTGCTTCCTGGGCCTTAGCCACCATACTAAGCTCTTCATGTGTCACTCTAAACCATTTC

core region

TGCTATAATGTTGTCATTATTATGCTCACTTGACAGATGAAGTTTAGAGAGACAAAGTAACTTACCCAAGGTCATGCAAT

tRNA-like region MIR →

GATAAAAGATGGAACTCGAACTATAGCAGTTATCTTCTGAGTAGTGAGCATTTAGATAAGTTTCTCTGACTGCATATTCT

← CAN SINE (Sku7-ins-1)

TTTTTTTTTTAAATATTTTATTTATTTGACAGAGAGAAACACAGCCAGAGAGGGAACACAAGCAGGGGGAGTGAGAGAGG

GAGAAGCAGGCTTCCTGCAGAGCAGGGAGCCCGATGTGGGACTCGATCCCGGGTCTCCGAGACCATGACCTGAGCCGAAG

CAN SINE (Sku-ins-1) → ← CAN SINE (Sku7-ins-2)

GCAGTCGCCCAACCAACTGAGCCACCCAGGCGCCCCTATATATTCTTTTTTTTTTTTTTAAGATTTTATTTATTTGACAG

AGAGAAACACAGCTAGAGAGGGAACACAAGCAGAGGGACTGAAAGAGGGAGAAGCAGGCTTCCTACAGAACAGGAAGCCA

CAN SINE (Sku-ins-2)

GATGTGGGGCTCAATCCCGGGTCTCTAGGATCATAACCTGAGCCGAAAGCAGTCACTTAACCAACTGAGCCACCTAGGCG

→

CCCCTCTGACTGTACATTCTAGTGTGTATATTGGAACCTTCATTTTGTTTTCCAGGGTTCTCTAATACTAAGGGAGCTGA

AAGCTGACTTGTCAGTTTAAAGAAACAAATGCAGATGAACTTGTCACCAGGCCCAGTTCTCTCTCTTTCTTGCTATAAGG

CAGAGAAGGTCTTTTGTGTCTTAGTTTGATTTAACTTGTAAAACTAGTGTGAAAGAGTACTGTGCACAGCACTCTTCTGT

GGGTATTTTGCAAAGAATTAACAGCACTGCAATTCTAATAGCCCCAAATATT

B Lutra lutra (otter) β-fibrinogen intron 4

ATATTTCTAGAGGGTTCTAGAACAAATAGTCTCTAAAAATAAGAGAACTTCAAAAAGCTTAGTGGCTCTTCCCCAACCAA

←

CCCTAATGACAAAAGAGCACATCATGAAGATATCCCTAGCCTGCTGTGTTCTGACTTAGCCTAATGTATTTGCCAGTTTG

CAN SINE (Otter4-ins)

TTTTTTTTTAAGATTATTTATTTATTTGACAGAAATCACAAGTAGGCAGAGAGGCAGACAGAGAGAGAGAGGAGGAAGCA

GGCTCCCTGCCAAGCAGAGAGCCCGATGTGGGACTCCATCCCAGGACCCTGGGATCATGACCTGAGCCAAAGGCAGAGAC

CAN SINE (Otter4-ins) →

TCAACCCCCTGAGCCACCCAGGTGCCCCTATTTGCCAGTTTTTGATGAATAGAATATAATGTAATGATGATGATAGCTCC

ACCTGGCTAATGCACATTTTTGCTTGGCTAGGTGGCAGAAGCATGGCGGGGAGGAGGGGTATGGAGTTTCTCCATCCCTT

GTGTTCTGGCAGGTTATGTATATCCTAGGATATAAAAGGTGAAGAAGGGCTTGTTTCATCCCTTCGGGAAATGGAACTCA

AAACTGAGTCTGATTTTCAGTAAAAAGATGATTCAGTTTCTATAATTTATTCTCAAATGTCTAGGATAAAACATTCCTCA

GTAACCTGTGTTATAAAATTGATGACTAAAGACCAAGGAGTTATGTTGTAGTTCCCTGAATATATGTTATGTTATGTTTG

CAGA

Fig. 1. Location and characterization of the new CAN SINEs found in **(A)** *Mephitis mephitis* (striped skunk) β-fibrinogen intron 7 and **(B)** *Lutra lutra* (otter) β-fibrinogen intron 4. Boxed-case letters in the sequence indicate the direct repeats flanking the SINEs.

two CAN SINE units, which was found to be a partial sequence of the 15-bp duplications, was the target-site duplication for Sku7-ins-1. Therefore, a scenario for the formation of this unusually large insertion in this intronic region can be proposed based on characterization of the two target-site duplications (Fig. 2). Sku7-ins-2 was first inserted near the MIR 5' B box region, producing the 15-bp duplication, and then the Sku7-ins-1 insertion occurred at the A+T rich tail of Sku7-ins-2, producing the 9-bp duplication. The two successive insertions thus gave rise to the seemingly “dimeric” CAN SINE structure discovered here. Indeed, comparisons of the two CAN SINEs with the SINEC1D_CF consensus sequence also revealed that Sku7-ins-2 has higher sequence divergence than Sku7-ins-1 (28.8% and 24.1% K2P distance for Sku7-ins-2 and Sku7-ins-1, respectively, calculated in MEGA3 [Kumar et al., 2004]), supporting the earlier insertion of Sku7-ins-2. This feature is consistent with the model we proposed.

Remarkably, besides β-fibrinogen intron 7, we also found a new CAN SINE in another intronic sequence of the same gene, in β-fibrinogen intron 4 (alignment positions 165–366), from *Lutra lutra* (otter), a representative of Lutrinae. Like Sku7-ins-1 and Sku7-ins-2, this 195-bp full-length CAN SINE was identified to belong to sub-subfamily SINEC1D_CF in subfamily SINEC1 of CAN SINEs (Repbase Update Release 9.11, December 2004). There were 13-bp target-site duplications (TATTTGCCAGTTT) flanking it (Fig. 1B), and this identified CAN SINE sequence differed from the consensus by 17.4% divergence.

Phylogenetic analyses of two new nuclear data sets

Fig. 3 shows trees based on separate MP analyses of

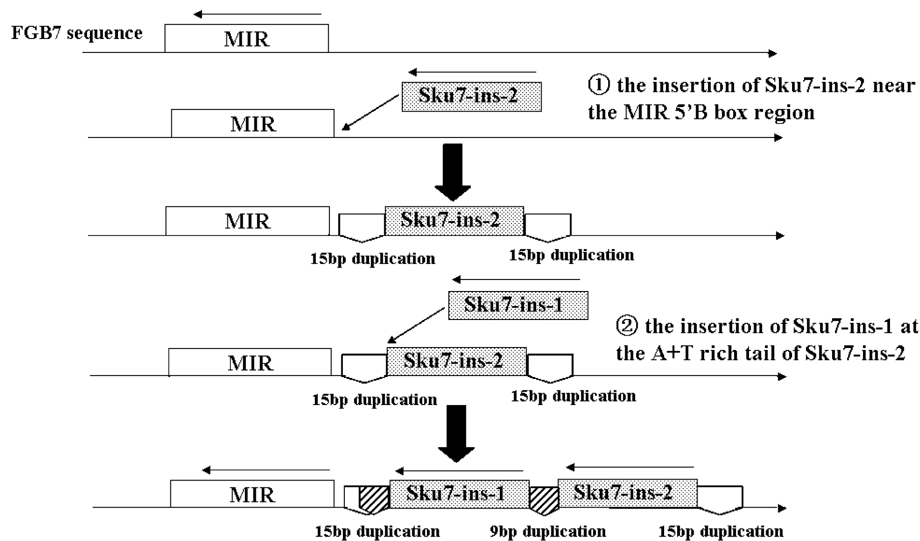


Fig. 2. A proposed model for the formation of the unusually large insertion in *Mephitis mephitis* intron 7.

β-fibrinogen introns 7 and 4 (FGB7 and 4). Groups that appeared in 50% or more of the trees were retained. Of the 618 aligned nucleotides for FGB7, 275 (44.50%) were variable and 178 (28.80%) were phylogenetically informative. The branch-and-bound search found three most parsimonious trees with a tree length of 380. The ML (model=HKY+G) and pBI analyses recovered a tree topology nearly identical to the MP tree, with similar nodal support. Within Mustelidae, genus *Martes* of Mustelinae branches off first, followed by Melinae and the clade containing Lutrinae and genus *Mustela* of Mustelinae. This result indicates the paraphyly of Mustelinae. In contrast to the well-resolved relationships among the three subfamilies (MP and ML BS≥90%; PP=1.00), the phylogenetic position of Mephitinae in Caniformia was ambiguous. Mephitinae, the pinnipeds (including three aquatic carnivoran families, Phocidae, Odobenidae and Otariidae), Procyonidae/non-mephitine Mustelidae, and Ursidae (plus Ailuridae) collapse into an unresolved polytomy.

The ML (model=HKY+G) and pBI trees based on β-fibrinogen intron 4 had nearly the same topology as the MP tree and showed similar support values. Of the 514 aligned nucleotides, 207 (40.27%) were variable and 114 (22.18%) were phylogenetically informative. The branch-and-bound search found six most parsimonious trees 273 steps long. As shown in Fig. 3B, Lutrinae and *Mustela* were most closely related, with high support (MP and ML BS>85%; PP=0.98), while the placements of Melinae and *Martes* were not resolved, although they formed a robust clade with Lutrinae and *Mustela* (MP and ML BS>95%; PP=1.00). The striking feature of the FGB4 gene tree in contrast to that of FGB7 was the sister-grouping of Mephitinae and Ailuridae (the red panda), but this relationship was poorly supported in all tree-building methods (MP BS=53%; ML BS<50%; PP=0.65).

Phylogenetic information in orthologous MIRs

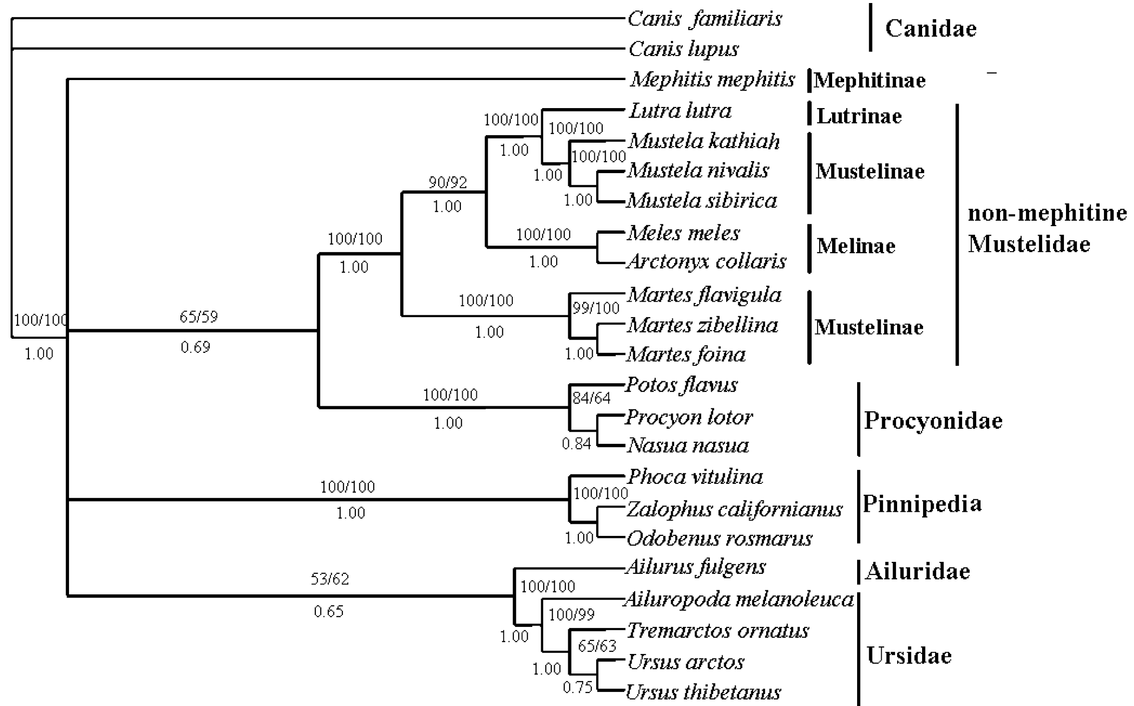
MP analysis of the orthologous MIRs embedded in β-fibrinogen intron 7 (alignment positions 82–275) recovered a similar, but less-resolved and lower-supported tree (Fig. 4A) compared to the trees based on non-MIRs sequences in FGB7 (Fig. 4B) and the entire FGB7 sequences (Fig. 3A). Of the 194 aligned nucleotides, 86 (44.33%) were variable and 57 (29.38%) were phylogenetically informative. The ML (model=K80) and pBI analyses produced the same topologies as the parsimony analysis. As shown in Fig. 4A, most interfamilial relationships across Caniformia were not resolved due to extremely low nodal support and formed a bush-like tree topology. With regard to the Mustelidae, only the monophyly of the non-mephitine mustelid clade (MP BS=76%; ML BS=71%; PP=0.70) and the close affinity of Lutrinae and *Mustela* within the clade (MP BS=97%; ML BS=97%; PP=1.00) were supported.

It appears that the non-MIR sequences retained more phylogenetic signal (Fig. 4B) and thus contributed more to the whole FGB7 tree and resolution of Caniformia phylogeny. The only exception is that all analyses of non-MIR sequences weakly supported Mephitinae as the sister taxon to the pinnipeds (MP BS=59%; ML BS=58%; PP=0.80), a relationship absent from the whole FGB7 tree. This relationship has not been reported in previous studies.

Phylogenetic analyses of combined data set

We retrieved most sequence data for two other nuclear genes, IRBP exon 1 (1186 bp in the alignment) and TTR intron 1 (910 bp in the alignment), and one mitochondrial protein-coding gene, ND2 (1044bp in the alignment), for the same set of taxa sampled in this study from the GenBank database and complemented them with sequences newly produced here (Table 1). These sequence data were combined with our new data sets for phylogenetic analyses. The combined aligned character matrix comprised 4272 nucleotides, of which 1850 (43.31%) were variable and 1335 (31.25%) were parsimony informative.

Compared with either individual gene tree, pooling four nuclear genes and one mt gene demonstrated a clear increase in the resolution of the overall Caniformia tree topology (Fig. 5). Identical relationships among the currently



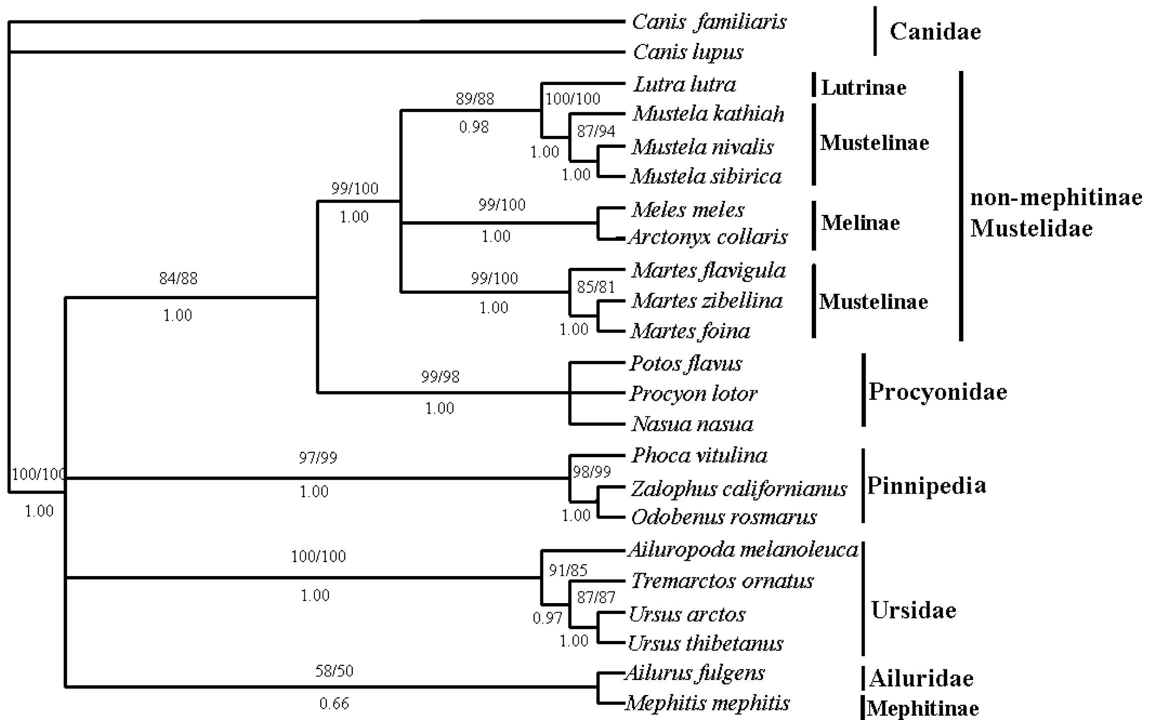


Fig. 3. Consensus phylogenetic trees (50% majority-rule) based on MP analyses of **(A)** β-fibrinogen intron 7 and **(B)** β-fibrinogen intron 4 sequences. Groups that appeared in 50% or more of the trees were retained. ML and pBI analyses recovered tree topologies nearly identical to those from the MP analyses. Bootstrap support (MP/ML) and posterior probability (PP) are shown above and below branches, respectively.

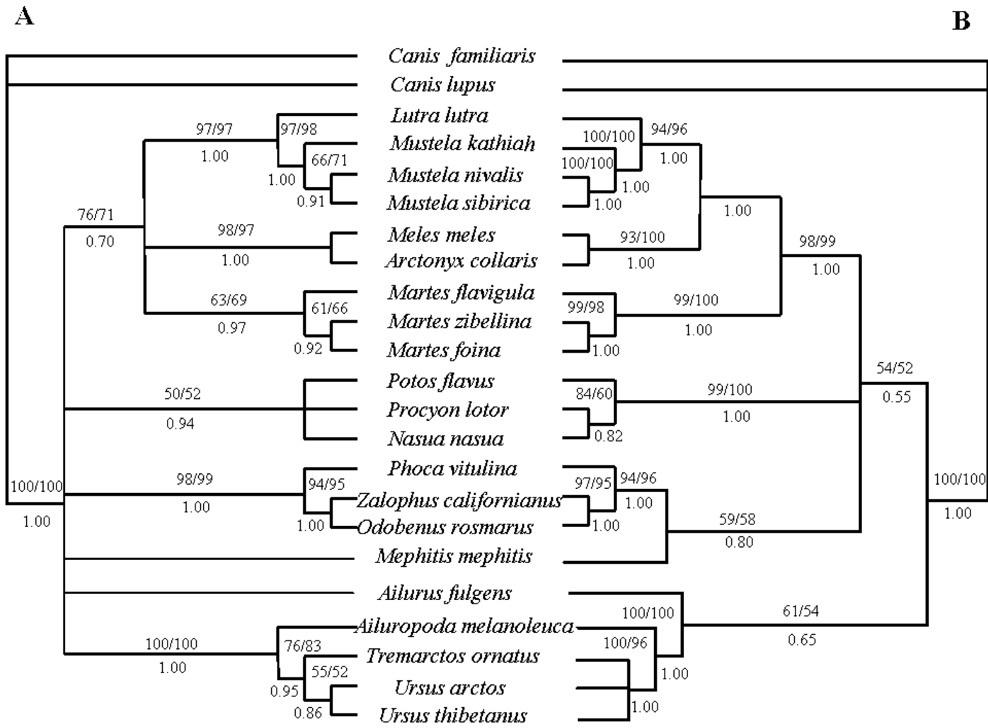


Fig. 4. Phylogenetic trees based on MP analyses of **(A)** orthologous MIR sequences embedded in β-fibrinogen intron 7 (only a single most parsimonious tree was found) and **(B)** non-MIR sequences in β-fibrinogen intron 7 (only a single most parsimonious tree was found). Groups that appeared in 50% or more of the trees were retained. ML and pBI analyses recovered tree topologies nearly identical to those from the MP analyses. Bootstrap support (MP/ML) and posterior probability (PP) are shown above and below branches, respectively.

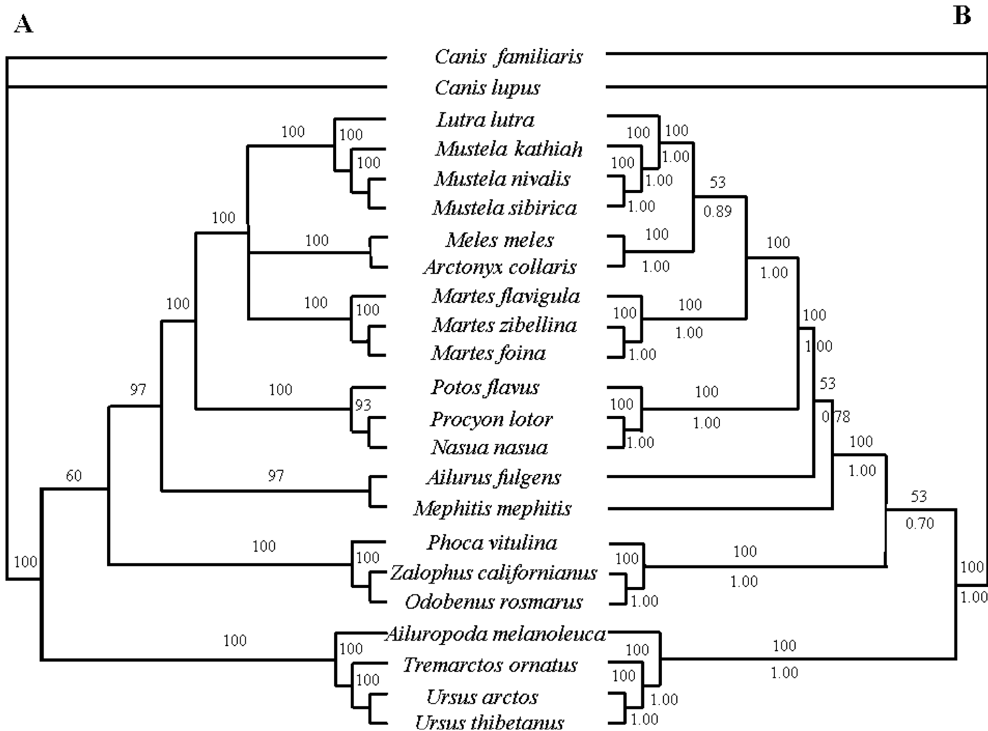


Fig. 5. Phylogenetic trees based on **(A)** MP (only a single most parsimonious tree was found) and **(B)** ML/pBI analyses of concatenated sequences from four nuclear genes and one mt gene. Groups that appeared in 50% or more of the trees were retained. Bootstrap support (MP/ ML) and posterior probabilities (PP) are shown.

recognized caniform families were obtained, regardless of the analytical approach. Ailuridae was placed as the closest taxon to the Procyonidae/non-mephitine mustelid clade, followed by the pinnipeds and Ursidae (including Ailuridae). However, for Mustelidae, the branching orders for subfamilies were different between the MP and ML/pBI analyses. The MP analysis (Fig. 5A; one most parsimonious tree 4402 steps long) resulted in a mustelid clade (including Mephitinae; groups that appeared in 50% or more of the trees were retained) identical to our FGB4 result (Fig. 3B) but with improved nodal supports, especially in the case of the close relatedness between Mephitinae and Ailuridae (MB BS=97%) The ML (model=GTR+I+G) and pBI analyses (Fig. 5B) produced a tree where relationships among Mustelinae, Lutrinae, and Melinae are the same as in the FGB7 tree (Fig. 3A). Mephitinae, however, was found to emerge after the divergence of Ursidae and the pinnipeds but before divergence of Ailuridae and Procyonidae/non-mephitine Mustelidae (ML BS=53%; PP=0.78), a relationship not recovered by our two nuclear single-gene analyses.

DISCUSSION

Here, we identified new members of the CAN SINE family, short interspersed nuclear elements specifically present in Order Carnivora. The most interesting feature is the presence of two perfect CAN SINEs joined in tandem (separated by a 9-bp spacer) in the alignment of *Mephitis mephitis* β-fibrinogen intron 7. Sequence analyses indicated that they (Sku7-ins-1 and Sku7-ins-2) arose by two successive insertion events, as evidenced by the existence of two sets of direct terminal repeats (Figs. 1, 2). This dimer-like structure is different from those resulting form previously reported primate *Alu*, rodent *MEN*, *B1-dID*, mosquito *Twin*, and galago SINE type II dimerization events, which involved a fusion of two monomers (related or unrelated) flanked by a set of short direct repeats. They retroposed into the host genome as a single element (Daniels and Deininger, 1985; Quentin, 1992; Jurka and Zuckerkandl, 1991; Serdobova and Kramerov, 1998; Feschotte et al., 2001; Kramerov and Vassetzky, 2001) rather than as two SINE units integrated nearby, as observed in the present study. We know of no examples of successive SINE insertions in such close proximity to a preexisting SINE. Our previous study on β-fibrinogen intron 7 showed that additional SINE sequences tend to insert into previously existing SINE elements (i.e., orthologous MIRs; Yu and Zhang, 2005b). The present interesting observation from the same intron of *Mephitis mephitis* corroborated the earlier finding, as illustrated by two tandemly arranged CAN SINE insertions and their obvious integration adjacent to an MIR (Figs. 1A, 2), and led us to speculate that the insertion mode of SINEs may be more complicated and diverse than previously recognized. Taken together, we are convinced that more interesting evolutionary features of the two intronic sequences will be discovered when more mammalian taxa are studied.

Our independent research on this intron in various mammals has revealed a high sporadic incidence of SINE elements in β-fibrinogen intron 7 (Yu and Zhang, 2005b). The finding of *Mephitis mephitis* CAN SINEs here attests again to the widely distributed nature of SINE amplification in this intronic region, though the possible impact of these abundant insertions on mammalian evolution and speciation remains to be determined. In addition, we also isolated a new CAN SINE located within another intronic region of the same gene (i.e., within β-fibrinogen intron 4) from *Lutra lutra*. Up to now, CAN SINE elements in *Mephitis mephitis* and *Lutra lutra* have only been discovered by analyzing the first intron of the transthyretin gene (Genbank Accession Nos. AF306948 and AF039734; Zehr et al., 2001; Vassetzky and Kramerov, 2002). We are the second group to detect new CAN SINEs present in other intron sequences of these two species. However, unlike the CAN SINEs reported here, the previously discovered CAN SINEs were produced by an orthologous insertion before the divergence of Caniformia. Sequence analysis showed the average sequence divergence between our new CAN SINEs and those previously discovered is 31.7% (Sku7-ins-1) and 30.7% (Sku7-ins-2) for *Mephitis mephitis* and 32.1% for *Lutra lutra*.

The MIR insertion in the orthologous locus of β-fibrinogen intron 7 allowed us to examine the potential phylogenetic signals harbored in MIR sequences. Zehr et al. (2001) made the first attempt to explore whether nucleotide sequence characters within SINEs contain useful information for the estimation of organismal phylogeny; they analyzed orthologous CAN SINEs from a phylogenetic point of view and compared the phylogenetic results with those based on the transthyretin intron 1 sequence in which the CAN SINE is located. Their study showed that the tree topology based on CAN SINE sequences was significantly different from that based on transthyretin intron 1, as a result of homoplasy introduced by an increased substitution rate for the CAN SINEs. Although the MIR elements in our case did not show a significant increase in substitution rate over the non-MIR sequences in β-fibrinogen intron 7 (data not shown), their analysis recovered a poor tree, in terms of both tree topology and nodal supports, compared to the non-MIR and whole FGB7 trees, especially in the case of the basal clades (Fig. 4A). It may be inferred that nucleotide sequence data from SINEs should be used with caution in phylogeny reconstruction, because phylogenetic hypotheses derived from their analyses are shown to be rather less sensitive and reliable, possibly due to short sequence length, limited informative content, or aberrant evolutionary patterns. Of course, more investigation with respect to this issue will be needed.

Our study not only explored the mode of evolution in the SINE elements isolated from the two new intronic sequences, but also provided several insightful results for mustelid phylogeny by separate and simultaneous analyses of the two new intronic sequences and two other nuclear and one mt genes previously reported, although the resulting tree topologies were not identical for different tree-building methods and gene partitions. In all analyses, the assemblage of Mustelinae, Lutrinae, and Melinae (i.e, non-mephitine Mustelidae) was recovered with confidence (BS≥99% and PP=1.00); moreover, two clades within Mustelinae were clearly recognized, genera *Mustela* and *Martes* (BS≥99% and PP=1.00). Notably, *Mustela* was found to consistently group with Lutrinae to the exclusion of *Martes* (BS>85% and PP>0.95), indicating the paraphyly of Mustelinae. The sister-grouping of *Mustela* with Mustelinae and Lutrinae recovered here was in accordance with the results of previous studies based on analyses of other mito-

chondrial and nuclear gene combinations (Sato et al., 2003, 2004, 2006; Koepfli and Wayne, 2003; Marmi et al., 2004; Delisle and Strobeck, 2005; Flynn et al., 2005; Fulton and Strobeck, 2006; Yonezawa et al., 2007). The traditional view from fossil evidence (Wolsan, 1999) and from morphological data (Hunt, 1974; Wozencraft, 1989; Wyss and Flynn, 1993; Bryant et al., 1993), as well as from mustelid supertree construction (Bininda-Emonds et al., 1999), in which Lutrinae was closer to Mephitinae, was not supported in this study. The monophyly of the subfamilies in Mustelidae has also been a subject of controversies arising from previous investigations. Among them, Mustelinae, the largest and most diverse subfamily in Mustelidae, has been gradually accepted to be a paraphyletic group (Bryant et al., 1993; Dragoo and Honeycutt, 1997; Koepfli and Wayne, 1998, 2003; Hosoda et al., 2000; Sato et al., 2003, 2004, 2006; Marmi et al., 2004; Delisle and Strobeck, 2005; Flynn et al., 2005; Fulton and Strobeck, 2006; Yonezawa et al., 2007), although there are still diverging views from molecular and morphological data (Bininda-Emonds et al., 1999). Our analyses confirmed non-monophyletic origin of this subfamily, as evidenced by its inclusion of Lutrinae.

Our analyses in this paper provide evidence that Melinae is more closely associated with Lutrinae/*Mustela* than is *Martes* (Figs. 3A, 5B). This relationship received especially good support from the β-fibrinogen intron 7 tree (BS≥90% and PP =1.00), a result that has not been obtained in previous analyses. Three synapomorphic substitutions were found to support this relationship in our FGB7 data set. The alternative hypotheses proposed in previous studies, including a closer affinity of *Martes* to the Lutrinae/*Mustela* clade inferred from the analysis of mitochondrial genes (Marmi et al., 2004; Delisle and Strobeck, 2005), nuclear genes (Sato et al., 2006; Fulton and Strobeck, 2006), and combined mitochondrial and nuclear genes (Sato et al., 2003; Yonezawa et al., 2007), and the possible sister status of Melinae and *Martes* inferred from the NJ analysis of Cyt B (Sato et al., 2003) and combined analyses of the five nuclear sequence data sets (Koepfli and Wayne, 2003), were not recovered by any of the present analysis. Evidence from additional genes is necessary to test the current phylogenetic hypothesis, considering that the ML and pBI inferences from the concatenated data set failed to convincingly retain this relationship (ML BS=53% and PP=0.89).

Our analyses unanimously support the rather distant association of Mephitinae to the rest of mustelids, an observation consistent with the increasing molecular evidence that Mephitinae should be elevated to a separate family (Vrana et al., 1994; Ledje and Arnason, 1996a, b; Dragoo and Honeycutt, 1997; Flynn et al., 2000; Marmi et al., 2004; Delisel and Strobeck, 2005; Flynn et al., 2005; Sato et al., 2006; Fulton and Strobeck, 2006), although its precise position in Caniformia remains unclear in our study. An earlier proposal, based on morphological evidence, that Mephitinae belongs in Mustelidae and shows closer affinity with Melinae (Simpson, 1945) or Lutrinae (Wozencraft, 1989; Wyss and Flynn, 1993; Bryant et al., 1993; Wolsan, 1999), was not recovered in any of our analyses. We found Mephitinae to diverge before the other mustelids and the monophyletic Procyonidae in all cases; however, it was placed as the sister taxon either to Ailuridae in the β-fibrinogen intron 4 (Fig. 3B) and concatenated MP trees (Fig. 5A) or to the clade containing Ailuridae and Procyonidae/non-mephitine Mustelidae in the concatenated ML/pBI trees (Fig. 5B). In recent nucleotide sequence studies of Caniformia, the interrelationship among Mephitinae, Procyonidae/non-mephitine Mustelidae, and Ailuridae has been an unresolved trichotomy. There are three resultant hypotheses: (1) Mephitinae is more closely related to Ailuridae than to Procyonidae/non-mephitine Mustelidae (Flynn et al., 2000; Delisle and Strobeck, 2005; Sato et al., 2006); (2) Mephitinae is more closely related to Procyonidae/non-mephitine Mustelidae than to Ailuridae (Marmi et al., 2004; Ledje and Arnason, 1996b; Flynn et al., 2005); and (3) Ailuridae and Mephitinae are successively more distant outgroups to Procyonidae/non-mephitine Mustelidae (Fulton and Strobeck, 2006; Sato et al., 2006). Both hypotheses (1) and (3) are supported in our analyses, although hypotheses (1) received more robust support from the concatenated MP tree (MP BS=97%). Therefore, more data are needed to test these alternative phylogenetic hypotheses in relation to Mephitinae.

Although neither intron from the β-fibrinogen gene alone has resolved all of the phylogenetic controversy concerning Mustelidae, our results indicate that these two nuclear regions are potentially serviceable in resolving some relationships among mustelids. The β-fibrinogen intron 7 region provides good resolution in the non-mephitine mustelid part of the Caniformia tree, while β-fibrinogen intron 4 contains information informative for the placement of Mephitinae, albeit with less robust support. A recent similar work on higher-level caniform phylogeny has revealed that these two introns of the β-fibrinogen gene are informative for carnivore relationships at the genus level or above (Yu and Zhang, 2006). Surprisingly, our study here, which included additional taxa of Mustelidae, finds that the relationships estimated among caniform families are rather limited, a result inconsistent with the conclusion drawn by Yu and Zhang (2006). This inconsistency may have been caused by the use of different outgroups (Canidae in Caniformia vs Felidae in Feliformia) or by the unbalanced sampling of representatives of caniform families (more mustelid taxa here compared to their study). We thus also selected two feliform species, *Panthera leo* and *Felis catus* (Accession nos. AY634374, AY634379, AY882043, and AY882044), which were used as outgroup taxa in Yu and Zhang (2006), for rooting in the β-fibrinogen intron 4 and 7 data sets. Phylogenetic analyses of 23 caniform carnivores using available felid species as outgroups gave a tree topology identical to that obtained using Canidae as the outgroup. Therefore, it seems that the inclusion of more mustelid taxa here likely explains the inconsistency. On the other hand, it is also important to note that although the combined data set containing four nuclear and one mitochondrial genes apparently provided phylogenetic signals useful for reconstructing the higher-level phylogeny of Caniformia, no significant improvement in the resolution of Mustelidae relationships was obtained compared with the individual analyses of the two new introns, possibly due to heterogeneous rates of evolution and levels of homoplasy between the gene partitions. Taken together, additional genetic markers are in urgent need to resolve the problematic regions that caused topological instability in the mustelid phylogeny in the present study. The supplementary

data for this article can be found online at “http://dx.doi.org/10.2108/zsj.25.662.s1”.

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