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Phylogeny of the caniform carnivora: evidence from multiple genes

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Abstract

The monophyletic group Caniformia in the order Carnivora currently comprises seven families whose relationships remain contentious. The phylogenetic positions of the two panda species within the Caniformia have also been evolutionary puzzles over the past decades, especially for *Ailurus fulgens* (the red panda). Here, new nuclear sequences from two introns of the β-fibrinogen gene (β-fibrinogen introns 4 and 7) and a complete mitochondrial (mt) gene (ND2) from 17 caniform representatives were explored for their utilities in resolving higher-level relationships in the Caniformia. In addition, two previously available nuclear (IRBP exon 1 and TTR intron 1) data sets were also combined and analyzed simultaneously with the newly obtained sequence data in this study. Combined analyses of four nuclear and one mt genes (4417 bp) recover a branching order in which almost all nodes were strongly supported. The present analyses provide evidence in favor of *Ailurus fulgens* as the closest taxon to the procyonid-mustelid (i.e., Musteloidea *sensu stricto*) clade, followed by pinnipeds (i.e., Otariidae and Phocidae), Ursidae (including *Ailuropoda melanoleuca*), and Canidae, the most basal lineage in the Caniformia. The potential utilities of different genes in the context of caniform phylogeny were also evaluated, with special attention to the previously unexplored β-fibrinogen intron 4 and 7 genes.

Introduction

The mammalian order Carnivora comprises 11 families and is traditionally grouped into two monophyletic assemblages, Caniformia (including family Canidae, Ursidae, Procyonidae, Mustelidae, Phocidae, Odobenidae, and Otariidae) and Feliformia (including family Viverridae, Herpestidae, Hyaenidae, and Felidae) (Eisenberg, 1989; Wozencraft, 1989; Wyss & Flynn, 1993). Phylogenetic relationships among the diverse families of living carnivorans have been hotly disputed in pioneer studies and are not well established yet. There are still many uncertainties about the interfamilial affinities within the Caniformia. Although there is a general consensus for the earliest divergence of the family Canidae (Wyss & Flynn, 1993; Vrana et al., 1994; Flynn & Nedbal, 1998), conflicting phylogenetic hypotheses exist for other caniform lineages that subsequently evolved. In addition, the systematic positions of the two pandas within the Caniformia have also been evolutionary puzzles over the past decades. While growing evidence supports the giant panda, *Ailuropoda melanoleuca*, as the most basal offshoot of the family Ursidae, the precise relationship of the red panda, *Ailurus fulgens*, to other caniform carnivores remains ambiguous (Zhang & Ryder, 1993; Vrana et al., 1994; Slattery & O'Brien, 1995; Flynn et al., 2000; Yu et al., 2004b).

Higher-level relationships within the Caniformia have already been investigated on morphological and molecular grounds. In these studies, most of phylogenetic information comes from mitochondrial (mt) DNA sequence data (Vrana et al., 1994; Ledje & Arnason, 1996; Dragoo & Honeycutt, 1997). Flynn and Nedbal (1998) first derived a higher-level carnivoran phylogeny based on an intron sequence of the nuclear transthyretin gene (TTR), and in Flynn et al.'s, (2000) study, the same intron, in concert with three mt genes, was utilized to resolve the placement of *Ailurus fulgens* within the Caniformia. Most recently, an analysis by us (Yu et al., 2004b) has also been presented by combining two nuclear genes, interphotoreceptor retinoid-binding protein (IRBP) and TTR genes, to further clarify the phylogeny of this group. Nuclear genes represent independent linkage loci of those genes from the mt genome, with a lower substitution rate and less subject to homoplasy. Despite the usefulness of the sequences from the nuclear genome, however, few of them other than IRBP and TTR genes have been available as phylogenetic characters to elucidate relationships among the caniform families so far.

In this study, we demonstrate the utility of two new nuclear sequences, the fourth and seventh introns of the fibrinogen gene (FGB; β-fibrinogen introns 4 and 7) from a diversity of caniform representatives. The β-fibrinogen intron 7 has been used at different taxonomic levels on birds (Prychitko & Moore, 1997) and reptiles (Creer, Malhotra & Thorpe, 2003), as well as on closely related cats (Yu & Zhang, 2005a), while the β-fibrinogen intron 4 has just been explored in avian phylogeny (Barker, 2004), but its use in mammalian groups has not been reported to date. Besides new nuclear DNA data, a mt protein-coding gene, NADH dehydrogenase subunit 2 (ND2), is also sequenced here as an unlinked locus. A comprehensive data set from 17 caniform carnivores and two feliform outgroups was thus generated by combining these new nucleotide sequences with our previous IRBP and TTR gene data to conduct analyses. Our objectives were to: (1) provide new insights into the relationships among the caniform families and the phylogenetic positions of the two pandas, and (2) examine the utilities and evolutionary dynamics of these genes in the context of caniform phylogeny, with special attention to the previously unexplored β-fibrinogen intron 4 and 7 genes.

Materials and methods

DNA samples and PCR amplifications

Seventeen caniform species representing six families of Caniformia (Canidae, Ursidae, Procyonidae, Mustelidae, Otariidae, and Phocidae) and two feliform outgroups were examined in this study as listed in Table 1. In addition, two panda species were also included. Unfortunately, of the three aquatic families known as pinnipeds, Odobenidae was not included here because samples were unavailable. For each sample, total genomic DNA was isolated from blood or frozen tissues using standard proteinase K, phenol/chloroform extraction (Sambrook, Fritsch & Maniatis, 1989).

The new nuclear and mt segments were amplified as follows. Target gene segments corresponding to the β-fibrinogen intron 7 gene (≈650 bp) and mt ND2 gene (=1044 bp) were amplified by PCR using previously reported primers (FGB-FelF/FGB-FelR primer set for β-fibrinogen intron 7, and ND2-FelF/ND2-FelR primer set for ND2; Yu & Zhang, 2005a). Primers FGB4-CarF and FGB-CarR used to amplify β-fibrinogen intron 4 gene (≈580 bp) were designed from conserved regions in flanking exons, based on comparisons of β-fibrinogen sequences from birds (Barker, 2004) and from available human and rat genomes. Additional internal primers for obtaining these three genes were also developed (Table 2). The optimal conditions adopted in PCRs were: 95°C initial hot start for 5 min, 35 cycles of 94°C denaturation for 1 min, 50°C (for β-fibrinogen intron 4 and 7 genes) or 56°C (for ND2 gene) annealing for 1 min and 72°C extension for 1 min, followed by a final 72 extension for 10 min. In all cases, three target segments were successfully amplified from each template. Forty-one out of 51 ingroup sequences were newly produced in this study (Table 1), as well as two feliform β-fibrinogen intron 4 sequences for outgroup representatives, *Felis catus and Panthera leo*. Some other sequences for these segments were retrieved from GenBank (Table 1).

Sequencing and data analysis

The amplified PCR products were purified and sequenced in both directions with an ABI PRISM™ 3700 DNA sequencer. Acquired

Table 1. List of taxonomic samples and sequences used in this study

Families	Taxon[a]			Newly established datasets			Previously available datasets	
	Scientific name	Common name	Sample source	Nuclear genes		Mt genes	Nuclear genes	
				FGB intron 4	FGB intron 7	ND2	IRBP	TTR
Canidae	*Canis lupus*	Gray wolf	China	AY882026 (this study)	AY726652 (Yu & Zhang, 2005b)	AY170044 (Yoder et al., 2003)	AY525044 (Yu et al., 2004b)	AF039732 (Flynn & Nedbal, 1998)
	Canis rufus	Red wolf	USA	AY882027 (this study)	AY882045 (this study)	AY882058 (this study)		
	Vulpes velox[b]	Swift fox		AF179293 (Springer et al., 2001)				
	Vulpes vulpes[b]	Red fox			AF039733 (Flynn & Nedbal, 1998)			
Procyonidae (Musteloidea sensu stricto clade)	*Procyon lotor*	Raccoon	San Diego Zoo, USA	AY882028 (this study)	AY726653 (Yu & Zhang, 2005b)	AY170046 (Yoder et al., 2003)	AY525029 (Yu et al., 2004b)	AF039736 (Flynn & Nedbal, 1998)
	Potos flavus	Kinkajou	San Diego Zoo, USA	AY882029 (this study)	AY882046 (this study)	AY882059 (this study)	AY525030 (Yu et al., 2004b)	AF039737 (Flynn & Nedbal, 1998)
	Nasua nasua	Coatimundi	San Diego Zoo, USA	AY882030 (this study)	AY882047 (this study)	AY882060 (this study)	AY525031 (Yu et al., 2004b)	AY525054 (Yu et al., 2004b)
Mustelidae (Musteloidea sensustricto clade)	*Martes flavigula*	Marten	Kunming Zoo, China	AY882031 (this study)	AY726654 (Yu & Zhang, 2005b)	AY882061 (this study)	AY525048 (Yu et al., 2004b)	AY525050 (Yu et al., 2004b)
	Martes zibellina	Sable	Haerbin Zoo, China	AY882032 (this study)	AY882048 (this study)	AY882062 (this study)	AY525047 (Yu et al., 2004b)	AY525051 (Yu et al., 2004b)
	Mustela kathia[b]	Yellow-bellied weasel	Yunnan Province, China	AY882033 (this study)	AY882049 (this study)	AY882063 (this study)	AY525046 (Yu et al., 2004b)	

Table 1. continued.

Families	Taxon[a]			Newly established datasets			Previously available datasets	
	Scientific name	Common name	Sample source	Nuclear genes		Mt genes	Nuclear genes	
				FGB intron 4	FGB intron 7	ND2	IRBP	TTR
	Mustela frenata[b]	Long-tailed weasel						AY039735 (Flynn & Nedbal, 1998)
	Arctonyx collaris	Hog badger	Yunnan Province, China	AY882034 (this study)	AY882050 (this study)	AY882064 (this study)	AY525049 (Yu et al., 2004b)	AY525053 (Yu et al., 2004b)
Ursidae	*Ursus arctos*	Brown bear	Heilongjiang Province, China	AY882035 (this study)	AY726655 (Yu & Zhang, 2005b)	AF303110 (Delisle & Strobeck, 2002)	AY303842 (Yu et al., 2004a)	AF039741 (Yu et al., 2004a)
	Ursus thibetanus	Asiatic black bear	Yunnan Province, China	AY882036 (this study)	AY882051 (this study)	AY882065 (this study)	AY303841 (Yu et al., 2004a)	AY303847 (Yu et al., 2004a)
	Tremarctos ornatus	Spectacled bear	San Diego Zoo, USA	AY882037 (this study)	AY882052 (this study)	AY170045 (Yoder et al., 2003)	AY303840 (Yu et al., 2004a)	AF039740 (Yu et al., 2004a)
Two pandas	*Ailuropoda melanoleuca*	Giant panda	Sichuan Province, China	AY882038 (this study)	AY882053 (this study)	AY882066 (this study)	AY303846 (Yu et al., 2004a)	AF039738 (Yu et al., 2004a)
	Ailurus fulgens	Red panda	Yunnan Province, China	AY882039 (this study)	AY882054 (this study)	AY882067 (this study)	AY525045 (Yu et al., 2004b)	AF039739 (Yu et al., 2004a)
Otariidae (pinnipeds)	*Otaria byronia*	Sea lion	USA	AY882040 (this study)	AY882055 (this study)	AJ428578 (Arnason et al., 2002)	AY525043 (Yu et al., 2004b)	AY039745 (Flynn & Nedbal, 1998)
	Callorhinus ursinus	Fur seal	Northern Pacific Ocean	AY882041 (this study)	AY882056 (this study)	AY882068 (this study)		
Phocidae (pinnipeds)	*Erignathus barbatus*	Bearded seal	China	AY882042 (this study)	AY882057 (this study)	AY170047 (Yoder etal., 2003)	AY170077 (Yoder et al., 2003)	AY039742 (Flynn & Nedbal, 1998)
Felidae (outgroup)	*Panthera leo*	Lion	Kunming Zoo, China	AY882043 (this study)	AY634374 (Yu & Zhang, 2005a)	AY170034 (Yoder et al., 2003)	AY525036 (Yu et al., 2004b)	AF039725 (Flynn & Nedbal, 1998)
	Felis catus	Domestic cat	Guangxi Province, China	AY882044 (this study)	AY634379 (Yu & Zhang, 2005a)	NC_001700	Z11811 (Stanhope et al., 1992)	AY525058 (Yu et al., 2004b)

[a]Taxonomic denomination followed classification of Nowak (1999).
[b]Taxa belonging to different species of the same genus, instead of the same specimen, were sequenced for target genes in previous studies, also see Yu et al. (2004b).

Table 2. List of PCR primers for amplification and sequencing of two nuclear introns of β-fibrinogen gene and mtND2 gene

Target gene	Primer name		Sequence (5′–3′)
β-fibrinogen intron 4	External primers	FGB4-CarF	TTGCAATATTCCTGTGGTGTCTGG
		FGB4-CarR	ATTTCAGATGTTTCACCTCCTTTCCT
β-fibrinogen intron 7	External primers	FGB-FelF	CACAACGGCATGTTCTTCAGCACG
		FGB-FelR	TACCACCATCCACCACCATCTTCTT
	Internal primers	FGB7-CarF	CTAAACCATTTCTGCTATAA
		FGB7-CarR	AGAACCCTGGAAAACGAAAT
mtND2	External primers	ND2-FelF	CCATACCCCGAAAATGTTGGTTTAT
		ND2-FelR	AGCTTTGAAGGCTCTTGGTCT
	Internal primers	ND2-CarF	CAAACACAACTACGAAAAATC
		ND2-CarR	GAGTATGCTAGGATTTTTCGT

sequences were then submitted to GenBank for BLAST searching (Altschul et al., 1997) to ensure the data validity.

The two nuclear FGB introns and mtND2 sequences were initially aligned separately with CLUSTAL X program (Thompson et al., 1997) and thereafter refined by eye. ND2 alignments were straightforward, while those of β-fibrinogen introns 4 and 7 exhibited sequence length variation due to multiple insertions/deletions. Notably, a species-specific insertion of the CAN SINE family (about 239 bp) in two Canidae taxa (*Canis lupus* and *Canis rufus*) was observed in the β-fibrinogen intron 7 alignment, one of which (CAN SINE in *Canis lupus*) has been reported recently by us elsewhere (Yu & Zhang, 2005b). The SINE regions were deleted prior to phylogenetic analysis, leaving 655 nucleotides in the final β-fibrinogen intron 7 alignment. Program MEGA (Kumar et al., 2001) was used to conduct sequence analyses of these three new genes. We also plot the number of transitions and transversions against Kimura 2-parameter distance to measure saturation patterns for the rapidly evolving ND2 gene using the software DAMBE (Xia, 2000).

Phylogenetic trees were reconstructed using PAUP*4.0b8 (Swofford, 2001) for maximum parsimony (MP) and maximum likelihood (ML) analyses, and MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003) for Bayesian analysis. In MP analyses, a branch-and-bound search was performed with tree-bisection-reconnection (TBR) branch swapping, random addition of taxa, and 1000 replicates per search. Only one of the best trees found during branch swapping was saved (MULTREES = NO in PAUP*) and zero length branches were collapsed. However, for the ND2 gene, a heuristic search was performed due to the time constraints. Because the saturation effect in transition substitutions of the third codon positions was indicated (plot not shown), parsimony analysis was also conducted by excluding fast evolving transitions in the third codon positions of the ND2 data set. Program ModelTest 3.06 (Posada & Crandall, 1998) was used to choose the best model of evolution and parameters for both ML and Bayesian analyses. In Bayesian analysis, four Metropolis-coupled Markov chain Monte Carlo (MCMCMC) were run for 2×10^6 generations and trees were sampled every 100 generations. The tree number discarded as a 'burn-in' was determined by checking the stationarity of likelihood values.

Bootstrap resampling analysis (Felsenstein, 1985) was performed to evaluate the reliability of internal branches in MP and ML tree topologies (1000 replicates for MP and 100 replicates for ML), with bootstrap support values (BS) higher than 70 interpreted as well-supported (Hillis & Bull, 1993). Bayesian posterior probabilities (PP) from the 50% majority-rule consensus tree were calculated to provide the estimates of nodal support in Bayesian phylogenies, with probabilities ≥90% judged as significant support (Shaffer, Meylan & McKnight, 1997).

Combination with other available data sets

Available sequence data from two other nuclear genes, IRBP exon 1 (1280 bp in alignment) and TTR intron 1 (857 bp in alignment) (Yu et al., 2004a, b), for the same set of taxa sampled in this

study except *Callorhinus ursinus*, were also obtained from the GenBank database and combined with our new data sets for phylogenetic analyses. First, we combined the two nuclear data sets from the IRBP and TTR genes with the new nuclear sequences, β-fibrinogen introns 4 and 7, from this study and analyzed them simultaneously as the four-part combined data set (3373 bp). In addition, because the partition homogeneity test (Farris et al., 1994, 1995) indicated the homogeneity of phylogenetic signal between the four nuclear and one mtND2 data sets ($p = 0.250$), a data set comprising all available characters for 16 caniform carnivore species and two feliform outgroups (4417 bp) was also constructed for phylogenetic estimation to make up our five-part combined data set.

Phylogenetic reconstructions for four-part combined data set (IRBP exon 1, TTR intron 1, β-fibrinogen introns 4 and 7) and five-part combined data set (four nuclear and one mt sequences) were performed as described above for the separate analyses (MP, ML, and Bayesian algorithms). However, in MP analysis, a heuristic search was run due to the time constraint for combined data. Characters were treated as equal weights for the four-part combined data set, while two MP weighting schemes were applied to the five-part combined data set similar to the ND2 analysis alone (i.e., exclusion of third position transitions in the ND2 gene partition). Each gene partition in two kinds of combined datasets was allowed to evolve under different rates in Bayesian inference. In addition, we use Partitioned Bremer support analysis (PBS; Bremer, 1988, 1994) to assess the respective contribution of each gene partition to the total nodal Bremer support as implemented by TreeRot.v2 (Sorenson, 1999).

Results

Sequence analyses

For our two new nuclear data sets, of the 581 aligned β-fibrinogen intron 4 nucleotides, 237 (40.8%) variable and 156 (26.9%) parsimony-informative sites were identified, and of the 655 aligned β-fibrinogen intron 7 nucleotides, 318 (47.8%) variable and 218 (32.8%) parsimony-informative sites were identified. Kimura 2-parameter (K2P) distances within the ingroup taxa ranged from 0 to 14.5% for β-fibrinogen 4 (9.3% on average) and from 0 to 19.7% for β-fibrinogen 7 (11.2% on average). There was an excess of AT residues in both β-fibrinogen introns (61.9% for intron 4 and 60.7% for intron 7). The mean ratio of transition and transversion (ti/tv ratio) was 2.04 and 2.01 from the β-fibrinogen intron 4 and 7 regions, respectively.

For the new mtND2 gene, comparison of 1044 base pairs recognized 575 (55.1%) variable and 474 (45.4%) parsimony-informative sites. K2P distances ranged from 0 to 35.3% (26.8% on average) between ingroup taxa. Base composition was AC-rich biased (63.3%) and the mean ti/tv ratio was 2.27.

Table 3 shows sequence characteristics for these three new genes, as well as for our previously published IRBP and TTR genes. Comparisons of the five data sets revealed that mt sequences, with the largest proportions of variable and informative sites, have a higher rate of substitutions (2.67 times on average) than nuclear ones, although the differences were not as significant as expected. Among the four nuclear gene regions, TTR intron 1 shows the highest percentage of variable and informative sites, similar to those of ND2, while IRBP has the lowest in both sites. In addition, the estimations of sequence divergences are largest for TTR, followed by β-fibrinogen intron 7, β-fibrinogen intron 4, and IRBP.

Phylogenetic analyses of two new nuclear datasets

The MP phylogenetic tree derived from a separate analysis of the β-fibrinogen intron 4 data sets is shown as a cladogram in Figure 1. Groups that appeared in 50% or more of the trees were retained. Parsimony analysis resulted in one tree with a length of 312 (CI = 0.856; RC = 0.744). Most of the recovered nodes in the tree were resolved with ≥84% MP BS, except for relationships among three species within the Procyonidae (MP BS < 50%), and the placement of *Ailurus fulgens* as sister taxon to a clade containing Mustelidae and Procyonidae (Musteloidea *sensu stricto* clade) (52% MP BS), as well as the sister grouping of Ursidae (including *Ailuropoda melanoleuca*) to them (52% MP BS). ML (model = HKY + G) and Bayesian analyses of the β-fibrinogen intron 4 gene recovered a similar tree topology to the MP analysis but differed in the

Table 3. Characteristics for nuclear and mitochondrial sequences data used in this study

	Nuclear data sets				Mt data set
	FGB intron 4	FGB intron 7	IRBP exon	TTR intron	ND2
Aligned sites (bp)	589	665	1280	857	1044
A%	31	28.6	18	26.7	35.9
C%	17.1	20.1	32.6	22.7	27.4
G%	21	19.1	32.1	22.4	9.8
T%	30.9	32.1	17.3	28.2	26.9
Variable sites (%)	237(40.8%)	318(47.8%)	284(22.2%)	444(51.8%)	575 (55.1%)
Parsimony-informative sits (%)	156 (26.9%)	218(32.8%)	201(15.7%)	307(35.8%)	474 (45.4%)
Ti:Tv ratio	2.04	2.01	2.57	1.82	2.27
Mean TN distance (%) within ingroup	9.3 (0–14.5%)	11.2(0–19.7)	7.6 (0.3–13.9)	14.4 (0.4–24.4)	26.8 (0–35.3)
Proportion of invariable sites (I)	0	0	0.5322	0	0.376305
Gamma-shape parameter (α)	3.002263	5.734468	0.7669	2.164428	1.894504

association of Ursidae with pinnipeds, although this relationship was poorly supported (< 50% ML BS, 50% PP). In addition, there was an obvious discrepancy in the level of support for the node uniting *Ailurus fulgens* to the Musteloidea *sensu stricto* clade among these three analyses of β-fibrinogen intron 4 (see Table 4).

The MP tree derived from a separate analysis of the β-fibrinogen intron 7 data set is shown as a cladogram in Figure 2. Groups that appeared in

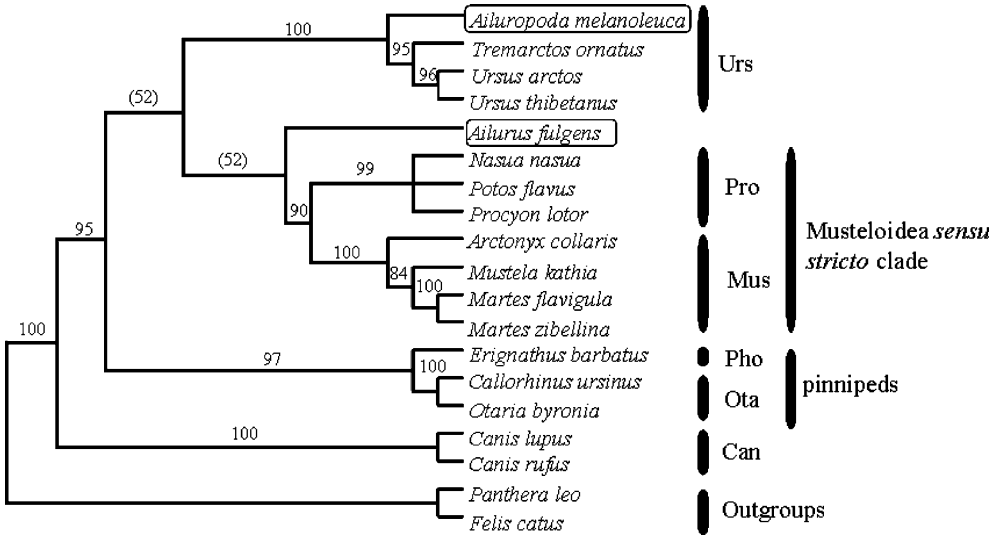

Figure 1. Cladogram resulting from parsimony analysis of nuclear β-fibrinogen intron 4 sequence data from 17 caniform carnivores and two feliform outgroups, with the two panda positions highlighted in boxes. Only the first three letters of the family names are shown. Numbers above the nodes are MP bootstrap support. ML and Bayesian analyses recovered overall similar tree topologies, but differed from the MP analysis in the placement of Ursidae as a sister group with the pinnipeds. Bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families under all three phylogenetic methods are summarized in Table 4.

50% or more of the trees were retained. Parsimony analysis resulted in one tree with a length of 434 (CI = 0.892, RC = 0.801). All nodes in the tree were resolved with ≥74% BS, except for the node connecting pinnipeds to the Musteloidea *sensu stricto* clade (54% MP BS). ML (model = TrN) and Bayesian analyses of this intron obtained identical tree topologies to the MP analysis, and the connection of pinnipeds and the Musteloidea *sensu stricto* clade was also poorly supported (< 50% ML BS, 77% PP). In addition, there was an apparent difference from the MP analysis in the level of support linking *Ailurus fulgens* to Ursidae (including *Ailuropoda melanoleuca*) (see Table 4).

The obtained tree topologies contrasted in several aspects, especially for the placement of *Ailurus fulgens*, pinnipeds and Ursidae, between the two new nuclear intron analyses, and even between the different tree-building methods in the β-fibrinogen intron 4 gene analyses. However, these differences were poorly supported in both two new nuclear intron analyses, except for two relationships: one is the sister grouping of *Ailurus fulgens* and the Musteloidea *sensu stricto* clade in the Bayesian analysis of the β-fibrinogen intron 4 gene (98% PP), and the other is the placement of *Ailurus fulgens* as the closest taxon to Ursidae in the parsimony analysis of the β-fibrinogen intron 7 gene (88% MP BS).

Phylogenetic analyses of the new mitochondrial data set

The equal-weight parsimony analysis of mtND2 gene resulted in one tree with a length of 2041 (CI = 0.462, RC = 0.217). The MP tree identified *Ailurus fulgens* as the closest taxon to pinnipeds, and they are the sister group of the Musteloidea *sensu stricto* clade. Canidae are placed at the basal position while Ursidae diverged subsequently. However, the close relatedness of *Ailurus fulgens* to pinnipeds and the sister grouping of them to the Musteloidea *sensu stricto* clade were both poorly supported.

Weighted parsimony analyses of the ND2 gene yielded one tree with a length of 1161 (CI = 0.488, RC = 0.280) that differed from the above tree by associating *Ailurus fulgens* with the Musteloidea *sensu stricto* clade. The weighted MP tree is shown as a cladogram in Figure 3. Groups that appeared in 50% or more of the trees were retained. Most of the nodes in the tree were resolved with ≥73% BS, except for the close relatedness of *Ailurus fulgens* to the Musteloidea *sensu stricto* clade (64% MP BS) and the sister grouping of pinnipeds with them (63% MP BS). ML (Model = TvM + I + G) and Bayesian analyses of ND2 data set obtained identical tree topologies to the weighted MP analysis, in which support for the placement of *Ailurus fulgens* was also weak, but support for pinnipeds and Ursidae was robust (see Table 4).

Compared to results of both new nuclear data sets, ND2 results imply different interfamilial relationships, suggesting that *Ailurus fulgens* was the closest taxon to the Musteloidea *sensu stricto* clade, followed by pinnipeds, Ursidae (including *Ailuropoda melanoleuca*), and Canidae. However, the placement of *Ailurus fulgens* in the mtND2 analysis was poorly supported under all algorithms, while those of pinnipeds and Ursidae were strongly supported in ML and Bayesian analyses.

Phylogenetic analyses of combined data sets

In the four-part combined data set analysis, one most parsimonious tree with a length of 1985 (CI = 0.799, RC = 0.654) was revealed (Figure 4). The resulting topology exhibited improved resolution and nodal support than either new nuclear gene region alone, especially regarding the association of *Ailurus fulgens* with the Musteloidea *sensu stricto* clade (100% MP BS), which was inconsistently resolved with low support in all separate new gene analyses. The close relationship between Ursidae (including *Ailuropoda melanoleuca*) and pinnipeds was identified here when four nuclear data sets were combined, which was only found in the separate analysis of the β-fibrinogen intron 4 data set with ML and Bayesian methods. However, this relationship was only weakly supported (56% MP BS), as with the β-fibrinogen intron 4 analysis. ML (model = K81 + G) and Bayesian estimations of the four-part combined data set produced different topologies from that of MP analysis. Interestingly, they showed identical results to the topologies derived from the ML, Bayesian, and weighted parsimony analyses of the mtND2 data set, in which pinnipeds were a sister group to the Musteloidea *sensu stricto* clade and *Ailurus fulgens*, albeit poorly supported (see Table 4). However, the ML and Bayesian trees

Table 4. Summary of bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families from the five data sets under three phylogenetic methods

FGB intron 4 analysis					
	(Pro, Mus)	(*Ailurus fulgens*, (Pro, Mus))	(Urs, (*Ailurus fulgens* , (Pro, Mus)))*	(Urs, Pinnipeds)*	basal position of Can
MP BS(%)	90	(52)	(52)		95
ML BS(%)	98	(58)		(< 50)	96
B PP (%)	100	98		(50)	99
FGB intron 7 analysis					
	(Pro, Mus)	(Pinnipeds, (Pro, Mus))	(*Ailurus fulgens* ,Urs)		basal position of Can
MP BS(%)	74	(54)	88		100
ML BS(%)	(66)	(< 50)	(64)		100
B PP (%)	93	(77)	(69)		100
mtND2 analysis					
	(Pro, Mus)	(*Ailurus fulgens*, (Pro, Mus))	(pinnipeds, (*Ailurus fulgens* ,(Pro, Mus)))		basal position of Can
Weighted MP BS(%)	86	(64)	(63)		98
ML BS(%)	97	(51)	98		100
B PP (%)	100	(73)	99		100
Four-part combined analysis					
	(Pro, Mus)	(*Ailurus fulgens*, (Pro, Mus))	(pinnipeds, (*Ailurus fulgens* ,(Pro, Mus)))*	(Urs, Pinnipeds)*	basal position of Can
MP BS(%)	99	100		(56)	100
ML BS(%)	100	100	(< 50)		100
B PP (%)	100	100	(58)		100
Five-part combined analysis					
	(Pro, Mus)	(*Ailurus fulgens*, (Pro, Mus))	(pinnipeds, (*Ailurus fulgens* ,(Pro, Mus)))		basal position of Can
Weighted MP BS(%)	100	100	(59)		100
ML BS(%)	100	100	83		100
B PP (%)	100	100	93		100

Note: *Different relationships obtained under three phylogenetic methods of a gene analysis.
Absence of a value = relationship not found; Pro = Procyonidae; Mus = Mustelidae; Urs = Ursidae; Can = Canidae; (number) = relationship not well-supported.

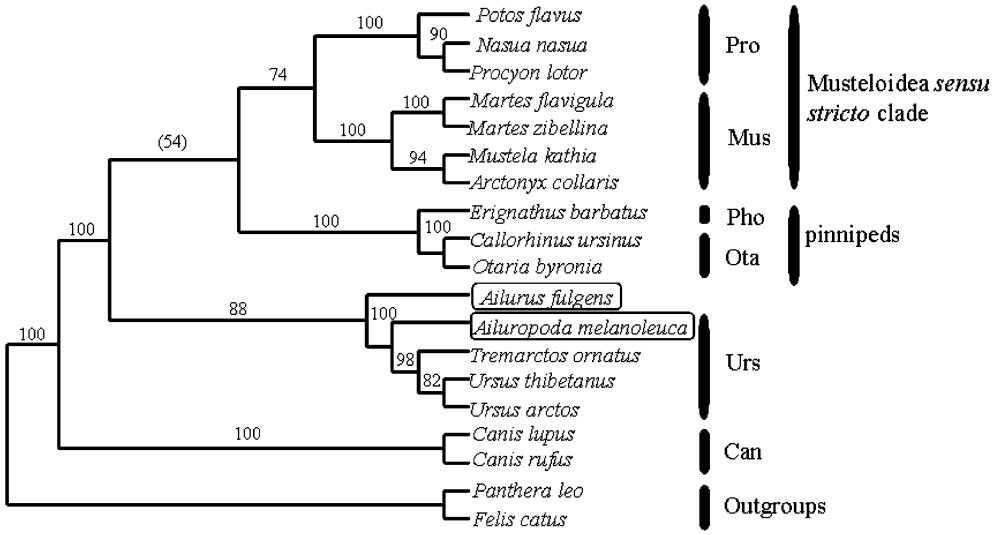

Figure 2. Cladogram resulting from parsimony analyses of nuclear β-fibrinogen intron 7 sequence data from 17 caniform carnivores and two feliform outgroups, with the two panda positions highlighted in boxes. Only the first three letters of the family names are shown. Numbers above the nodes are MP bootstrap support. ML and Bayesian analyses recovered identical tree topologies to the MP analysis. Bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families under all three phylogenetic methods are summarized in Table 4.

from the four-part combined data set and those from the mtND2 dataset differed in two ways: one is the levels of nodal support for the affinity of *Ailurus fulgens* with the Musteloidea *sensu stricto* clade (100% ML BS and PP in the four-part combined data set tree, 51% ML BS and 73% PP in mtND2 tree), and the other is for the position of pinnipeds as sister group to them (< 50% ML BS

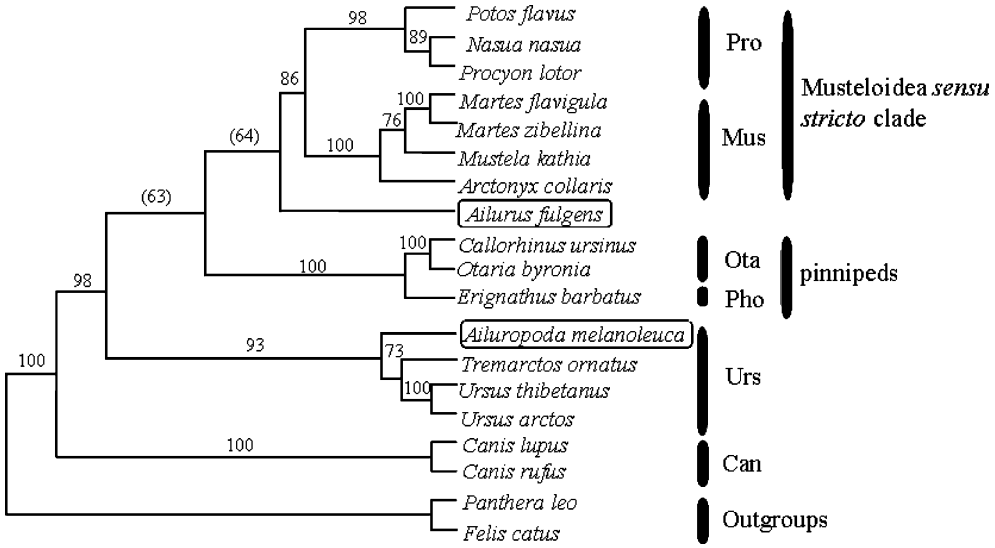

Figure 3. Cladogram resulting from weighted parsimony analysis of mtND2 sequence data from 17 caniform carnivores and two feliform outgroups, with the two panda positions highlighted in boxes. Only the first three letters of the family names are shown. Numbers above the nodes are MP bootstrap support. ML and Bayesian analyses recovered identical tree topologies to the weighted MP analysis. Bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families under all three phylogenetic methods are summarized in Table 4.

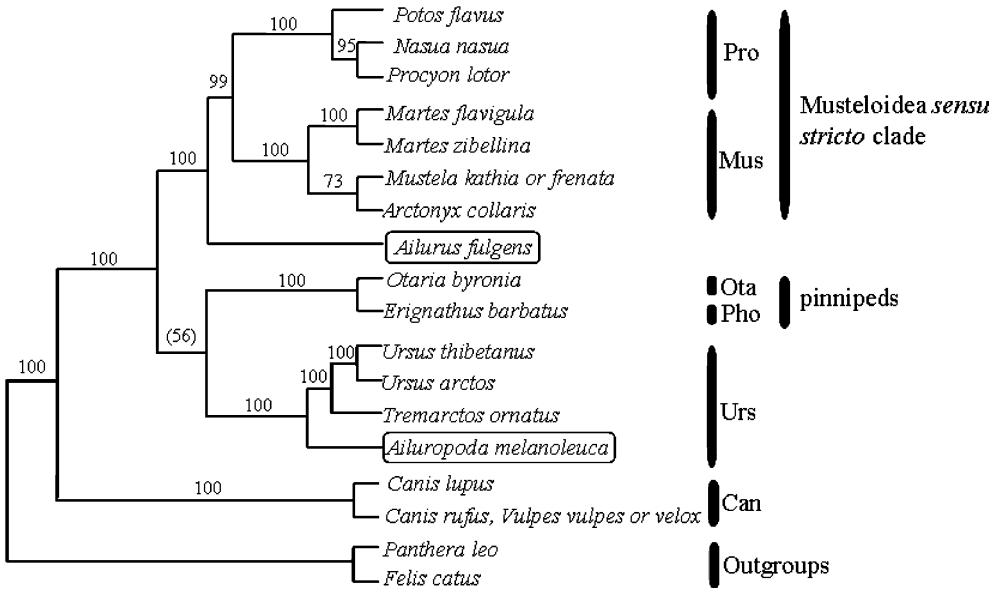

Figure 4. Cladogram resulting from parsimony analyses of four-part combined nuclear analysis (IRBP, TTR, β-fibrinogen intron 4 and 7 sequence data) from 16 caniform carnivores and two feliform outgroups, with the two panda positions highlighted in boxes. Only the first three letters of the family names are shown. Numbers above the nodes are MP bootstrap support. ML and Bayesian analyses recovered overall similar tree topologies, but differed from the MP analysis in the placement of Ursidae and pinnipeds diverging in succession after Canidae. Bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families under all three phylogenetic methods are summarized in Table 4.

and 58% PP in the four-part combined data set tree, 98% ML BS and 99% PP in mtND2 tree, see Table 4).

Equal-weight parsimony analysis of the five-part combined data set recovered two trees of equal length (3961 steps, CI = 0.634 and RC = 0.409). The two MP trees both identified the basal position of Canidae and the sister-grouping of *Ailurus fulgens* with the Musteloidea *sensu stricto* clade, but they differed in the placement of Ursidae (including *Ailuropoda melanoleuca*) and pinnipeds. One tree associates Ursidae and pinnipeds as a sister pair, in agreement with the MP analysis of the four-part combined data set and the β-fibrinogen intron 4 ML and Bayesian analyses. The other MP tree suggests, however, that they diverged in succession respectively after Canidae, identical to the phylogeny revealed by ML and Bayesian analysis of the four-part combined data set and the ND2 gene analyses alone.

Weighted MP analysis (tree length = 3110, CI = 0.687 and RC = 0.496) based on all five genes combined produced a tree topologically the same as one of the equal-weight trees, in which Ursidae and pinnipeds diverged in succession after Canidae (59% MP BS). The weighted MP tree is shown as a cladogram in Figure 5. Groups that appeared in 50% or more of the trees were retained. ML (model = GTR + I + G) and Bayesian analyses of the five-part combined data set produced identical interfamilial relationships to weighted MP analysis.

Compared with the individual and four-part combined trees, pooling all five genes demonstrated a clear increase in the level of support for almost all nodes. Although the placement of pinnipeds as the nearest lineage of *Ailurus fulgens* and the Musteloidea *sensu stricto* clade was not strongly supported in weighted parsimony analysis, this relationship was robustly resolved in both ML and Bayesian analyses (83% ML BS, 93% PP, Table 4).

The analyses of all five genes combined (Figure 5) represent the best estimate of caniform phylogeny in this study, with almost all nodes resolved and well-supported. The PBS analysis (data not shown) indicated that TTR gene has the highest contribution to the five-part combined tree resolution (26.15%), followed by mtND2 gene (25.25%). IRBP gene has the same contributions as the β-fibrinogen intron 7 gene (18.06%), while β-fibrinogen intron 4 gene contributed least

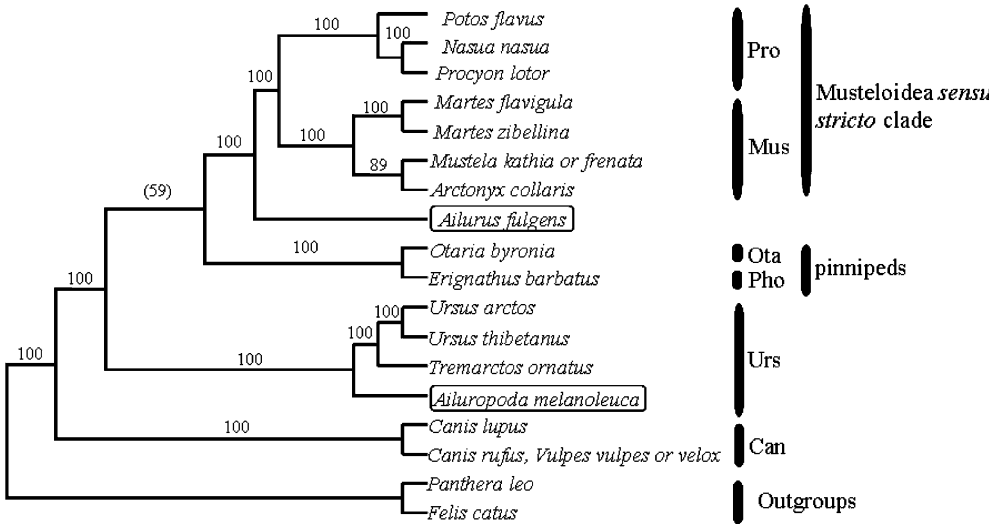

Figure 5. Cladogram resulting from weighted parsimony analyses of all five genes combined (IRBP, TTR, β-fibrinogen introns 4 and 7 as well as mtND2 sequence data) from 16 caniform carnivores and two feliform outgroups, with the two panda positions highlighted in boxes. Only the first three letters of the family names are shown. Numbers above the nodes are MP bootstrap support. ML and Bayesian analyses recovered identical tree topologies to the weighted MP analysis. Bootstrap support (BS) and posterior probability (PP) for the nodes connecting caniform families under all three phylogenetic methods are summarized in Table 4.

(12.48%). Here, we tentatively propose that in Caniformia, *Ailurus fulgens* is closely related to the Musteloidea *sensu stricto* clade, and pinnipeds are the sister lineage to them. Ursidae (including *Ailuropoda melanoleuca*) diverged prior to the pinnipeds while Canidae was the most basal lineage.

Discussion

Phylogenetic resolutions of the two pandas

All gene sequences in this study, analyzed individually or in combination, strongly favored the prevailing view that *Ailuropoda melanoleuca* was the most basal member within family Ursidae. In sharp contrast, the phylogenetic position of *Ailurus fulgens* varied widely with the gene regions and analytic methods used. In total, our analyses suggested two hypothetical relationships of *Ailurus fulgens* with other caniform carnivores: β-fibrinogen intron 7 gene associates *Ailurus fulgens* with Ursidae, a result which has also been recognized by previous morphological (Wozencraft, 1989) and mt cytochrome *b* (cytb) studies (Ledje & Arnason, 1996). However, although our parsimony analysis of β-fibrinogen intron 7 provides good support for it, both ML and Bayesian inferences failed to convincingly retain this association. Alternatively, mtND2, β-fibrinogen intron 4 gene, and the two combined data sets show affinity of *Ailurus fulgens* and the Musteloidea *sensu stricto* clade as a sister pair, which was especially robust in the Bayesian analysis of the β-fibrinogen intron 4 gene and the two combined data sets analyses.

Though analyses of single gene regions can not definitely resolve the placement of *Ailurus fulgens* within Caniformia, the two combined data sets provided more robust evidence for relating *Ailurus fulgens* to the Musteloidea *sensu stricto* clade, which has only been observed in recent reports (Flynn & Nedbal, 1998; Flynn et al., 2000; Yu et al., 2004b). Earlier proposals of *Ailurus fulgens* as a member within the family Procyonidae (Zhang & Ryder, 1993; Slattery & O'Brien, 1995) or as a sister taxon of Ursidae plus the pinnipeds (Wyss & Flynn, 1993; Vrana et al., 1994) were not recovered in any of our analyses.

Interfamilial relationships within caniformia

In all the individual and combined analyses, the family Canidae separates first, corroborating the traditional view that this family was imbedded within Caniformia as the earliest diverging lineage. However, an overview of our phylogenetic results revealed apparent topological discrepancy for the phylogenetic relationships among the other

caniform families. Only the concatenated analyses of all the five gene regions provide fully resolved trees with compelling bootstrap support and posterior probability for all nodes under three reconstruction approaches, except for the parsimony analysis uniting pinnipeds and the Musteloidea *sensu stricto* clade with *Ailurus fulgens*. Analyses of separate gene regions and four nuclear genes combined, however, have reduced resolving power.

Our favored estimates of interfamilial phylogeny suggested that Canidae was the most basal followed by Ursidae, pinnipeds and a sister grouping of *Ailurus fulgens* with the Musteloidea *sensu stricto* clade. Our conclusions are in agreement with some other sequence-based studies by Flynn and Nedbal (1998), Flynn et al. (2000) and Yu et al. (2004b), as well as with that from Bininda-Emond, Gittleman and Purvis (1999), who built tree topologies through supertree consensus methods. An older opinion based on some morphological and mtDNA evidence favoring the association of Ursidae and pinnipeds (Wyss & Flynn, 1993; Vrana et al., 1994; Hunt & Barnes, 1994) was revealed but poorly supported in our ML and Bayesian analyses of the β-fibrinogen intron 4, parsimony analysis of the four-part combined data set, and one MP tree of the five-part combined data set analysis. In addition, more close relatedness of Ursidae to the Musteloidea *sensu stricto* clade and *Ailurus fulgens* than pinnipeds also received poor support here in the parsimony analysis of the β-fibrinogen intron 4 gene, which agree with one of Flynn and Nedbal (1998)'s analyses combining TTR, cytb, and morphological characters. The relationship between the pinnipeds and the family Mustelidae proposed earlier (Arnason &Widergren, 1986; Arnason & Ledje, 1993) was not recovered by any present analysis.

Analyses from combining four nuclear genes greatly increase support levels of recovered nodes, especially for *Ailurus fulgens*, which leaves only the placement of pinnipeds ambiguous. When mtND2 is added to the four nuclear genes, the analyses demonstrated an improved resolution of the affinity of pinnipeds with other caniform carnivores. Thus, the combined nuclear gene and the mtND2 gene seem to possess compensatory rather than conflicting phylogenetic signals. As a result, they contribute significant information to different parts of the five-part combined tree. Four nuclear gene sequences seem to be necessary for definitive resolution of placement of *Ailurus fulgens*, while the mtND2 gene is useful for lending support to pinnipeds' divergence. We find that this higher-level branching order of Caniformia favored by our study has been recovered in almost all sequence-based analyses since Flynn and Nedbal (1998)'s study (Flynn et al., 2000; Yu et al., 2004b), as well as in the supertree analysis from Bininda-Emonds, Gittleman and Purvis (1999). Therefore, our work, from independent evidence, corroborates other recently published phylogenies of Caniformia, based on combined analysis of multiple, newly explored genes.

Utility of different genes for caniform phylogenetics

The majority of our analyses of individual nuclear and mt genes yielded different tree topologies from each other and from the combined gene trees. It is important to note that in these individual analyses, the problematic regions causing topological instability are exactly where previous phylogenies are also contentious, but resolution was improved in this study when all characters were combined.

Our two new nuclear introns from the β-fibrinogen gene, especially in combined analysis were shown to be potentially serviceable in contributing to interfamilial phylogenetic reconstruction within Caniformia. The β-fibrinogen intron 7 gene has been well used in avian phylogenetic studies at all taxonomic levels. A recent work of low-level feline phylogeny in the order Carnivora (Yu & Zhang, 2005a) finds that the relationships estimated using the β-fibrinogen intron 7 gene are rather limited due to low sequence divergence over a short speciation time. However, our results from this study provide persuasive evidence that this intron sequence is more valuable to carnivoran relationships at the genus level or above. The other nuclear intron, β-fibrinogen intron 4 gene, has only shown its potential in recovering avian phylogeny recently at the generic level (Barker, 2004). Our analyses here, as its first application to the mammalian group, demonstrate that this gene region is a useful tool with which to construct an interfamilial phylogeny in Caniformia.

The ND2 gene from the mt genome contains more phylogenetically informative sites and evolves at a faster rate than either nuclear gene.

However, its utility in this study was somewhat discounted by the fact that the ND2 sequence was more homoplasious than the nuclear ones as a result of its fast substitution rate, as evidenced by lower CI and RC values and the obvious saturation in its third position transitions. Therefore, for the higher-level carniform phylogeny, the mtND2 gene alone does not present much better resolving power than the two introns of the β-fibrinogen gene.

Although the combined analyses including four nuclear and one mt genes in our study recover a branching order with improved resolution and nodal support compared to separate data analyses, the relationship among Ursidae, pinnipeds, and the Musteloidea *sensu stricto* clade, was not strongly supported in the parsimony analysis of the five-part combined data set. Therefore, evidence from additional genes was still necessary to strengthen or oppose current estimate of higher-level relationships within the Caniformia.

Supplementary materials

The sequences reported in this paper have been deposited in GenBank database. Accession numbers: AY882026–AY882068

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