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Phylogenetic Utility of Nuclear Introns in Interfamilial Relationships of Caniformia (Order Carnivora)

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Abstract.—The monophyletic group Caniformia (dog-like carnivores) in the order Carnivora comprises 9 families. Except for the general consensus for the earliest divergence of Canidae and the grouping of Procyonidae and Mustelidae, conflicting phylogenetic hypotheses exist for the other caniformian families. In the present study, a data set comprising >22 kb of 22 nuclear intron loci from 16 caniformian species is used to investigate the phylogenetic utility of nuclear introns in resolving the interfamilial relationships of Caniformia. Our phylogenetic analyses support Ailuridae as the sister taxon to a clade containing Procyonidae and Mustelidae, with Mephitinae being the sister taxon to all of them. The unresolved placements of Ursidae and Pinnipeds here emphasize a need to add more data and include more taxa to resolve this problem. The present study not only resolves some of the ambiguous relationships in Caniformia phylogeny but also shows that the noncoding nuclear markers can offer powerful complementary data for estimating the species tree. None of the newly developed introns here have previously been used for phylogeny reconstruction, thus increasing the spectrum of molecular markers available to mammalian systematics. Interestingly, all the newly developed intron data partitions exhibit intraindividual allele heterozygotes (IIAHs). There are 115 cases of IIAHs in total. The incorporation of IIAHs into phylogenetic analysis not only provides insights into the interfamilial relationships of Caniformia but also identifies two potential hybridization events occurred within Ursidae and Otariidae, respectively. Finally, the powers and pitfalls of phylogenetics using nuclear introns as markers are discussed in the context of Caniformia phylogeny. [Caniformia; intron; intraindividual allele heterozygotes; phylogeny; transposable elements.]

The monophyletic group Caniformia (dog-like carnivores) in the order Carnivora includes families Canidae (dogs), Ursidae (bears), Ailuridae (red panda), Procyonidae (raccoons), Mustelidae (weasels), Mephitinae (skunks), and aquatic Pinnipeds, that is, families Odobenidae (walrus), Otariidae (sea lions), and Phocidae (true seals). These families are characterized by great morphological, ecological, and behavioral variation (Gittleman 1989) and have had many controversial issues surrounding their phylogenies. Currently, with the exception of the general consensus for the earliest divergence of Canidae and the grouping of Procyonidae and Mustelidae as Musteloidea *sensu stricto* (Wyss and Flynn 1993; Vrana et al. 1994; Flynn and Nedbal 1998; Yu et al. 2004, 2008; Yu and Zhang 2006), conflicting phylogenetic hypotheses exist for the other caniformian families (Fig. 1). For example, it is generally recognized that Ailuridae, Mephitinae, and Musteloidea *sensu stricto* together form the Musteloidea. However, there had long been controversy over the sister group relationships among them (Flynn et al. 2000, 2005; Yu et al. 2004, 2008; Delisle and Strobeck 2005; Fulton and Strobeck 2006, 2007; Sato et al. 2006, 2009; Arnason et al. 2007; Yonezawa et al. 2007). In addition, the trichotomy between Ursidae, Pinnipeds, and Musteloidea also remain unresolved (Yu et al. 2004; Delisle and Strobeck 2005; Arnason et al. 2006, 2007; Fulton and Strobeck 2006; Sato et al. 2006; Yu and Zhang 2006; Rybczynski et al. 2009; Schröder et al. 2009).

Recently, several studies have shown that relative to the commonly used nuclear protein-coding and mitochondrial (mt) genes, the noncoding intron sequences can be an equally fruitful source of phylogenetic characters as they possess a number of traits that are desirable for molecular phylogenetics (Creer et al. 2006; Benavides et al. 2007; Matthee et al. 2007; Möller-Krull et al. 2007; Dalebout et al. 2008; Schröder et al. 2009), for example, lack of functional constraints, a high substitution rate and less homoplasy (Friesen et al. 1997; Friesen 2000; Creer et al. 2006). In these studies, the nuclear introns offer powerful complementary data to address the ambiguous relationships of different taxonomic levels, including the beaked whale species (Dalebout et al. 2008), the Asian pitvipers genus (Creer et al. 2006), the carnivoran families (Schröder et al. 2009), and the eutherian orders (Matthee et al. 2007). Here, therefore, we aim to add more than 22 kb nuclear intron sequences to resolve the interfamilial relationships of Caniformia.

The completion of genome sequences of *Canis lupus familiaris* (domestic dog), a representative of the Canidae family in Caniformia, creates an opportunity to identify a wealth of single-copy nuclear genes for building a robust Carnivora phylogeny. By combining bioinformatics

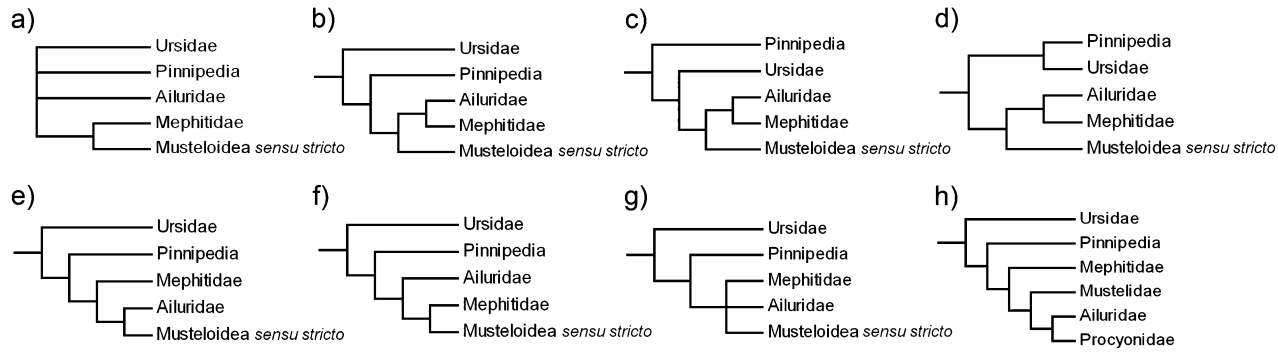

FIGURE 1. Alternative hypotheses of interfamilial relationships in Carnivora a) Based on 2 mt gene fragments (Ledje and Arnason 1996a,b) b) Based on 3 mt sequences and 1 nuclear intron (Flynn et al. 2000) and 4 nuclear loci and 1 mt gene (MP analyses, Yu et al. 2008). c) Based on 12 complete protein-coding mt genes (ML analyses, Delisle and Strobeck 2005). d) Based on 12 complete protein-coding mt genes (MP analyses, Delisle and Strobeck 2005). e) Based on 5 nuclear genes (Fulton and Strobeck 2006), 3 nuclear genes (Sato et al. 2006), 4 nuclear loci and 1 mt gene (ML/Bayesian anlyses, Yu et al. 2008), and 5 nuclear loci (Sato et al. 2009). f) Based on 3 nuclear loci and 3 mt sequences (Bayesian anlyses, Flynn et al. 2005) and 12 complete protein-coding mt genes (Arnason et al. 2007). g) Based on 3 nuclear loci and 3 mt sequences (MP analyses, Flynn et al. 2005). h) Based on 3 mt sequences and morphological characters (Dragoo and Honeycutt 1997).

and polymerase chain reaction (PCR)-targeted experimental approaches, we succeeded in obtaining 19 novel nuclear intron regions from 15 orthologous single-copy genes of 16 representative taxa across distantly related caniformian families. These 19 introns, together with 3 other previously reported single-copy nuclear introns, constitute our data set. The present study not only helps to resolve some of the intractable questions bearing on Caniformia interfamilial phylogeny but also contributes to better understanding of the evolutionary dynamics of the nuclear introns involved. Besides the occurrence of substantive transposable element (TE) insertions, a large numbers of intraindividual allele heterozygotes (IIAHs) are also observed in these introns (115 cases in total). Introns of diploid organisms will either be heterozygotic or be homozygotic (Creer et al. 2006). However, in most studies using introns as phylogenetic markers (Johnson and Clayton 2000; Pitra et al. 2000; Jenkins et al. 2001; Braband et al. 2002; Matthee et al. 2007; Dalebout et al. 2008; Schröder et al. 2009), heterozygotic introns were seldom mentioned or detected. Until recently, IIAHs have gradually received attention due to the identification of them in several studies of insects, reptiles, and mammals (Palumbi and Baker 1994; Beltrán et al. 2002; Sota and Vogler 2003; Pons et al. 2004; Creer et al. 2006). Remarkably, in the present study, all the newly developed intron data partitions exhibit IIAHs. The discovery of such a large numbers of IIAHs provides an opportunity for us to evaluate the phylogenetic utility of IIAHs and investigate the appropriate methodologies concerning their treatment of IIAHs in the analyses. Our results show that incorporating IIAHs into phylogenetic analysis not only provides insights into the relationships among caniformian families but also identifies two potential hybridization events occurred within Ursidae and Otariidae, respectively. Hence, our results exemplify the utility of incorporating IIAHs into phylogenetic frameworks when nuclear introns are used as genetic markers.

MATERIALS AND METHODS

Data Set Collection

The strategy of developing potential nuclear single-copy introns for Caniformia phylogenetic studies was similar to that used by Townsend et al. (2008) in which 25 novel nuclear protein-coding loci were mined from genome databases for higher level phylogenetics of squamate reptiles. It involves three major steps. Step 1 was to identify the nuclear genes likely to be present in all the genomes of mammalian representatives, including those of *Homo sapiens* (build 36.1), *Mus musculus* (build 36.1), and *Canis lupus familiaris* (build 2.1), with the reciprocal best hits criterion performed by a BLAST-based algorithm. Among these nuclear genes, those that are found to have potential paralogs or very close gene family relatives were removed. Step 2 was to determine the intron locations in each putative single-copy gene identified in Step 1 by examining the exon–intron boundary using the UCSC genome browser (http://genome.ucsc.edu/) and the SPIDEY program (http://www.ncbi.nlm.nih.gov/spidey/). Intron regions were considered for subsequent steps if 1) the size of sequence fragments fell between 700 and 1500 bp; 2) no large TEs were inserted. TEs are checked by the RepeatMasker program (Smit, Hubley, and Green 1996–2004, RepeatMasker Open-3.0, http://www.repeatmasker.org); and 3) by aligning *H. sapiens*, *M. musculus* and *Canis lupus familiaris* orthologs for each gene of interest, a conserved exon-primed region could be found to allow primer design. There were about 2000 introns identified by the above criteria. We randomly selected 30 for laboratory work

TABLE 1. Species used in this study

Superfamily	Family	Scientific name	Common name	Sample source
	Canidae	*Canis lupus*	Grey wolf	Mongolia, China
		C. familiaris	Dog	The UCSC Genome Browser Database
	Ursidae	*Ursus arctos*	Brown bear	Heilongjiang Province, China
		U. thibetanus	Asiatic black bear	Yunnan Province, China
		Ailuropoda melanoleuca	Giant panda	Sichuan Province, China
	Mephitidae	*Mephitis mephitis*	Striped skunk	San Diego Zoo, United States
	Ailuridae	*Ailurus fulgens*	Red panda	Kunming Zoo, China
Pinnipedia	Otaeiidae	*Zalophus californianus*	California sea lion	Qingdao, Shandong Province, China
		Callorhinus ursinus	Northern fur seal	Qingdao, Shandong Province, China
	Odobenidae	*Odobenus rosmarus*	Walrus	San Diego Zoo, United States
	Phocidae	*Phoca vitulina*	Harbor seal	Qingdao, Shandong Province, China
Musteloidae	Procyonidae	*Procyon lotor*	Raccoon	San Diego Zoo, United States
sensu stricto		*Potos flavus*	Kinkajou	San Diego Zoo, United States
	Mustelidae	*Martes flavigula*	Yellow-throated marten	Kunming Zoo, China
		Mustela kathiah	Yellow-bellied weasel	Yunnan Province, China
		Arctonyx collaris	Hog badger	Yunnan Province, China

conducted in Step 3. In Step 3, the introns were amplified from a set of Caniformia "test taxa" consisting of representatives from a range of Caniformia families to assess each intron's potential taxonomic breadth of amplification. Finally, 19 intron regions from 15 genes could be successfully amplified and sequenced across all the test taxa. They were developed as novel loci for this study. These 19 introns, along with 3 other single-copy nuclear introns (Ttr1, Fgb4, and Fgb7), discussed in our previous published studies (Yu et al. 2004; Yu and Zhang 2005a, 2006), constitute our data set. The pertinent information about these 22 intron regions is in Supplementary material table 1.

For each caniformian species, total genomic DNA was isolated from blood or frozen tissues. Applying an exon-primed, intron-crossing primer design strategy (Lessa 1992; Slade et al. 1993), 19 external and 93 internal primer pairs were used to amplify the newly developed nuclear intron regions from 16 caniformia species. The species used in the present study are shown in Table 1. (Primer sequence information is available as Supplementary Table 2, see Supplementary Material Online at http://www.sysbio.oxfordjournals.org.). A "touch-down" PCR amplification was carried out using the following parameters: 95 °C hot start (5 min), 10 cycles of 94 °C denaturation (1 min), 60–50 °C annealing (1 min), 72 °C extension (1 min), and finally 25 cycles of 94 °C denaturation (1 min), 50 °C annealing (1 min), 72 °C extension (1 min). The amplified DNA fragments were purified and sequenced in both directions with an ABI PRISM™ 3730 DNA Analyzer following the manufacturer's protocol. In the case of poor performance of direct sequencing resulting from complex DNA structures, tandem repeats or intron heterozygotes, the amplified PCR products were gel purified and cloned into the pMD18-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) and transformed into ultracompetent *Escherichia coli* cells (TaKaRa Biotechnology Co., Ltd.). Thirty positive clones per ligation reaction were sequenced. All sequences obtained were checked carefully and queried in BLAST searches of GenBank to assess homology. Partial sequences were obtained from intron 6 of *Impal* gene (*Impal*-6) of three mustelid species (i.e., the Marten *Martes flavigula*, the Yellow-bellied weasel *Mustela kathia*, and the Hog badger *Arctonyx collaris*) because of sequencing difficulties resulting from the unexpected occurrence of extremely long tandem repeats. Also, several PCR attempts using different primer pairs and cloning methods failed to produce sequence data for the Raccoon *Procyon lotor Guca1b*-3 region. This sequence was therefore excluded from the Guca1b-3 independent gene analyses and treated as missing data in the combined analyses. Several previous studies on introns have reported the discovery of allelic introns of identical length or variable length in diploid organisms (Palumbi and Baker 1994; Beltrán et al. 2002; Sota and Vogler 2003; Pons et al. 2004; Creer et al. 2006). In the present study, 115 cases of IIAHs were detected from all the newly determined introns. In total, 400 newly determined intron sequences are deposited in the GenBank database under accession numbers FJ692614–FJ693013.

Sequence Characterizations and Alignments

Statistical attributes of the nucleotide sequence data were estimated using MEGA 4.0 (Tamura et al. 2007). The calculation of pairwise maximum likelihood (ML) sequence divergence among Caniformia ingroup species was performed with the PAML package (Yang 2007). Given high A + T content and lack of functional constraint, nuclear introns are favorable chromosomal regions for integration of TEs (Yu and Zhang 2005b), which comprise a ubiquitous source of indels in eukaryotes (Edwards et al. 2004). Therefore, the introns were also screened for interspersed repeats known to exist in mammalian genomes by using the program RepeatMasker (Smit, Hubley, and Green 1996–2004, RepeatMasker Open-3.0, http://www.repeatmasker.org). Sequences were aligned using the MUSCLE v3.6 software with default settings (Edgar 2004). The ambiguous areas of alignment were located and removed by using the program Gblocks 0.91b (Castresana 2000) with default parameters. For the individual sequence

alignments, we use the relaxed gap selection criterion (allowed gap positions = all) because the simulation studies suggested that this criteria is applicable for short alignments in Gblocks (Talavera and Castresana 2007; Hultgren and Stachowicz 2008). For the combined sequence alignment, besides the relaxed gap option, we also use two more stringent gap selection criteria (allowed gap positions = no and with half) to test whether the strength of the phylogenetic inferences was influenced by choice of gaps. These alignments have been submitted to TreeBASE (Accession number: S10901).

Phylogenetic Analyses

Phylogenetic reconstruction of individual introns was performed using PAUP* 4.0b10 (Swofford 2002) for ML analyses and using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian inference. In ML analysis, the model of sequence evolution was optimized using Akaike information criterion (AIC; Akaike 1974; Posada and Buckley 2004) as implemented in Modeltest version 3.7 (Posada and Crandall 1998). The chosen model and its parameters were used to infer the ML trees with the heuristic algorithm, 10 random-addition sequence replicates, and tree bisection-reconnection branch swapping in PAUP*. Bootstrap support under ML analysis was assessed using a nonparametric bootstrap resampling of 100 replicates (Felsenstein 1985). The parameters estimated by Modeltest were also used in the priors of Bayesian inference with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Four Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for 10^6 generations, sampling trees every 100 generations. The first 10^5 generations were discarded as the burn-in. At the end of the run, the average standard deviation of split frequencies was less than 0.01 in all the cases, indicating a good convergence level (MrBayes 3.1.2 manual). A 50% majority rule consensus of post burn-in trees was constructed to summarize posterior probabilities (PPs) for each branch.

In addition to individual analyses of each introns, analyses were also conducted with combined data sets. Phylogenetic analysis of combined data sets was performed using RAxML Web-Servers (Stamatakis et al. 2008) for ML analysis with 1000 bootstrap replications. In addition, partitioned Bayesian analyses, which use the best fitting models chosen using the AIC in Modeltest 3.7 (Posada and Crandall 1998), were run for 2×10^6generations. The average standard deviation of split frequencies was close to 0.002 (no gap; with half gap: 0.0007; all gap: 0.0002) when the run ended. The first 10^5 generations were discarded as the burn-in. Based on partitioned Bayesian analyses, we performed Bayesian concordance analysis (BCA; Ané et al. 2007) to estimate primary concordance trees. Tree reliability was evaluated by sample concordance factors (CFs). The Metropolis-coupled MCMC sampled with 2×10^6generations was employed (4 runs and 4 chains) and a priori level of discordance $\alpha = 2.5$ was used in BCA. In all analyses, trees were rooted with Canidae as the outgroup.

Intraindividual Allele Heterozygotes

For the individual intron analyses, both copies of alleles from a species were included, but such a treatment of IIAHs is intractable for the combined intron analysis because in order to include all the identified IIAHs (115 cases), 2^{115} combinations of alleles from each species in total would be included as independent terminals in the analysis. Alternatively, IIAHs can be incorporated into the combined analyses by the program POFAD v1.03 (Phylogeny Analysis From Allelic Data; Joly and Bruneau 2006). POFAD is a recently developed method of constructing organismal phylogeny from multiple data sets that contain allelic information. It converts a distance matrix of alleles into a distance matrix of organisms so that individuals become the terminals of the analyses.

In order to investigate potential effect of the inclusion of IIAHs in phylogenetic analysis and appraise the utility of the software POFAD, we performed phylogenetic analyses of our combined data set by 1) choosing randomly an allele per species for ML analysis and Bayesian inference (without the inclusion of IIAHs) and 2) using POFAD to incorporate IIAHs. In POFAD analysis, we calculated the average uncorrected pairwise distances in PAUP* (Swofford 2002). The resulting distance matrices served as the input for the calculation of standardized pairwise distances between species in POFAD (Joly and Bruneau 2006). The standardized distances were then used as input for the neighbor-joining analysis conducted using PAUP* (Swofford 2002) to produce a phylogenetic tree.

Testing Tree Incongruence

The incongruence among different tree topologies was evaluated using the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa 1999) and the approximately unbiased (AU) test (Shimodaira 2002), as implemented in the CONSELV0.1i program (Shimodaira and Hasegawa 2001) with default scaling and replicate values. The site-wise log-likelihood values were estimated by PAUP* (Swofford 2002).

RESULTS

Characteristics of the Nuclear Intron Data

The 22 nuclear introns of 16 Carnivora species varied in length from 541 (Fgb-4) to 2804 (Impa1-6) aligned positions. The removal of ambiguous areas resulted in the aligned sequence length varying from 540 (Fgb-4) to 1318 (Ccng2-2) positions. In addition, these introns differed in the number of parsimony-informative sites, ranging from 99 sites (Fgb-4) to 420 sites (Tbc1d7-6). According to different gap selection criteria in Gblocks,

TABLE 2. Summary characterizations of introns examined in the present study and published mt and nuclear protein-coding genes

Sequence type	Fragments name	TEs	Aligned[a]	Final date[b]	Persimony-informative	Nucleotide composition				Ti/Tv	Best fit model	Among-site Rate variation	
						A	T	G	C			I	α
Introns	Atp5d-2	Yes	927	668	316	0.17418	0.22546	0.32484	0.27552	1.4	TVM + G	0	1.6144
	Ccng2-2	Yes	2024	1318	288	0.29212	0.35588	0.20194	0.15006	2.0	TVM + G	0	0.9131
	Ccng2-6	Yes	1766	1260	378	0.28627	0.35388	0.18896	0.17089	1.9	TVM + G	0	1.4343
	Cidea1	Yes	2056	1057	362	0.29833	0.24440	0.23092	0.22636	1.7	TVM + G	0	0.9711
	Coro1c-4	Yes	1533	1367	404	0.24177	0.27902	0.23912	0.24009	2.3	TrN + G	0	0.9927
	Coro1c-5	Yes	1380	1070	243	0.30313	0.32797	0.21399	0.15491	2.4	TVM + G	0	1.4059
	Fgb4	No	541	540	99	0.30018	0.31494	0.20888	0.17600	1.9	K81uf + G	0	2.9369
	Fgb7	Yes	1227	604	147	0.28079	0.32907	0.19299	0.19715	1.8	GTR + G	0	3.3784
	Guca1b-3	Yes	1075	752	291	0.23899	0.19239	0.29441	0.27422	1.3	K81uf + G	0	1.7242
	Impa1-6	Yes	2804	1021	288	0.34631	0.29854	0.21304	0.14211	1.3	TrN + G	0	1.0229
	Ociad1-4	Yes	2207	1024	275	0.33106	0.39224	0.14635	0.13034	2.0	TVM + G	0	2.2675
	Plod2-13	No	1336	1220	301	0.29267	0.31800	0.23544	0.15388	1.4	TVM + G	0	2.8394
	Plod2-14	Yes	2623	1125	301	0.34620	0.33169	0.17434	0.14777	1.8	TVM + G	0	1.5644
	Ssr1-5	Yes	2019	798	206	0.32352	0.37572	0.16380	0.13696	2.3	GTR + G	0	2.3589
	Tbc1d7-6	Yes	1166	1111	420	0.25034	0.29405	0.26154	0.19407	1.5	TVM + G	0	1.1715
	Tbk1-8	Yes	1130	838	175	0.33061	0.37317	0.15182	0.14440	2.0	GTR + G	0	2.7828
	Tinagl1-1	Yes	1264	1156	273	0.21028	0.20409	0.29921	0.28642	2.1	HKY + G	0	0.6368
	Tinagl1-3	Yes	1147	1017	278	0.18447	0.24606	0.27772	0.29175	1.8	TrN + G	0	0.8891
	Ttr1	Yes	1079	995	306	0.26467	0.27922	0.21783	0.23827	1.5	GTR + G	0	1.3240
	Wasf1-3	Yes	1353	1026	234	0.31627	0.35086	0.16424	0.16863	1.8	GTR + G	0	0.9993
	Wasf1-6	Yes	1271	1120	267	0.31237	0.34998	0.18873	0.14892	2.2	TVM + G	0	1.8401
	Wasf1-7	Yes	1380	1164	282	0.31646	0.35513	0.16392	0.16449	2.0	GTR + G	0	1.0658
	Con1[c]			16,459	3292	0.28019	0.31041	0.21658	0.19282	1.8			
	Con2			21,570	4565	0.28470	0.31243	0.21381	0.18906	1.8			
	Con3			22,695	4702	0.28502	0.31153	0.21399	0.18946	1.8			
mtDNA	ATP6			681	288	0.29672	0.28527	0.12119	0.29682	2.2	HKY + I + G	0.4	0.7413
	ATP8			205	100	0.37946	0.25744	0.08243	0.28067	1.5	HKY + I + G	0.3	0.9952
	COX1			1545	558	0.27439	0.24043	0.17924	0.30593	2.4	TrN + I + G	0.6	0.4916
	COX2			684	247	0.32846	0.24854	0.14561	0.27739	2.5	TrN + I + G	0.5	0.5879
	COX3			784	289	0.27241	0.27862	0.15768	0.29129	1.9	TVM + I + G	0.6	0.7661
	CYTB			1140	433	0.29684	0.28854	0.13801	0.27661	1.9	TVM + I + G	0.5	0.8936
	ND1			957	334	0.30582	0.29223	0.12456	0.27739	1.9	TVM + I + G	0.5	0.4289
	ND2			1044	453	0.35824	0.27535	0.09815	0.26826	1.4	TVM + I + G	0.4	1.2045
	ND3			348	150	0.30688	0.27325	0.13355	0.28632	1.8	TVM + I + G	0.5	0.8970
	ND4			1378	578	0.31592	0.28002	0.11892	0.28515	2.0	TVM + I + G	0.4	0.4908
	ND4L			297	128	0.28866	0.25006	0.13221	0.32907	2.2	TVM + I + G	0.5	0.4956
	ND5			1836	782	0.32569	0.27635	0.11896	0.27900	1.8	GTR + I + G	0.4	1.0881
	Com12P[d]			10,215	4093	0.30818	0.27368	0.13188	0.28626	1.9			
Exons	IRBP1			1188	204	0.17570	0.32494	0.32280	0.17656	2.3	HKY + I + G	0.5	0.6645
	APOB			963	97	0.32499	0.22191	0.18121	0.27189	2.7	HKY + G	0	0.4864
	RAG1			1095	204	0.26063	0.25346	0.27109	0.21481	2.0	HKY + G	0	0.4864
	TBG			442	32	0.26939	0.25582	0.21477	0.26002	2.7	K80	0	equal

Notes: If TEs were detected in introns, it indicated yes, otherwise no. Transition/transversion (Ti/Tv), proportion of invariant sites (*I*), and gamma-shape parameters (α) are shown.

[a]The length of sequences that were aligned using the MUSCLE 4.0 software with default settings (Edgar 2004).

[b]The length of analyzed data after the ambiguous areas of alignment removed by using the program Gblocks 0.91b (Castresana 2000) with default parameters.

[c]The length of concatenated all introns after the ambiguous areas of alignment removed by using the program Gblocks 0.91b (Castresana 2000) with no gap parameters (with half gap : Con2; all gap : Con3).

[d]The length of concatenated 12 mt protein-coding genes.

the alignment of the combined data set consisted of 22,695 (allowed gap positions = all), 21,570 (allowed gap positions = with half), 16,459 (allowed gap positions = none) positions, respectively. The parsimony-informative sites in the three data sets are 4702 (20.72%), 4565 (21.16%), and 3292 (20.00%), respectively (Table 2). An A-T bias was apparent in most introns, as typically observed in noncoding regions (Pecon-Slattery et al. 2004; Pons et al. 2004; Yu et al. 2004; Yu and Zhang 2005a, 2006; Matthee et al. 2007). The optimal model of nucleotide substitution varied by intron regions, suggesting different evolutionary processes among loci. In comparison, most introns showed gamma shape parameters (α) larger than 1.0 with an estimated proportion (I) = 0 of invariant sites, indicating the nearly complete absence of among-site rate variation. The estimation of the relative substitution rate shows that it ranged from 7.4% (Ccng2-2) to 26.8% (Atp5d-2), averaging 11.16% (Fig. 2). One of these introns, Atp5d-2, is considerably more variable than the others. In addition, low transition (Ti)/transversion (Tv) rate ratios (between 1.3 and 2.4) were observed, consistent with the findings from the other animal group intron studies (Drovetski 2002).

Occurrence of IIAHs

The overall incidence of IIAHs in our 19 newly developed introns appears universal. There were 115 cases of

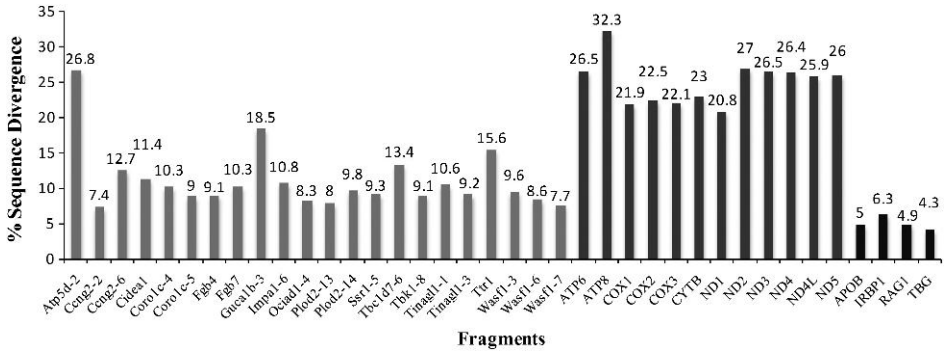

FIGURE 2. Sequence divergences among Caniformia ingroup species for 22 intron fragments in the present study, mt and nuclear protein-coding genes.

IIAHs in total. Of the 16 carnivores that were examined, 3 to 9 cases of IIAHs were detected from each of the introns. In addition, each of the species had IIAHs at between 3 and 15 introns (Table 3). IIAHs will either be of equal or variable length. Fourteen of 19 introns had allele length variant heterozygotes (Creer et al. 2006) due to indels ranging in size from 1 to 17 bp, with the other nucleotide sites either the same or the distinct at 1–14 bp (Table 3), whereas IIAHs of identical length were discovered in all 19 introns, exhibiting 1–11 substitutional differences. Sequence divergence between IIAHs (average 0.58%) was much lower than those between species (average 11.13%), generally by a factor of 20 or more. Generally, IIAHs form monophyletic pairs on the phylogenetic trees as expected (see Supplementary Material fig. 1). A few cases of the nonmonophyletic IIAHs were illustrated by the close relatedness of one allele of Asiatic black bear *Ursus thibetanus* to Brown bear *Ursus arctos* (Plod2-13), and vice versa (Guca1b-3), as well as one allele of sea lion *Zalophus californianus* to fur seal *Collorhinus ursinus* (Tbc1d7-6). For these alleles, the lack of reciprocal monophyly between closely related species most likely indicates the incomplete lineage sorting or introgressive hybridization occurring in these species (Rosenberg 2002, 2003; Degnan and Salter 2005; Joly and Bruneau 2006).

Occurrence of TEs

In our intron data sets, there are pervasive TEs insertions, with insertion frequencies ranging from low (0) in FGB-4 and Plod2-13 to high (8) in Impal1-6 across the carnivores sampled (Table 4).

TEs discovered here were dominated by a variety of non-long-terminal repeat retrotransposons, including long interspersed elements (LINEs), short interspersed elements (SINEs), mammalian-wide interspersed repeats (MIRs), and DNA transposons. MIRs and DNA transposons (e.g., Tc1/Mariner, Tc2/Kangal, and hAT-like superfamilies) were found to integrate into the orthologous loci of all examined carnivores, which suggests an ancient origin. The result was consistent with the earlier finding that these two classes of TEs represented remnants or "fossils" of TEs, predating the radiation of mammalian orders, and had long ago become inactive in mammalian lineages (Jurka et al. 1995; Smit and Riggs 1995; Lander et al. 2001; Waterston et al. 2002). The great majority of LINEs and SINEs identified here were members of the L1_Canid (Fc) and CAN SINE groups that have been exclusively found in Carnivora (Minnick et al. 1992; Coltman and Wright 1994; van der Vlugt and Lenstra 1995; Das et al. 1998; Zehr et al. 2001; Vassetzky and Kramerov 2002; Pecon-Slattery et al. 2004; Yu and Zhang 2005b). Most of them were characterized by sporadic locations within the introns and restricted taxonomic distributions, suggesting that they emerged after the divergence of the species in whose they had embedded in and are likely to have retained the ability to retrotranspose. A few of them were also found to insert at orthologous sites of three intron loci across Carnivora (Ccng2-5, Impal1-6, and TTR-1), suggesting their invasion of the genomes before the carnivoran radiation.

The insertions of TEs at new genomic sites are often considered irreversible and random (Nikaido et al. 2001), which suggests that they may be excellent homoplasy-free markers in phylogenetic analyses (Kawai et al. 2002; Salem et al. 2003; Sasaki et al. 2004; Nikaido et al. 2006; Nishihara et al. 2006). Therefore, we also address interfamilial Caniformia relationships based on TE insertions.

Phylogenetic Analyses

Individual intron analyses.—A variety of tree topologies concerning interfamilial relationships of Caniformia were produced by the individual analyses of 22 introns (see Supplementary material fig. 1; Tree topologies have been submitted to TreeBASE [accession number: S10861]). These trees differed primarily at the sister group relationships among Ailuridae, Mephitinae, and Musteloidea *sensu stricto*, and those among Ursidae, Pinnipeds, and Musteloidea. As seen from the results, 11 analyses support a Ailuridae/Musteloidea *sensu stricto* clade, whereas 4 analyses support a Ailuridae/Mephitinae clade. On the other hand, 9 analyses suggest a Ursidae/Pinnipeds clade and 7 analyses suggest a Musteloidea/Pinnipeds clade. These alternative

TABLE 3. IIAHs detected in the present study

Intron fragments	Species														
	Harbor seal	Walrus	Sea lion	Fur seal	Red panda	Skunks	Hog badger	Yellow-bellied weasel	Marten	Raccoon	Kinkajou	Giant panda	Asiatic black bear	Brown bear	Wolf
Atp5d-2	./.	./.	./.	./.	./.	./.	/2/.	1/.	1/.	1/.	./.	./.	./.	./.	./.
Cng2-2	./.	./.	5/1/.	2/.	./.	./.	2/./1	./.	./1	1/1/.	1/.	./.	1/./1	./.	./.
Cng2-6	./.	./.	1/.	./.	./.	./5	./.	./.	4/4/17[b]	./.	1/.	./.	./.	./.	1/./6
Cidea1	./.	1/1/.	1/.	1/2/.	./.	1/.	3/./1	./.	1/1/.	/1/.	1/.	./.	./.	./.	1/./.
Coro1c-4	./.	./.	/1/.	./1	3/1/.	3/1/.	./.	./.	./.	/1/.	./.	./.	4/3/.	./.	1/./.
Coro1c-5	./.	./.	./.	./.	./.	./.	1/1.	./.	./.	./.	./.	1/1/.	4/1/.	./.	1/./.
Guca1b-3	./.	1/./.	./.	./.	./.	./.	5/6/.	./.	5/./.	N/A	./1	./.	./.	5/./5	4/./.
Impa1-6	/1/.	/1/.	./.	./.	./.	./.	./.	./.	./.	1/./.	./.	./.	./.	2/./.	./.
Ociad1-4	./.	./.	./.	./.	./.	./.	2/./.	3/2/.	./1/8	/1/2	3/./.	1/./.	./1	1/./.	./.
Plod2-13	1/1/1	./.	./.	./.	1/1/.	./.	./.	1/./.	./1	4/1/2	./.	./.	11/1/4	./.	2/1/.
Plod2-14	./.	./.	./.	./.	./.	1/./5	./.	./.	1/2/.	6/1/4	./.	./.	11/3/1	./.	./.
Ssr1-5	./.	1/./1	1/./5[a]	./.	./.	./.	./.	1/./.	./.	/1/6	1/./1	./.	4/./.	/1/.	./.
Tbc1d7-6	/1/.	./.	5/3/1	./.	./6	1/2/1	./.	./.	1/./.	2/1/.	./.	3/2/.	3/2/4[a]	./.	./.
Tbk1-8	./.	./.	./.	./.	./.	./.	./.	./.	./.	/1/.	./.	./.	1/./.	1/1/.	./.
Tinag1-1	./.	./.	./.	./.	./.	./.	./.	3/./.	3/1/6	3/./.	./.	./.	1/./.	./.	./.
Tinagl1-3	/1/.	./.	./.	./.	1/./.	./.	1/1/.	4/1/.	1/./.	5/3/1	./.	./.	5/./.	2/1/.	./.
Wasf1-3	./.	./.	1/2/.	./4	./.	./.	./.	./.	./.	1/./2	./.	./.	./.	./.	1/./.
Wasf1-6	./.	./.	./.	2/1/.	./.	./.	./.	./.	./.	2/./.	3/1/.	./.	/1/.	./.	./.
Wasf1-7	./.	./.	1/1/.	2/./.	1/4	./1	./.	./.	1/./.	3/./.	2/./2	./.	4/2/3[a]	./.	1/./3

Notes: The information of IIAHs were represented as the number of transitions/ the number of transversions/ the length of indels. The dot means zero.

[a]two independent indels;

[b]three independent indels.

hypotheses recovered by the individual intron analyses were also the most frequent competing phylogenies in previous Caniformia analyses (Flynn and Nedbal 1998; Yu et al. 2004; Flynn et al. 2005; Delisle and Strobeck 2005; Sato et al. 2006, 2009; Yu and Zhang 2006; Arnason et al. 2007).

Combined intron analyses.—Phylogenetic trees reconstructed from the combined data set using three gap selection criteria in Gblocks (allowed gap positions = none, with half, and all) and two tree-building methods (ML and Bayesian analyses) all strongly supported the sister grouping of Ailuridae and Musteloidea *sensu stricto* (ML BS = 100%; PP = 1.00), with Mephitinae being the sister taxon to them (ML BS = 100%; PP = 1.00). All the combined intron analyses showed the sister grouping of Ursidae and Pinnipeds (Fig. 3) with the exception of the Bayesian analyses with all the gaps removed in which Pinnipeds and Musteloidea was grouped together; however, these two relationships both received very low BS (ML analyses: no gap allowed, BS < 50%; half gaps allowed, BS = 66%; all gaps allowed, BS = 68%) and CF (BCA analyses: no gap allowed, CF = 65; half gaps allowed, CF = 56; all gaps allowed, CF = 54). Therefore, the relationships among Musteloidea, Ursidae, and Pinnipeds were unclear.

Interestingly, the inclusion of IIAHs in the combined analysis by using POFAD grouped Ursidae and Pinnipeds, regardless of the methods of recovering allelic distances and combining multiple matrices.

Phylogenetic content of TEs.—Of the 57 cases of TE insertions, 17 were present at othologous sites of all carnivores under study, supporting the monophyly of Caniformia. The analyses of the remaining 40 TE insertions gave independent supports for 8 of 14 internal branches of the combined intron tree (Fig. 3): 1) the monophyly of Canidae (4 TEs), Ursidae (1 TE), Procyonidae (1 TE), Mustelidae (3 TEs), Otariidae (1 TE), and Pinnipeds (8 TEs); 2) the monophyly of supergroup Musteloidea that comprises Mustelidae, Procyonidae, Ailuridae, and Mephitidae (1 TE); 3) the sister group relationship of *Ursus arctos* and *Ursus thibetanus* within Ursidae (3 TEs). Of the TEs identified here, only two TEs contradicted the species relationships within Mustelidae in our combined intron tree. Both TEs were found in *Martes flavigula* and *Arctonyx collari* to the exclusion of *Mustela kathiah*, whereas in the combined intron tree, there is weak support for the grouping of *Martes flavigula* and *Mustela kathiah*.

DISCUSSION

Incorporation of IIAHs in Phylogenetic Analyses

The incorporation of IIAHs into phylogenetic analysis not only provides insights into the interfamilial relationships of Caniformia but also identifies two potential hybridization events occurred within Ursidae

Table 4. TEs discovered in the present study

Intron fragments	TEs						
	Species specific				Orthologus		
	Species	TEs	Class	Length (bp)	TEs	Class	Length (bp)
Atp5d-2	Wolf/Dog	SINEC_a1	SINE/Lys	163			
Ccng2-2	Marten/Hog badger/Weasel[a]	SINEC_b2	SINE/Lys	178-187	L2b	LINE/L2	57-91
	Striped skunk/Red panda/Raccoon/Kinkajou/ Marten/Hog badger/Weasel	SINEC_b2	SINE/Lys	170-196			
	Fur seal/Sea lion	SINEC_b1	SINE/Lys	199			
Ccng2-6	Raccoon	SINEC_b1	SINE/Lys	190	L1_Carn7	LINE/L1	191-204
	Raccoon	SINEC_b1	SINE/Lys	93			
Cidea1	Giant panda/Brown bear /Asiatic black bear	SINEC_b1	SINE/Lys	197-199			
	Brown bear /Asiatic black bear	L1_Carn7	LINE/L1	362-363			
	Striped skunk	SINEC_Mv	SINE/Lys	187			
	Marten/Hog badger/Weasel	L1_Canid_	LINE/L1	74-81			
	Hog badger	SINEC_Mv	SINE/Lys	193			
Coro1c-4					Tigger12c	DNA/TcMar-Tigger	80-82
					MIRb	SINE/MIR	110-127
Coro1c-5	Brown bear /Asiatic black bear	SINEC_b1	SINE/Lys	196-197	L1M5	LINE/L1	66-82
					tRNA-Lys-AAG	tRNA	64-73
FGB7	Wolf/Dog	SINEC_a1	SINE/Lys	182	MIRb	SINE/MIR	197-218
	Striped skunk	SINEC_b1	SINE/Lys	194			
	Striped skunk	SINEC_b2	SINE/Lys	194			
Guca1b-3	Giant panda	SINEC_b1	SINE/Lys	197	MIRb	SINE/MIR	61-75
Impa1-6	Brown bear /Asiatic black bear	SINEC_a2	SINE/Lys	196	L1_Carn2	LINE/L1	46-58
	Striped skunk	SINEC_b1	SINE/Lys	197			
	Red panda	SINEC_b1	SINE/Lys	168			
	Marten/Hog badger	L1_Carn2	LINE/L1	42-43			
	Marten/Hog badger/Weasel	SINEC_b1	SINE/Lys	180-195			
	Marten/Hog badger	SINEC_b1	SINE/Lys	188-191			
	Weasel	SINEC_Mv	SINE/Lys	184			
Ociad1-4	Marten	SINEC_Mv	SINE/Lys	174-178	MIRc	SINE/MIR	113-125
	Striped skunk	SINEC_b2	SINE/Lys				
	Harbor seal/Walrus/Fur seal/Sea lion	L1_Canid_	LINE/L1	275-283			
	Harbor seal/Walrus/Fur seal/Sea lion	L1_Carn2	LINE/L1	266-270			
Plod2-14	Harbor seal/Walrus/Fur seal/Sea lion	L1_Canid_	LINE/L1	270-281	Kanga1	DNA/Tc2	156-188
	Harbor seal/Walrus/Fur seal/Sea lion	L1MA6	LINE/L1	302-314	Kanga1	DNA/Tc2	270-349
	Harbor seal/Walrus/Fur seal/Sea lion	L1MA6	LINE/L1	248-252			
	Raccoon/Kinkajou	SINEC_b1	SINE/Lys	189-196			
Ssr1-5	Harbor seal/Walrus/Fur seal/Sea lion	SINEC_b1	SINE/Lys	191-193			
	Harbor seal/Walrus/Fur seal/Sea lion	SINEC_b2	SINE/Lys	200-217			
	Wolf/Dog	SINEC_Cf	SINE/Lys	182			
	Wolf/Dog	SINEC_Cf2	SINE/Lys	154			
	Red panda	SINEC_b1	SINE/Lys	197			
Tbc1d7-6					MIR	SINE/MIR	173-210
TBK1-8	Striped skunk	SINEC_b1	SINE/Lys	189			
Tinagl1-1					MIR	SINE/MIR	73
Tinagl1-3					MIR	SINE/MIR	74-95
TTR1					SINEC_b1	SINE/Lys	136-152
Wasf1-3	Harbor seal/Walrus/Fur seal/Sea lion	SINEC_b1	SINE/Lys	191-204	L2c	LINE/L2	82-91
Wasf1-6					MER58A	DNA/hAT-Charlie	187-224
Wasf1-7	Hog badger	L1_Fc	LINE/L1	104			

and Otariidae. Our study demonstrates the importance of identifying and incorporating IIAHs in phylogenetic intron analyses. Introns of diploid organisms will either be heterozygotic or be homozygotic (Creer et al. 2006). However, only a few earlier studies using introns as phylogenetic markers detected and mentioned the occurrence of IIAHs (Palumbi and Baker 1994). Until recently, the identification and incorporation of IIAHs in phylogenetic framework have received attention because growing studies showed that IIAHs can contribute to phylogenetic inferences and facilitate detection of potential hybridization events (Beltrán et al. 2002; Sota and Vogler 2003; Pons et al. 2004; Creer et al. 2005, 2006; Creer 2007). However, investigations of the optimal incorporation methodology and the potential phylogenetic utility of IIAHs in intron data analyses remain limited. The analysis of IIAHs within a single locus analysis is simple, but analysis of the multilocus data sets is challenging (Creer 2007). Currently, the common strategy is to duplicate homozygotic loci alongside heterozygotic loci to incorporate IIAHs simultaneously as independent terminals in the data matrices (Sota and Vogler 2003); however, such an approach is only manageable with small numbers of independent terminals. Joly and Bruneau (2006) recently employed a distance-based algorithm, implemented in the program POFAD (Joly and Bruneau 2006), whereby a distance matrix of alleles was converted to a distance matrix among organisms. This approach has been shown to be very useful for reconstructing the phylogenetic history of closely related organisms from multiple genes (Joly and Bruneau 2006; Leaché et al. 2009; Wallace et al. 2009), but the phylogenetic performance of this approach for studies at a high taxonomic level has not yet been explored. Our

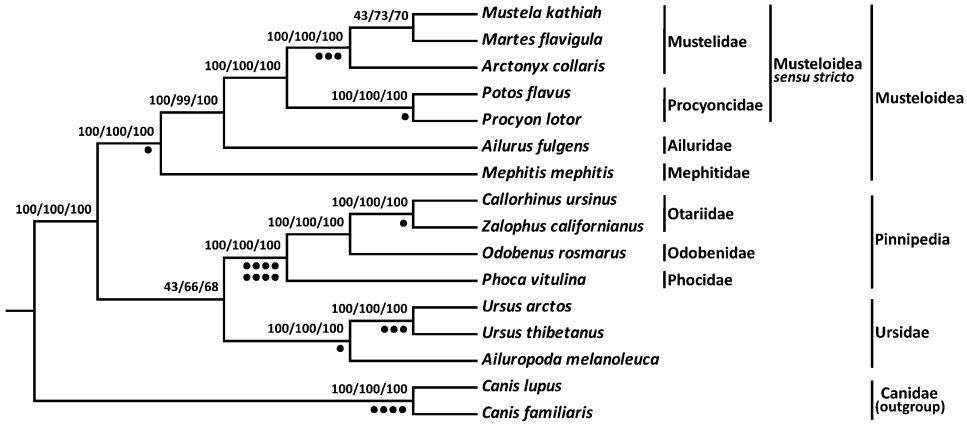

FIGURE 3. Phylogenetic tree topology based on combined ML intron analyses. Bootstrap values under three gap treatments in Gblocks (allowed gap positions = none/with half/all) are shown above nodes. The parsimony-informative TEs are mapped on the tree and indicated as circles.

work is the first to report such a large numbers of IIAHs (115 cases) by analyzing 19 introns from 16 carnivores. In this article, the use of POFAD for the first time empirically proved that it is also useful at the interfamilial level where it recovered a single tree in favor of the combined ML intron trees.

In addition to helping reconstructing interfamilial phylogeny, the incorporation of IIAHs identified potential cases of likely hybridization events. From the individual intron analyses, we can see that 98 of the 115 IIAHs formed monophyletic pairs. The remaining nonmophyletic 17 IIAHs, interestingly, did not occur randomly, and are only found in the representatives of Ursidae (Asiatic black bear *Ursus thibetanus* and Brown bear *Ursus arctos*) and Otariidae (sea lion *Zalophus californianus* and fur seal *Collorhinus ursinus*). Further inspection revealed that the nonmonophyletic IIAHs resulted from the close relatedness of one allele of one species to the other closely related species within the two families, implying that introgressive hybridization or incomplete lineage sorting may cause the lack of reciprocal IIAH monophyly between these recently diverged species. In fact, viable hybrids between *Ursus thibetanus* and *Ursus arctos* in Ursidae and between *Zalophus californianus* and *Collorhinus ursinus* in Otariidae have been documented (Gray 1971; Sinclair 1994). Thus, introgressive hybridization is a more likely explanation of the results, although we cannot rule out the possibility that incomplete lineage sorting may also play a role (Rosenberg 2002, 2003; Degnan and Salter 2005). In sum, our present work makes a good case suggesting that the inclusion of IIAHs in phylogenetic studies will be indispensable for a better understanding of evolutionary processes that have occurred in the past.

Interfamilial Relationships of Caniformia

Previous analyses failed to resolve relationships among Ailuridae, Mephitidae, and Musteloidea *sensu stricto* within Musteloidea. Contradictory results were obtained from mt versus nuclear genes. Studies of four nuclear introns and one nuclear coding gene by Fulton and Strobeck (2006) and five nuclear coding genes by Sato et al. (2009) supported the sister grouping of Ailuridae and Musteloidea *sensu stricto* to the exclusion of Mephitidae, whereas studies of complete mt genomes suggested the close relationship either between Ailuridae and Mephitidae (Delisle and Strobeck 2005) or between Musteloidea *sensu stricto* and Mephitidae (Arnason et al. 2007), depending on the taxa examined and analytical methods used. The concatenated analyses of mt and nuclear genes result in the almost equal supports to each of the three hypotheses (Yonezawa et al. 2007).

Phylogenetic analyses of the more than 22 kb data set of noncoding intron DNA provided unambiguously strong support for the grouping of Musteloidea *sensu stricto* and Ailuridae to the exclusion of Mephitidae (Fig. 3). The other two alternative hypotheses were both rejected by the AU and SH tests ($P < 0.05$; data not shown). This result is in contradiction to the mt studies (Ledje and Arnason 1996a, b; Delisle and Strobeck 2005; Arnason et al. 2007), but in agreement with the nuclear studies (Fulton and Strobeck 2006; Sato et al. 2009). In addition, earlier proposals of the red panda as a member of the family Procyonidae (Zhang and Ryder 1993; Slattery and O'Brien 1995) or as a sister taxon of Ursidae plus Pinnipeds (Wyss and Flynn 1993; Vrana et al. 1994) were not supported here ($P < 0.05$). To our knowledge, the present work provides the first large-scale intron data to resolve the trichotomy in Musteloidea.

Unfortunately, our results cannot resolve the problematic relationship among Musteloidea, Ursidae, and Pinnipeds, possibly due to limited taxon sampling. Their relationships are particularly sensitive to the treatment of gaps, whereas all the other branches are not affected. The Ursidae/Pinnipeds monophyly tree and Ursidae-basal tree were both discovered here with weak support, although most combined analyses

and POFAD analyses support the Ursidae/Pinnipeds monophyly tree. The clustering of Ursidae and Pinnipeds is congruent with the traditional morphological point of view (Flower 1869; Wyss and Flynn 1993; Hunt and Barnes 1994) and a most recent study on cytochrome b sequences of 243 taxa in Carnivora (Agnarsson et al. 2010), whereas the placement of Ursidae as a basal branch is supported in most current analyses of mt genomes (Fulton and Strobeck 2006; Arnason et al. 2007) and nuclear genes (Yu et al. 2004; Flynn et al. 2005; Yu and Zhang 2006; Sato et al. 2009; Schröder et al. 2009). We expect that the analyses with a denser taxonomic sampling might help to address this problem.

Powers and Pitfalls of Phylogenetic Studies Using Introns as Markers

In earlier phylogenetic practices, nuclear intron sequences have often been either avoided in the initial step of primer design or discarded in the tree-building methods. The major problem with intron regions stems from the fact that they add to difficulties in data acquisition, alignment, and analysis, as a result of their higher rates of variation and frequencies of indels. However, several recent intron studies have indicated that introns might also hold considerable signals for resolution of difficult phylogenies (Benavides et al. 2007; Matthee et al. 2007; Möller-Krull et al. 2007; Dalebout et al. 2008; Schröder et al. 2009). Our analyses of 22 intron sequences support their contentions and show that the noncoding intron genes offer powerful complementary data indispensable for further understanding of ambiguous phylogenies that had been difficult to resolve conclusively with the most commonly used mt or nuclear coding genes. For example, the results of the present study gave strong support for the clustering of Musteloidea *sensu stricto* and Ailuridae to the exclusion of Mephitidae.

The availability of published mt genomes and four nuclear protein-coding markers (IRBP, TBG, RAG1, and APOB) across all Caniformia families (Delisle and Strobeck 2005; Flynn et al. 2005; Fulton and Strobeck 2006; Sato et al. 2006; Arnason et al. 2007) allowed us to compare the phylogenetic utility of different classes of genetic markers at interfamilial level. Compared with mt and nuclear coding genes, noncoding introns show lower levels of character homoplasy (introns: consistency index of 0.837; mt coding genes: consistency index of 0.474; nuclear coding genes: consistency index of 0.826) and larger gamma-shape parameters (introns: $\alpha = 1.658$; mt coding genes: $\alpha = 0.744$; nuclear coding genes: $\alpha = 0.546$), suggesting their apparent absence of significant site-to-site rate variation. Comparisons of the relative rate of evolution among introns, mt, and nuclear coding genes revealed that introns and mt coding genes are more variable than nuclear coding genes, with the nuclear coding genes showing a roughly two and five times lower rate of nucleotide variation than introns and mt coding genes, respectively (Fig. 2). Interestingly, one of the introns examined here (Atp5d-2) displays nucleotide variation comparable to mt coding genes (Fig. 2). The distinct rates of evolution observed in these introns make them potentially useful to resolve relationships over a range of taxonomical levels. In conclusion, these newly developed introns are likely to be also useful for other investigations within Carnivora and mammals more broadly.

Although introns as phylogenetic markers have received considerable attention in recent years, it must be recognized that working with introns is not as straightforward as working with mt and nuclear protein-coding data (Creer 2007; Sang 2002). IIAHs and indels, including TEs, smaller gaps and tandem repeats, all appear to be commonplace in intronic sequence data and have long been considered as much of a hindrance as help in the phylogenetic reconstruction. They make experimental work labor intensive by virtue of the additional time and money required to isolate alleles and optimize PCR amplification and sequencing. Moreover, they can create positional homology problems associated with areas of ambiguous alignment (Creer 2007). These issues are central to the appropriate application of intron data in phylogenetic reconstruction and they should be comprehensively and explicitly addressed in the future studies (Creer et al. 2006).

Conclusions

The increasing availability of genomic sequence data not only advances our understanding of organismal phylogenies but also allows development of new molecular markers in nonmodel species as well. Here, the phylogenetic utility of introns was assessed using Caniformia as a model. The results showed that some of the most intractable issues in Caniformia phylogeny have been resolved. In addition, our study demonstrates the importance of identifying and incorporating IIAHs in phylogenetic intron analyses. None of the newly developed introns here has previously been used for phylogeny reconstruction, increasing the spectrum of molecular markers available to mammalian systematics.

Supplementary Material

Supplementary material, including data files and/or online-only appendices, can be found at http://www.sysbio.oxfordjournals.org/.

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