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Pattern and timing of diversification of the mammalian order Carnivora inferred from multiple nuclear gene sequences

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ABSTRACT

The mammalian order Carnivora has attracted the attention of scientists of various disciplines for decades, leading to intense interest in defining its supra-familial relationships. In the last few years, major changes to the topological structure of the carnivoran tree have been proposed and supported by various molecular data sets, radically changing the traditional view of family composition in this order. Although a sequence of molecular studies have established a growing consensus with respect to most inter-familial relationships, no analysis so far has included all carnivoran lineages (both feliform and caniform) in an integrated data set, so as to determine comparative patterns of diversification. Moreover, no study conducted thus far has estimated divergence dates among all carnivoran families, which is an important requirement in the attempt to understand the patterns and tempo of diversification in this group. In this study, we have investigated the phylogenetic relationships among carnivoran families, and performed molecular dating analyses of the inferred nodes. We assembled a molecular supermatrix containing 14 genes (7765 bp), most of which have not been previously used in supra-familial carnivoran phylogenetics, for 50 different genera representing all carnivoran families. Analysis of this data set led to consistent and robust resolution of all supra-familial nodes in the carnivoran tree, and allowed the construction of a molecular timescale for the evolution of this mammalian order.

1. Introduction

The mammalian order Carnivora exhibits a remarkable diversity of form and function, evolved as adaptations to widely different habitats, ranging from equatorial deserts and forests to temperate mountains and polar marine environments. Carnivorans are very widespread geographically, and demonstrate one of the most extreme cases of size variation among all mammalian orders (from a ~45 g weasel to a 3700 kg elephant seal). Members of this group range from charismatic species well known to the general public (e.g. cats, dogs, bears) to mysterious organisms about which almost nothing is known beyond museum materials used in original taxon descriptions (MacDonald, 2001; Nowak, 1999).

There are currently 286 recognized living carnivoran species, classified into 125 different genera (Wilson and Mittermeier, 2009) traditionally placed in 11 families (Nowak, 1999; Wozencraft, 1993). Due to their diversity, public and scientific appeal, and rich fossil record, carnivorans have been historically the subject of extensive evolutionary studies, including numerous attempts to resolve phylogenetic relationships among some or all of their lineages (e.g. Bininda-Emonds et al., 1999; Flynn et al., 2000, 2005; Wozencraft, 1989). Phylogenies of the order Carnivora have been used to make inferences on processes involved in taxon diversification patterns, tempo and mode of character evolution, and conservation-related issues. As many carnivoran species have suffered tremendous anthropogenic impact on their populations and habitats, many of them are endangered or likely to become so in the future (Gittleman et al., 2001). A phylogenetic framework (Bininda-Emonds et al., 1999) has been used to make assessments of biological and geographic features related to extinction vulnerability, which were proposed to serve as guides in the design of conservation strategies. For such purposes, as well as for other

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biological applications, it is critical to assess whether the underlying phylogenies are accurate, and to obtain a stable evolutionary framework for this group. Likewise, insights from the rich carnivoran fossil record can be greatly augmented by synergistic interaction with a well-established evolutionary timescale derived from molecular data.

The order Carnivora is divided into two main evolutionary lineages: the suborders Feliformia and Caniformia. The suborder Feliformia has traditionally comprised four families (Felidae, Herpestidae, Hyaenidae and Viverridae), while Caniformia would contain the terrestrial families Canidae, Mustelidae, Procyonidae and Ursidae, along with the marine carnivores (Pinnipedia, which include the families Otariidae, Odobenidae and Phocidae). Caniformia is further subdivided into the Cynoidea (containing the family Canidae, thought to be the deepest divergence in this group) and Arctoidea (with the six remaining families in this suborder). This taxonomic arrangement was first proposed by Flower (1869) on the basis of the form and structure of the auditory bulla, and has since been consistently supported by numerous phylogenetic studies employing other anatomical/morphological and molecular characters (e.g. Flynn and Wesley-Hunt, 2005). Despite this consistency with respect to higher level relationships, extensive controversy has dominated the evolutionary literature regarding the phylogenetic relationships among families in each suborder, the placement of enigmatic taxa (e.g. giant and red pandas, walrus, and the Malagasy fossa [*Cryptoprocta*]) and even the monophyly of several families. The extensive literature covering these controversies will not be described here in detail (see Flynn and Wesley-Hunt (2005) and Eizirik and Murphy (2009) for reviews), and we will focus only on the most recent developments regarding these issues.

Recent challenges to the monophyly of traditional families have included the following propositions: (i) the African Palm civet (*Nandinia binotata*) is a basal feliform, and not included in the Viverridae (Flynn, 1996; Hunt, 1987); (ii) Asian linsangs (genus *Prionodon*) are also removed from the Viverridae, and constitute the sister-group to felids (Gaubert and Veron, 2003); (iii) Malagasy carnivores usually placed in the Herpestidae and Viverridae actually form a separate feliform clade, not included in either family (Yoder et al., 2003); (iv) skunks do not belong in the Mustelidae, and form a separate arctoid clade (Dragoo and Honeycutt, 1997; Wayne et al., 1989). These hypotheses have been proposed on the basis of morphological (i) and molecular (i–iv) data, with the latter based on DNA–DNA hybridization results (iv), mtDNA sequences (i–iv) and DNA sequences from 1 to 3 nuclear loci (iii). Recent papers have corroborated one or more of these phylogenetic propositions (e.g. Arnason et al., 2007; Flynn et al., 2005; Fulton and Strobeck, 2006; Johnson et al., 2006; Perelman et al., 2008), but none has addressed all of them simultaneously, nor used independent, multigene data sets to specifically test these hypotheses.

Phylogenetic relationships among most families have remained unresolved or tentative for decades. Recent studies have clarified several portions of the tree (e.g. sister-group relationship between Procyonidae and Mustelidae – Flynn et al., 2000), primarily using DNA sequences from one or a few mtDNA or nuclear segments. Most of these papers have focused on a single or a few phylogenetic issues, and so far no molecular study has attempted to address all outstanding problems using a single data set that covers all living families. Such a data set would allow for simultaneous and comparable testing of multiple phylogenetic hypotheses, and could also be used in divergence dating analyses of the whole order. This joint assessment would permit the construction of a unified phylogenetic framework and molecular timescale for the order Carnivora.

In this study we aimed to resolve the phylogeny of living carnivoran families and to date all the included divergence events, by generating a large, multi-gene data set composed exclusively of segments from the nuclear genome. Nuclear sequences have been found to be more informative than mtDNA at different phylogenetic levels (e.g. Koepfli and Wayne, 2003; Springer et al., 2001), and have been successfully used to resolve various portions of the mammalian phylogeny (e.g. Amrine-Madsen et al., 2003; Eizirik et al., 2001, 2004; Johnson et al., 2006; Koepfli et al., 2006, 2007, 2008; Koepfli and Wayne, 2003; Murphy et al., 2001a,b; Sato et al., 2006; Yu et al., 2004; Janecka et al., 2007). Concatenation of multiple independent segments has been shown to produce an amplification of the phylogenetic signal, usually leading to well-resolved and supported trees (e.g. Rokas et al., 2003; de Queiroz and Gatesy, 2007). In particular, we selected a novel set of genes, most of which have not been used previously in higher-level carnivoran phylogenetics (e.g. Flynn et al., 2005; Gaubert and Veron, 2003; Yoder et al., 2003; Sato et al., 2004, 2006; Yu et al., 2004), thus providing an independent test for many recently proposed supra-familial hypotheses. Using this data set and multiple inferential approaches, we arrived at a well-resolved phylogeny presenting congruence among methods and high support for all higher-level nodes. Divergence dating analyses based on this data set produced an evolutionary timescale of living carnivoran lineages, and led to inferences on historical processes involved in the diversification of this mammalian order.

2. Materials and methods

2.1. Taxon sampling

The major goal of our taxon-sampling scheme was to represent all extant carnivoran families, as well as the most basal divergence within each family (i.e. the base of each crown-group). For that purpose, we included divergent genera (one species for each) from all traditionally recognized carnivore families, as well as all additional lineages whose membership in traditional families had been questioned by previous studies (e.g. Dragoo and Honeycutt, 1997; Flynn and Nedbal, 1998; Gaubert and Veron, 2003; Yoder et al., 2003). With this scheme, we aimed to (i) sample all known or suggested extant carnivore clades; (ii) break long branches so as to maximize phylogenetic accuracy with all methods; and (iii) perform divergence dating analyses addressing the origin and diversification of each lineage. We used the previous studies performed by McKenna and Bell (1997), Flynn et al. (2000, 2005), Koepfli and Wayne (2003) and Yoder et al. (2003) as guides for the choice of the most divergent living representatives within each lineage, and attempted to sample at least two genera per putative clade (Table 1). A pangolin (*Manis pentadactyla*, order Pholidota) was used as the outgroup, given the strong recent evidence that this mammalian lineage is the closest living relative of carnivores (Murphy et al., 2001a,b; Amrine-Madsen et al., 2003).

Biological samples (blood, tissue, DNA) from the selected species were obtained from collaborators working in field ecology projects, zoological parks and museums. Genomic DNA was extracted from these materials using a standard phenol–chloroform method (Sambrook et al., 1989) or a QIAmp DNeasy Mini Kit (Qiagen), and evaluated using spectrophotometer quantification and/or agarose gel analysis.

2.2. Selection of gene segments and data collection

We aimed to select nuclear segments exhibiting amplification performance and substitution rates (i.e. information content) appropriate for evolutionary studies of the order Carnivora. We also avoided marker overlap with previous supra-familial studies (e.g. Flynn et al., 2000, 2005; Gaubert and Veron, 2003; Yoder

Table 1
Samples analyzed in this study.

Family		Species
Traditional[a]	Updated	
Felidae	Felidae	*Felis catus*
Felidae	Felidae	*Acinonyx jubatus*
Felidae	Felidae	*Lynx lynx*
Felidae	Felidae	*Leopardus pardalis*
Felidae	Felidae	*Panthera onca*
Viverridae	Prionodontidae[b]	*Prionodon linsang*
Hyaenidae	Hyaenidae	*Crocuta crocuta*
Hyaenidae	Hyaenidae	*Hyaena hyaena*
Hyaenidae	Hyaenidae	*Parahyaena brunnea*
Hyaenidae	Hyaenidae	*Proteles cristatus*
Herpestidae	Herpestidae	*Suricata suricatta*
Herpestidae	Herpestidae	*Helogale parvula*
Herpestidae	Herpestidae	*Herpestes javanicus*
Herpestidae	Herpestidae	*Ichneumia albicauda*
Herpestidae	Herpestidae	*Rhynchogale melleri*
Viverridae	Eupleridae[c]	*Cryptoprocta ferox*
Viverridae	Eupleridae[c]	*Fossa fossana*
Herpestidae	Eupleridae[c]	*Galidia elegans*
Viverridae	Viverridae	*Civettictis civetta*
Viverridae	Viverridae	*Genetta genetta*
Herpestidae	Viverridae	*Paradoxurus hermaphroditus*
Viverridae	Nandiniidae[d]	*Nandinia binotata*
Canidae	Canidae	*Canis familiaris*
Canidae	Canidae	*Nyctereutes procyonoides*
Canidae	Canidae	*Urocyon cinereoargenteus*
Mustelidae	Mephitidae[e]	*Mephitis mephitis*
Mustelidae	Mephitidae [e]	*Spilogale putorius*
Mustelidae	Mephitidae[e]	*Conepatus chinga*
Mustelidae	Mephitidae[e]	*Mydaus marchei*
Mustelidae	Mustelidae	*Eira barbara*
Mustelidae	Mustelidae	*Enhydra lutris*
Mustelidae	Mustelidae	*Lontra canadensis*
Mustelidae	Mustelidae	*Ictonyx striatus*
Mustelidae	Mustelidae	*Meles meles*
Mustelidae	Mustelidae	*Mustela vison*
Mustelidae	Mustelidae	*Martes americana*
Mustelidae	Mustelidae	*Taxidea taxus*
Otariidae	Otariidae	*Arctocephalus forsteri*
Otariidae	Otariidae	*Zalophus californianus*
Phocidae	Phocidae	*Phoca vitulina*
Phocidae	Phocidae	*Mirounga angustirostris*
Odobenidae	Odobenidae	*Odobenus rosmarus*
Procyonidae	Ailuridae[f]	*Ailurus fulgens*
Procyonidae	Procyonidae	*Bassariscus astutus*
Procyonidae	Procyonidae	*Nasua nasua*
Procyonidae	Procyonidae	*Potos flavus*
Procyonidae	Procyonidae	*Procyon lotor*
Procyonidae	Procyonidae	*Bassaricyon alleni*
Ursidae	Ursidae	*Ailuropoda melanoleuca*
Ursidae	Ursidae	*Ursus arctos*
Outgroup	Pholidota	*Manis pentadactyla*

[a] Based on traditional sources such as Wozencraft (1993), McKenna and Bell (1997), Nowak (1999).
[b] Gaubert et al. (2005).
[c] Yoder et al. (2003), Wozencraft (2005).
[d] McKenna and Bell (1997), Wozencraft (2005).
[e] Dragoo and Honeycutt (1997), Wozencraft (2005).
[f] Wozencraft (2005).

et al., 2003), so as to produce a molecular data set that was independent from previous ones. This is relevant in the context of testing phylogenetic hypotheses generated with existing molecular data sets (e.g. position of the red panda and Asian linsangs, monophyly of Malagasy carnivores, and distinctiveness of Mephitidae). While we were working on this study, a parallel paper addressing arctoid relationships was published which independently employed three of the same segments used here (Fulton and Strobeck, 2006), thus resulting in a minor degree of overlap with our data set. In addition, two recent studies focusing on the internal phylogeny of families Procyonidae and Mustelidae also included markers that overlapped with those used in this study (three segments in Koepfli et al. (2007) and nine segments in Koepfli et al. (2008)).

Gene segments were selected from the following sources: (i) markers used in previous phylogeny studies (Eizirik et al., 2001; Murphy et al., 2001a,b); (ii) markers used in recent phylogenies of the carnivore families Felidae, Hyaenidae and Mustelidae (Johnson et al., 2006; Koepfli and Wayne, 2003; Koepfli et al., 2006); and (iii) novel markers developed in our laboratories for evolutionary and genome mapping studies (Murphy and O'Brien, 2007). A total of 33 candidate segments were empirically evaluated for use in this study, through PCR amplification (conditions given below) and sequencing in a panel of species representing several feliform and caniform families. Fourteen segments presenting consistent amplification, good-quality sequence, sufficient variability among taxa and no evidence of paralogy (as assessed by phylogenetic consistency among segments and also relative to well-established portions of the topology) were included in the study. The selected segments were *ADORA3*, *APOB*, *APP*, *ATP7A*, *BDNF*, *CHRNA1*, *FBN1*, *FES*, *GHR*, *PLP1*, *PNOC*, *PTPRG*, *RAG2* and *RASA2*, for which primer sequences are given in the following source papers: Venta et al. (1996) [*FES, GHR*]; Lyons et al. (1997) [*CHRNA1*]; Murphy et al. (1999) [*PLP1*]; Eizirik et al. (2001) [*ADORA3, APP, ATP7A, BDNF, PNOC, RAG2*]; Amrine-Madsen et al. (2003) [*APOB*]; Johnson et al. (2006) [*RASA2*]; Janecka et al. (2007) [*FBN1*]. The *PTPRG* segment (Murphy and O'Brien, 2007) was amplified with primers PTPRG-F (5′-AAATGGAAATGGTCCCATGA-3′) and PTPRG-R (5′-GCAGTAACACT-GATCATATAGTGCAAA-3′).

We strived to minimize missing data by repeating PCR and/or sequencing reactions multiple times until high-quality data could be obtained. In some cases, sequences for the same species were obtained from GenBank and/or from previous data sets generated by our groups (Amrine-Madsen et al., 2003; Johnson et al., 2006; Koepfli et al., 2006, 2007, 2008; Koepfli and Wayne, 2003; Murphy et al., 2001a – see Supplementary Material for accession numbers of previously published sequences, along with additional information on our supermatrix composition). Of the 474 new taxon-segment combinations sequenced specifically for this study (deposited in GenBank under Accession Nos. GU930839-GU931312), all except four were generated from the same species representing each genus (Table 1). The four exceptions were cases where the target species could not be amplified, but a closely related representative of the same genus could: *Canis lupus* instead of *C. familiaris* for *APOB* (thought to be conspecific in this case), *Conepatus leuconotus* instead of *C. chinga* for *APOB* and *FBN1*; and *Ursus americanus* instead of *U. arctos* for *APOB*. Since the monophyly of each of these genera is widely accepted, the use of these four instances of within-genus chimerization should have no effect on the results, especially given the deeper phylogenetic scope of our analyses.

PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) and a touchdown profile for all segments (annealing temperature decreasing from 60 to 50 °C in the first 10 cycles, with 2 °C lowered every two cycles). PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase, and sequenced using BigDye chain terminator chemistry (Applied Biosystems). Sequencing products were purified using Sephadex G-50 plates, and analyzed with an ABI 3700 or ABI 3730 automated DNA sequencer. Sequences were obtained for both DNA strands of each segment, with at least one read per strand. Up to eight total sequencing passes (including multiple forward and reverse reads) were performed per segment, allowing for in-depth verification of sequence accuracy and features such as heterozygous sites. The two or more sequencing reads of each segment were combined and then manually checked and corrected using Sequencher (GeneCodes). Verified sequences were aligned with ClustalX (Thompson et al., 1997), and the resulting alignment was checked

and improved by eye using Se–Al (http://tree.bio.ed.ac.uk/software/seal). As a further checking and adjustment step, in some cases alignments of exon sequences were verified by translating these sequences into amino acids and comparing them with the orthologous human gene sequence. Regions of ambiguous alignment were removed from all analyses (see Section 3).

2.3. Phylogenetic analyses

Phylogenetic analyses using maximum parsimony (MP) and distance-based (employing the neighbor-joining [NJ] algorithm [Saitou and Nei, 1987]) approaches were performed using PAUP*4.0b10 (Swofford, 2002) for each nuclear segment separately, to evaluate its information content and to assess the occurrence of any conflict among segments. Since no conflict was detected and extensive congruence was observed among single-gene phylogenies (results not shown), all segments were concatenated into a single data set, which was used for all subsequent phylogenetic and dating analyses.

Final phylogenetic analyses were performed with the following methods: (i) Maximum likelihood (ML) with PAUP*; (ii) ML using the program PHYML (Guindon and Gascuel, 2003); (iii) ML using the genetic algorithm implemented in MetaPIGA (Lemmon and Milinkovitch, 2002); (iv) Bayesian Inference (BI) using the program MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001); (v) Bayesian relaxed phylogenetics as implemented in BEAST 1.5.2 (Drummond and Rambaut, 2007); (vi) Maximum Parsimony (MP) with PAUP*; and (vii) Distance-based, using a Minimum Evolution (ME) heuristic search in PAUP*.

For the likelihood-based analyses, a model of DNA sequence evolution must be assumed, and selection of a model that best fits the data while minimizing the number of free parameters is critical (Whelan et al., 2001). Model selection was performed for the full concatenated data set in PAUP* with a top-down approach, using likelihood-ratio tests (LRT) and the Akaike Information Criterion (AIC) to contrast multiple alternative (simpler) models with the most complex model available (GTR + Γ + I: General-Time-Reversible with gamma correction for rate heterogeneity among sites and an estimated proportion of invariant sites). The goal was to select the simplest possible model whose fit was not significantly worse than GTR + Γ + I, thus eliminating unnecessary parameters that contribute variance to the overall estimates. The selected model (using the AIC as the tie-breaking criterion) was a special case of GTR + Γ + I with four substitution rates instead of six (rAG and rCT [transitions] are compressed into a single rate, as are rAC and rCG). This model and its estimated parameters were used in all ML analyses with PAUP*, and the closest possible available model was used for the other likelihood-based analyses: GTR + Γ + I (with parameters estimated during the run) for PHYML and MrBayes, and HKY85 + Γ + I for MetaPIGA. In the case of BEAST, each of the 14 gene segments was allowed to have an independent GTR + Γ + I model and evolutionary rate per branch (i.e. its own clock model), with parameters estimated during the analysis (see section on 'Divergence Dating' below for more details of the BEAST analysis).

The ML analysis in PAUP* used a heuristic search starting from an NJ tree, followed by unconstrained NNI branch swapping. To assess whether NNI was sampling tree space appropriately (so it could be confidently applied in subsequent searches), an equivalent search using unconstrained TBR branch swapping (much more thorough and computationally intensive) was also performed, achieving an identical tree and likelihood score. Therefore, ML-PAUP* support values for the observed clades were calculated using 100 nonparametric bootstrap replications with NNI branch swapping and all settings identical to the original search for the optimal tree.

PHYML searches used the program defaults, and support was assessed using 100 bootstrap replications. This was conducted through the generation of replicate data sets with the program SeqBoot (from the PHYLIP package), followed by the PHYML algorithm for each of them, and the computation of a consensus tree with CONSENSE. The MetaPIGA analysis used 4 populations of 4 trees each, with the majority-rule consensus tree being derived from the final 400 trees.

The MrBayes analyses used the Metropolis-coupled MCMC approach, with random starting trees, uniform prior distributions and four separate chains (one cold, three heated) that could exchange states periodically. The following set of priors was used in all analyses: Dirichlet priors for six substitution rates of the GTR model (1, 1, 1, 1, 1, 1); a Dirichlet prior for base frequencies (1, 1, 1, 1); a uniform prior for the proportion of invariant sites (0, 1); a uniform distribution prior for the shape parameter of the gamma distribution of rate heterogeneity among sites (0, 200); all topologies equally probable; and unconstrained branch lengths with an exponential probability density. Chains were run for 500,000 generations, with samples taken every 100 generations. Convergence onto a stable range of likelihood scores, evaluated visually with the program Tracer (Rambaut and Drummond, 2007), was achieved after *ca.* 30,000 generations; allowing for a conservative cutoff, trees generated in the first 50,000 generations were excluded as burn-in. The remaining 4500 trees were used to produce a majority-rule consensus in PAUP*, from which clade posterior probabilities were assessed. This analysis was run twice to confirm convergence between independent runs. A third MrBayes analysis was performed allowing each of the 14 nuclear segments to be a separate data partition, for which all model parameter values were estimated separately. This MCMCMC run was performed with four chains as above, and run for 5 million generations, with samples taken every 100 generations (total of 50,000 trees produced). Majority-rule consensus trees were produced from the 45,000 trees remaining after removal of the first 500,000 generations as burn-in.

The MP phylogeny was obtained with a heuristic search using 50 replicates of random taxon addition, equally weighted characters, gaps counted as missing data and tree bisection–reconnection (TBR) branch swapping. Support was assessed with 1000 nonparametric bootstrap replicates, each including 10 replications of random taxon addition. The ME (distance-based) tree was calculated using ML distances and a heuristic search consisting of TBR branch swapping on a starting NJ phylogeny; support for the observed groups was estimated with 1000 nonparametric bootstrap replicates.

To further test the stability of the two most difficult nodes in Caniformia (base of the Mustelida [Mephitidae, Ailuridae, Musteloidea] – node 39 in Fig. 1 [see Section 4 for clade name definition]; and base of the Arctoidea [Ursidae, Pinnipedia, Mustelida] – node 45), we performed additional phylogenetic analyses focusing on these portions of the tree, and varying the immediate outgroups to each clade of interest. All taxa in the respective ingroup were maintained and all possible combinations of immediate outgroups were used in separate searches: different sets of ursids and pinnipeds were used in the analyses assessing stability of relationships within Mustelida; and different canids were used for those addressing the basal nodes in the Arctoidea. Both sets of analyses were performed using 100 bootstrap replicates of an ML heuristic search in PAUP*, started from an NJ tree and performing NNI branch swapping. In the case of the most difficult node (relative positions of Mephitidae, Ailuridae and Musteloidea), this outgroup jackknifing analysis was complemented by a Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) contrasting the three alternative resolutions of this node.

2.4. Divergence dating

Dating analyses were performed using two different approaches: (i) the relaxed molecular clock method implemented in

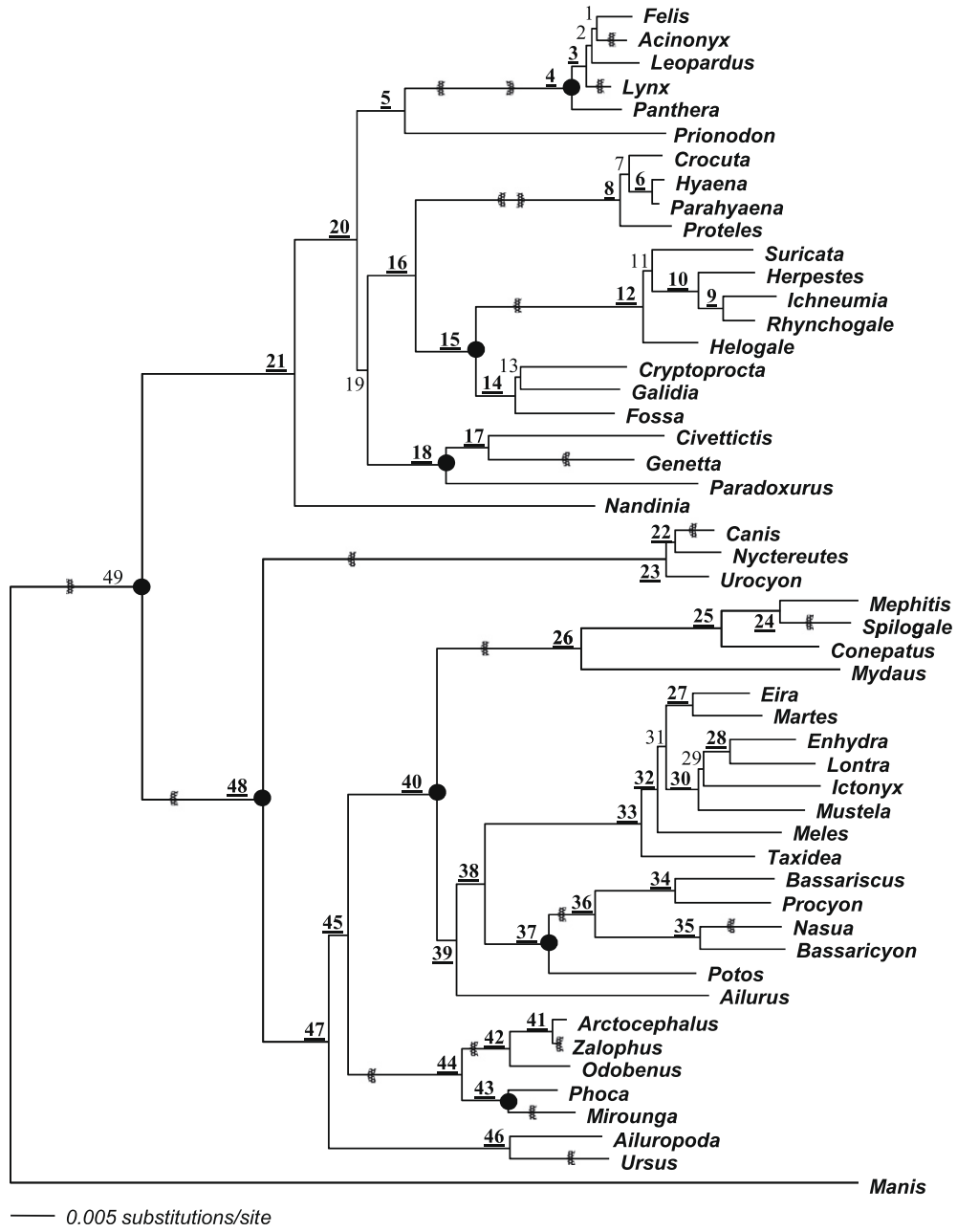

Fig. 1. Maximum likelihood phylogram depicting the evolutionary relationships of major extant lineages of the order Carnivora. Nodes are numbered sequentially for cross-reference with Tables 2–6. Numbers in bold, underlined types indicate nodes supported by >90% bootstrap values (or posterior probabilities) for all likelihood-based phylogenetic methods (see Table 4 for detailed results). Hatched arrowheads indicate the phylogenetic position of the 21 fossil constraints used in our final divergence dating analysis with the program Divtime (see Table 2 for details): right-pointing arrowheads are maximum ages for the subsequent node, while left-point arrowheads are minimum ages for the previous node. Black circles on nodes indicate the eight calibrations used in the BEAST divergence dating analysis that presented the best posterior probability (Run 2 – see text for details).

the program package Divtime (Kishino et al., 2001; Thorne et al., 1998), and (ii) the uncorrelated relaxed molecular clock method implemented in BEAST. Although both approaches employ Bayesian methods that allow branches to have variable evolutionary rates while incorporating multiple fossil constraints, each employs different model assumptions and calibration strategies, rendering their comparison interesting from a methodological standpoint. For the Thorne/Kishino method, the maximum likelihood tree obtained in PAUP* was used as the starting point for branch-length estimation (along with a rate variance–covariance matrix) using the program *estbranches* and the F84 model of sequence evolution (the closest available to our best-fit model), with parameters re-estimated from the data set. Results from *estbranches* were used as input for the dating estimation with the program *divtime5b*, based on 1 million generations computed after exclusion of a 100,000-generation burn-in. These analyses incorporated various sets of fossil constraints on node ages, using up to 25 different calibrations. The reliability of the fossil constraints was evaluated through cross-comparison among calibration points, aiming to test for consistency across the carnivoran tree. This was performed by successively removing or relaxing one or more constraint at a time, and comparing the estimated age of the relevant node (based on the remaining calibrations) and the fossil-based age. This allowed us to identify some instances where the fossil age was incompatible with its assumed phylogenetic position and the overall age estimate of its containing clade, and to fine-tune our set of fossil

calibrations to produce a conservative ensemble that was internally consistent. Overall, 12 different runs of this dating exercise were performed, varying the set of fossil constraints or the mean of the distribution of the root age prior, to assess their impact on our posterior estimates of nodal ages. Our final set of analyses was run with 21 conservative fossil constraints (Table 2), and three alternative priors for the root age. The main final run used a mean of 55 million years ago (MYA) for the prior probability distribution of the ingroup root age, based on the oldest caniform fossils (McKenna and Bell, 1997) and previous molecular estimates (e.g. Springer et al., 2003). To assess whether the posterior estimate for ages across the tree might be biased by this prior probability, two extreme final variants were also performed, in which this mean root prior was either doubled (i.e. 110 MYA) or halved (i.e. 27 MYA).

For the BEAST analyses, the uncorrelated lognormal model was used, and each of the 14 genes was allowed to have separate substitution and clock parameters (see above). The underlying topology was kept linked for all segments, and was estimated from the alignments during the run, independently of any prior topological assumption (i.e. no clade was constrained to be monophyletic). The MCMC procedure was run for 10^8 generations, with data sampled every 5000 steps to allow for adequate mixing given the complexity of the partitioned model. The first 10^7 generations (i.e. 2000 out of 20,000 samples) were removed from all analyses as burn-in. Three different BEAST runs were performed, each incorporating a different set of fossil calibrations used as boundaries in uniform prior probabilities for specific node ages. Fossil constraints were adapted from those listed in Table 2, and in some cases complemented by conservative counterparts based on calibrations for additional nodes, or on the limits of credibility intervals observed in the Divtime analyses. Run 1 incorporated three constraints (in MYA): (i) 37–50 for node 48 (see Table 2 and Fig. 1 for node numbers); (ii) 5.3–16.4 for node 4; and (iii) 50–65 for node 49 (carnivoran root). Run 2 used the same calibrations, while adding five others: (iv) 16.4–40 for node 40; (v) 11.2–30 for node 43; (vi) 11.2–30 for node 37; (vii) 11.2–40 for node 18; and (viii) 16.4–40 for node 15. Finally, Run 3 used these eight calibrations plus four others: (ix) 3–16.4 for node 8; (x) 25–40 for node 5; (xi) 28.5–50 for node 45; and (xii) 15–40 for node 44. Results of each run were assessed and compared using Tracer 1.4.8 (including estimates of the TMRCA [time to the most recent common ancestor] for every familial and supra-familial node), and also analyzed graphically using the programs TreeAnnotator 1.5.2 and FigTree 1.3 (http://tree.bio.ed.ac.uk/software/figtree). The resulting posterior distribution of trees obtained from this analysis was employed in the phylogenetic inference along with the other six methods described above.

3. Results

3.1. Supermatrix characteristics

Nucleotide sequences were obtained for 14 nuclear gene segments in a sample of 50 carnivoran genera and one pangolin (*Manis pentadactyla)* used as the outgroup (Tables 1 and 3). This data set included 714 segments (gene-taxon combinations), of which only 55 (7.7%) were missing (i.e. data could not be collected). The taxon with the most missing data (*Mydaus*) was represented by only three out of the 14 segments, yet its phylogenetic position could be robustly inferred with all methods (see below). Of the remaining 50 taxa, the mean number of missing segments was 1.1, with the upper limits being six and five segments missing for *Potos* and *Galidia*, respectively.

The 14 nuclear segments included exonic, intronic and 3′ UTR regions, with varying degrees of molecular divergence among taxa (Table 3). Single-gene phylogenetic analyses using MP and NJ were consistent with each other, and no meaningful conflict among segments was observed (results not shown). Each individual segment resolved several inter-familial nodes, albeit usually with low or moderate support, and some of them (e.g. *GHR*, *APP*) produced remarkably resolved carnivoran trees with less than 1000 bp. Concatenation of the 14 segments produced a data set of 8493 bp, which after exclusion of ambiguously aligned sites led to a final alignment of 7765 bp, used in all analyses reported here. This data set contained 3294 variable and 2084 parsimony-informative sites,

Table 2
Fossil constraints applied in the Thorne–Kishino relaxed molecular clock divergence dating analysis. Some of these same calibrations were incorporated in the BEAST analyses (see text for details). Values indicate the age (in millions of years ago [MYA]) of fossils placed as a maximum or minimum constraint on specific nodes, labeled as in Fig. 1. All calibrations were obtained from McKenna and Bell (1997), employing conservative phylogenetic assumptions regarding fossil placement, as well as conservative usage of temporal boundaries for a given fossil age.

Node	Maximum	Minimum	Calibration/assumption
1		3.5	Oldest *Acinonyx* fossils – Early Pliocene
3		5.3	Oldest *Lynx* fossils - Late Miocene
4	16.4		Fossils of stem felids – Early Miocene
5		28.5	Oldest felid fossils – Early Oligocene
8	16.4		Oldest hyaenid fossils – Early Miocene
15		16.4	Oldest herpestid fossils – Early Miocene
16		16.4	Oldest hyaenid fossils – Early Miocene
17		11.2	Oldest *Genetta* fossils – Middle Miocene
22		5.3	Oldest *Canis* fossils – Late Miocene
24		1.8	Oldest *Spilogale* fossils – Late Pliocene
35		3.5	Oldest *Nasua* fossils – Early Pliocene
37		11.2	Assumes that Middle Miocene procyonid fossils assigned to *Bassariscus* or *Arctonasua* are contained in the crown Procyonidae, i.e. post-dating the divergence of *Potos*
40		16.4	Assumes that the Early Miocene *Miomephitis* fossils are contained in the mephitid lineage, post-dating its divergence from other arctoids
41		1.8	Oldest *Zalophus* fossils – Late Pliocene
43		11.2	Assumes monophyly of phocid tribe Monachini (and placement of *Mirounga* therein), whose oldest fossils are dated at the Middle Miocene
44		16.4	Oldest odobenid fossils – Early Miocene
45		28.5	Pinniped fossils – Early Oligocene
46		3.5	Oldest *Ursus* fossils – Early Pliocene
48		37	Oldest canid fossils – Middle Eocene
49	65		Assumes that the split between Caniformia and Feliformia occurred after the K–T boundary
49		50	Caniform fossils – Early Eocene

Table 3
Characterization of the gene segments employed in this study, including length of the final alignment, number of variable nucleotide sites, number of Parsimony-informative (P.I.) sites, and number of phylogenetically informative insertion/deletion events (indels).

Gene segment	Length (bp)[a]	Segment type	Variable sites[b]	P.I. sites[b]	Informative indels[c]
ADORA3	368 (368)	Exon	167	121	1d
APOB	942 (942)	Exon	384	243	1d
APP	665 (634)	Exon/UTR	201	109	5d, 4i
ATP7A	670 (670)	Exon	244	150	–
BDNF	563 (563)	Exon	121	75	–
CHRNA1	397 (382)	Exon/intron	253	186	6d, 1i
FBN1	731 (634)	Exon/intron	177	98	1d
FES	509 (360)	Exon/intron	220	167	5d
GHR	959 (653)	Exon/intron	332	207	1d
PLP1	1017 (953)	Exon/intron	529	323	13d, 1i
PNOC	289 (289)	Exon	118	67	1i
PTPRG	294 (294)	Exon/UTR	46	30	–
RAG2	464 (464)	Exon	162	92	–
RASA2	625 (559)	Exon/intron	340	216	13d, 1i
Total	8493 (7765)		3294	2084	46d, 8i

[a] Numbers in parentheses are final segment lengths after exclusion of ambiguously aligned sites.
[b] Estimated after exclusion of ambiguously aligned segments.
[c] Values indicate the number of inferred phylogenetically informative indels: d, deletion; i, insertion.

respectively, along with a minimum of 58 phylogenetically informative insertions/deletions (indels) (Table 3).

3.2. Phylogenetic relationships

Relationships within and among families were consistently resolved by the concatenated data set (Figs. 1 and 2; Table 4). All family-level and inter-familial nodes were congruent and received considerably high bootstrap and posterior probability support with all phylogenetic approaches (84–100% for likelihood-based methods; 71–100% for MP and ME), indicating considerable stability of the derived topology. Within-family nodes were also congruent across methods, but did not always receive similarly high support (see Table 4). Overall, 35 out of 48 nodes had support values >90% for all methods.

Consistent resolution of the phylogenetic relationships among carnivoran families sheds light onto several outstanding issues. Families Viverridae, Herpestidae and Mustelidae are not monophyletic considering their traditionally recognized membership of genera (compare Table 1 and Fig. 2), but in each of them a stable core can still be discerned, warranting continued usage of these taxonomic entities for this restricted monophyletic subset (Fig. 2). In addition to the 11 traditional families (including those with modified membership), five other major carnivoran lineages can be clearly delimited (Fig. 2), leading to a proposition of a total of 16 families in this mammalian order.

In the feliform clade, the Asian linsang (genus *Prionodon*, traditionally placed in the Viverridae) was the sister-group of the Felidae, warranting family-level status (Prionodontidae). Malagasy carnivores formed a monophyletic clade, including genera previously placed in the Viverridae and Herpestidae (see Table 1), and should also comprise a separate family (Eupleridae). The African palm civet (*Nandinia binotata*) is indeed very divergent from all other feliforms, which corroborates the evidence put forth by previous authors (e.g. Flynn et al., 2000, 2005; Flynn and Nedbal, 1998; Hunt, 1987; Koepfli et al., 2006) arguing that it represents its own monotypic family (Nandiniidae). Overall, the inferred phylogenetic structure of Feliformia is markedly different from the traditional view of this suborder (e.g. Nowak, 1999), with seven major clades instead of the usually recognized four families, and taxa previously assigned to the Viverridae now divided among four different families. Inter-familial relationships in Feliformia were also well supported with the present data set (Figs. 1 and 2; Table 4). The most basal lineage is Nandiniidae, followed by the (Felidae + Prionodontidae) clade; the next to diverge was Viverridae, followed by Hyaenidae, and leaving an internal clade composed of (Eupleridae + Herpestidae).

In the caniform clade, most outstanding issues have been resolved with high support by the present analyses (Figs. 1 and 2, Table 4). Skunks and the stink badger (genus *Mydaus*) formed a monophyletic clade separate from the Mustelidae (where they were traditionally placed), warranting family-level recognition as Mephitidae. The sister-group relationship between *stricto sensu* Mustelidae (skunks excluded) and *stricto sensu* Procyonidae (red panda excluded) was strongly supported, forming a clade that we will refer to henceforth as Musteloidea (see Section 4). The red panda (*Ailurus*) was found to be the sister-group to the Musteloidea, supporting its placement in a separate, monotypic family (Ailuridae). The relationships among Ailuridae, Mephitidae and Musteloidea, which have been unresolved or contentious in previous studies (e.g. Flynn et al., 2005, 2000; Fulton and Strobeck, 2006; Sato et al., 2006), have achieved stability with this concatenated nuclear data set (Fig. 2), with Mephitidae placed as the most basal clade in this group. We will refer to this whole clade (Musteloidea + Ailuridae + Mephitidae) as Mustelida (see Section 4).

Our analyses strongly supported the monophyly of Pinnipedia (Figs. 1 and 2, Table 4), and within it the sister-group relationship between Otariidae and Odobenidae. The relative relationships among Ursidae, Pinnipedia and Mustelida were also resolved, with the former being the most basal clade in the Arctoidea (see Fig. 2).

Given the historical difficulty in resolving the relative positions of the red panda (*Ailurus*), skunks (Mephitidae) and Musteloidea (e.g. Delisle and Strobeck, 2005; Flynn et al., 2005; Flynn and Wesley-Hunt, 2005; Fulton and Strobeck, 2006), the short branch observed prior to this split (Fig. 1), and the somewhat lower support estimated here relative to most other nodes (Table 4), we performed additional analyses to further verify the stability of these relationships. These analyses focused on a restricted data set containing all mustelids, procyonids, mephitids and the red panda, as well as a varied set of outgroups drawn from other arctoid clades. The goal was to test the stability of the relationships among the main branches of the Mustelida (defined by nodes 38, 39 and 40 in Fig. 1), given an outgroup jackknifing procedure. Seven different outgroups were tested, and the results of all runs strongly supported the Musteloidea + *Ailurus* clade, with bootstrap support >95% (Table 5). The Shimodaira–Hasegawa (SH) test also provided support for the resolution of this node shown in Figs. 1

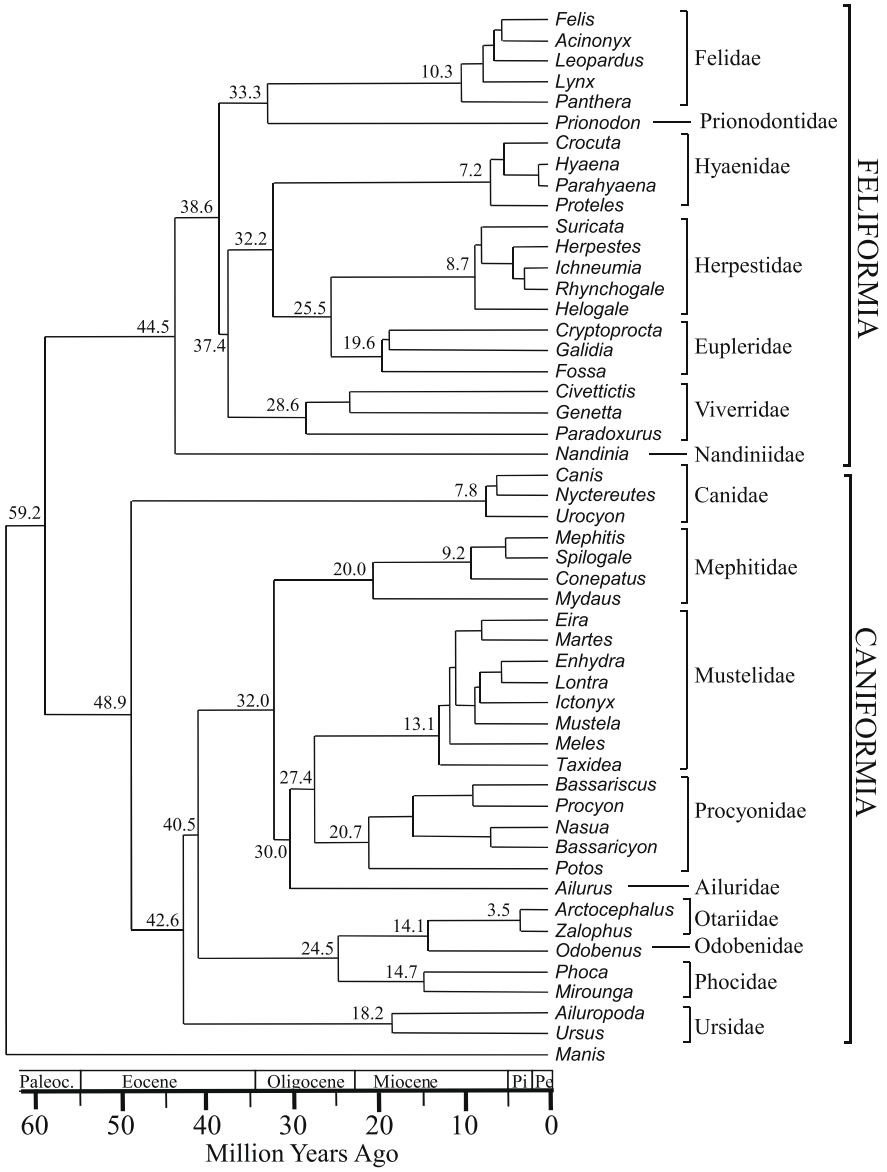

Fig. 2. Timescale of carnivoran diversification, based on the Thorne/Kishino relaxed molecular clock method, incorporating 21 fossil calibrations (similar results were obtained with BEAST – see Fig. 3, Table 6 and Supplementary Information). The tree is a cladogram with node depths (branch lengths) drawn proportional to time, with a timescale shown at the bottom, including the paleontological epochs of the Cenozoic Era (Paleoc. = Paleocene; Pi = Pliocene; Pe = Pleistocene). Family names are indicated on the right, as are the major suborders Feliformia and Caniformia. Numbers above nodes are divergence ages in MYA (millions of years ago); only nodes defining families or higher clades are labeled (the only exception is the New World sub-clade of the Mephitidae, mentioned in the main text); see Table 6 for point estimates and credibility intervals for node ages estimated with Divtime and BEAST.

and 2. Even though the SH test is conservative, the alternative topology with *Ailurus* grouped with Mephitidae was significantly worse than our best tree ($p = 0.0367$), while the third possibility (*Ailurus* as the most basal divergence in Mustelida) was marginally non-significant ($p = 0.06$).

The outgroup jackknifing test was also employed to assess another portion of the caniform phylogeny that has been historically difficult to resolve, i.e. the relationship between Pinnipedia, Ursidae and Mustelida. In this case we used 12 ingroup taxa that maximized lineage and character coverage (*Meles*, *Enhydra, Taxidea, Bassariscus, Bassaricyon, Ailurus, Conepatus, Zalophus, Odobenus, Mirounga, Ailuropoda, Ursus*), and four alternatives for the outgroups (the three canids together or each of them separately). Using the three canids jointly, support for the Pinnipedia + Mustelida clade was 98%; when each was used separately, support for this clade ranged from 90% to 96%.

3.3. Divergence dating

Divergence dates for all major nodes in the crown Carnivora were estimated using two different relaxed molecular clock methods (Table 6). Although some nodes presented discrepant dates between the two approaches (e.g. nodes 4, 42, 44 – see Table 6), most of the estimated ages were quite congruent, to the extent that point estimates for all 27 familial and supra-familial nodes were highly correlated ($r^2 = 0.95$) between the Divtime and BEAST final runs (Fig. 3A). Moreover, almost all credibility intervals overlapped between the two estimates, with the only exceptions being nodes 42 and 44 (see Table 6).

Considering each dating method separately, robust estimates seem to have been achieved as assessed by comparing multiple runs with varying parameters. In the case of Divtime, point estimates and credibility intervals for node ages were very consistent

Table 4
Support values obtained with different phylogenetic methods for each node indicated in Fig. 1. Nodes defining family-level clades are shown in bold underlined font. Nodes involved in the definition of monotypic family-level lineages are in bold italic font.

Node[a]	ML-PAUP	MetaPIGA	PHYML	MrBayes[b]	BEAST	MP	ME
1	74	98	78	1.0	1.0	85	<50
2	68	93	68	1.0	0.99	62	<50
3	100	100	98	1.0	1.0	99	99
4$^{(3)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
5$^{(2)}$	***100***	***99***	***100***	***1.0***	***1.0***	***100***	***100***
6$^{(1)}$	100	99	100	1.0	1.0	100	<50
7	66	97	70	0.95	1.0	82	<50
8$^{(7)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
9$^{(2)}$	100	96	100	1.0	1.0	100	<50
10	100	100	100	1.0	1.0	100	100
11	63	94	59	0.94	<0.5	78	64
12$^{(3)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
13	60	78	60	0.76	0.85	<50	<50
14$^{(1)}$	**100**	**97**	**100**	**1.0**	**1.0**	**100**	**92**
15$^{(1)}$	100	100	100	1.0	1.0	100	99
16$^{(1)}$	100	100	100	1.0	1.0	100	100
17	100	99	100	1.0	1.0	100	100
18	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
19	84	91	91	1.0	1.0	82	78
20$^{(1)}$	***100***	***98***	***100***	***1.0***	***1.0***	***100***	***100***
21$^{(6)}$	***100***	***98***	***100***	***1.0***	***1.0***	***100***	***100***
22	95	90	97	1.0	1.0	87	<50
23$^{(10)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
24	100	99	100	1.0	1.0	100	100
25$^{(1)}$	100	100	100	1.0	1.0	100	100
26	**100**	**93**	**100**	**1.0**	**1.0**	**100**	**97**
27	100	100	100	1.0	1.0	100	100
28	100	100	100	1.0	1.0	100	100
29	72	97	68	0.99	0.99	72	66
30	100	100	100	1.0	1.0	100	100
31	84	97	86	1.0	1.0	71	<50
32$^{(1)}$	99	100	100	1.0	1.0	98	99
33$^{(3)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
34	100	100	100	1.0	1.0	100	100
35$^{(2)}$	100	100	100	1.0	1.0	100	100
36	100	100	100	1.0	1.0	100	97
37	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
38	***99***	***98***	***99***	***1.0***	***1.0***	***97***	***81***
39	***93***	***92***	***96***	***1.0***	***1.0***	***71***	***99***
40$^{(2)}$	100	100	100	1.0	1.0	100	97
41	**100**	**97**	**100**	**1.0**	**1.0**	**100**	**96**
42$^{(2)}$	***100***	***100***	***100***	***1.0***	***1.0***	***100***	***100***
43$^{(1)}$	**100**	**99**	**100**	**1.0**	**1.0**	**100**	**100**
44$^{(1)}$	100	100	100	1.0	1.0	100	100
45	100	97	100	1.0	1.0	99	96
46$^{(2)}$	**100**	**100**	**100**	**1.0**	**1.0**	**100**	**100**
47	100	98	100	1.0	1.0	100	96
48$^{(1)}$	100	94	100	1.0	1.0	100	97

[a] Superscript values are the number of phylogenetically informative indels supporting the node.
[b] The lowest value observed among the three MrBayes runs (see Section 2) is indicated here.

Table 5
Maximum likelihood bootstrap support values for three nodes in the arctoid phylogeny, obtained by varying the outgroup taxa employed.

Outgroup	Musteloidea[c]	Musteloidea[c] + *Ailurus*	Mustelida[d]
Ursidae[a]	100	98	100
Pinnipedia[b]	100	95	100
Zalophus, *Ursus*	100	97	100
Ursus	98	100	N/A[e]
Zalophus	99	95	N/A[e]
Mirounga	100	96	N/A[e]
Odobenus	100	98	N/A[e]

[a] Includes both ursid genera sampled here: *Ursus* and *Ailuropoda*.
[b] Includes all pinnipeds sampled in this study: *Zalophus*, *Arctocephalus*, *Odobenus*, *Phoca* and *Mirounga*.
[c] Musteloidea [node 38 in Fig. 1] = Mustelidae (skunks excluded) + Procyonidae.
[d] Mustelida [node 40 in Fig. 1] = Musteloidea + *Ailurus* + Mephitidae.
[e] Bootstrap values are not derived for this node when a single outgroup is used.

Table 6
Divergence dates among carnivoran lineages, based on relaxed molecular clock analyses. Node numbers are identified in Fig. 1, and clade names are indicated when the node refers to a recognized taxon. Two relaxed clock approaches were employed (see text for details), namely the Thorne/Kishino method implemented in the program Divtime (assuming a single clock model for the whole data set), and the uncorrelated lognormal model implemented in BEAST (allowing each of the 14 segments to have independent clocks and substitution models). For each approach, the point estimate of the node age is indicated, followed by its Credibility Interval (CI). Only family-level and supra-familial nodes are included (see Supplementary Information for more detailed results obtained with each method).

Node	Clade	Thorne/Kishino		BEAST	
		Age	CI	Age	CI
4	Felidae	10.3	7.3–14.6	6.2	5.3–7.3
5		33.3	28.9–39.1	27.4	23.4–31.4
8	Hyaenidae	7.2	4.5–11.3	5.1	3.9–6.4
12	Herpestidae	8.7	6.1–12.3	10.0	8.3–11.6
14	Eupleridae	19.6	15.1–25.0	14.3	11.7–17.1
15		25.5	20.7–31.2	21.2	18.3–23.9
16		32.2	27.2–38.1	27.4	24.1–30.6
18	Viverridae	28.6	23.5–34.5	24.0	20.5–27.4
19		37.4	32.2–43.4	32.7	29.0–36.5
20		38.6	33.4–44.6	33.9	30.0–37.6
21	Feliformia	44.5	38.6–50.6	39.8	35.0–44.3
23	Canidae	7.8	5.9–11.5	5.5	4.1–7.0
26	Mephitidae	20.0	14.6–26.0	23.3	18.6–28.2
33	Mustelidae	13.0	9.6–17.1	15.6	13.5–17.8
37	Procyonidae	20.7	16.1–25.8	22.6	19.4–25.5
38	Musteloidea	27.4	22.3–32.9	29.4	26.2–32.5
39		30.0	24.7–35.6	31.9	28.3–35.0
40	Mustelida	32.0	26.6–37.7	33.8	30.3–37.1
41	Otariidae	3.4	1.9–6.3	1.4	0.7–2.3
42		14.1	9.9–19.5	7.5	5.7–9.4
43	Phocidae	14.7	11.4–20.3	11.6	11.2–12.3
44	Pinnipedia	24.5	19.5–30.5	15.9	13.9–18.0
45		40.5	34.8–46.3	39.6	35.7–43.0
46	Ursidae	18.2	12.9–24.5	12.7	9.6–15.9
47	Arctoidea	42.6	36.8–48.3	41.4	37.4–44.9
48	Caniformia	48.9	42.4–54.9	47.6	43.8–50.0
49	Carnivora	59.2	51.6–64.7	58.1	52.5–63.4

among the three final runs (with root prior ages of 55, 110 and 27 MYA), indicating that the posterior distributions of these divergence dates were robust, and not biased by the prior probabilities set for the root age (see Supplementary Information). In the case of BEAST, age estimates were very similar between Run 2 (incorporating eight fossil calibrations) and Run 3 (incorporating 12 fossil calibrations), which is also reflected in their very similar posterior probability ($-\ln L = 46{,}829.15$ *vs.* 46,841.64, respectively). Run 1 (incorporating only three fossil calibrations) produced less concordant results, and provided a significantly worse fit to the data (as assessed by the posterior probability [$-\ln L = 47{,}060.75$], whose distribution was largely non-overlapping with those of Runs 2 and 3). Given these observations, we show here (Figs. 1 and 3; Table 6) only the results obtained with Run 2, which provided a slightly better fit to the data than Run 3 while requiring fewer assumptions (i.e. fossil calibrations).

Given the overall concordance between the two methods, we used the point estimates obtained with the main Divtime run (with the root prior set at 55 MYA) to construct a timescale of carnivoran evolution, in which the age of the crown group for each extant family and the divergence time among families can be assessed and compared (Fig. 2). A similar timescale was constructed with the BEAST results (see Supplementary Information), in which the same overall patterns can be discerned.

The integrated assessment of divergence times across the order Carnivora led to some interesting observations. Comparisons could

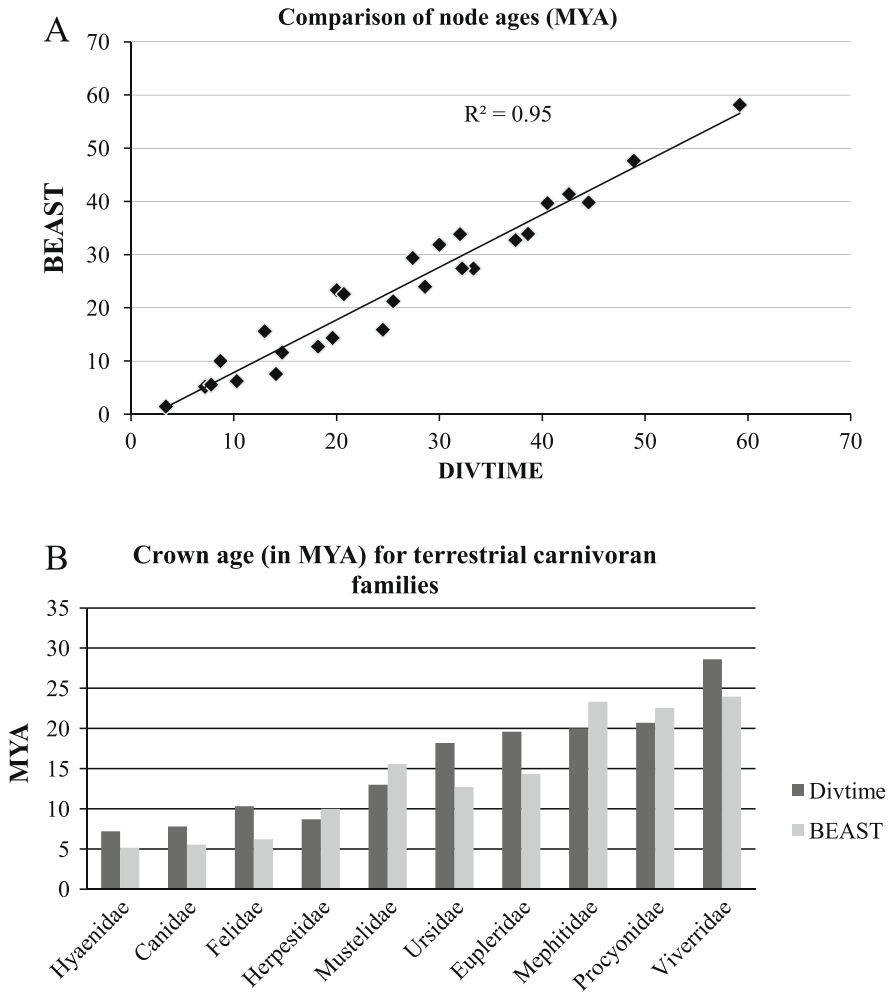

Fig. 3. (A) Comparison of point estimates for the age of 27 carnivoran familial and supra-familial nodes obtained from two different relaxed molecular clock methods, implemented in the programs Divtime and BEAST, respectively. The Divtime analysis assumed a single model for the full concatenated data set, whereas BEAST allowed each of the 14 genes to have independent substitution and molecular clock models. The Divtime approach incorporated 21 different fossil constraints (minimum or maximum) applied to nodes throughout the Carnivora tree, while the BEAST run used calibrations for eight nodes (see Fig. 1 and text for details). (B) Estimated age for the crown-group of each terrestrial carnivoran family, inferred with the Divtime and BEAST approaches. Pinniped dates are not shown here as these dates do not seem to have been as robustly estimated (given the observed discrepancy between the two methods – see Table 6), along with the fact that the base of Otariidae was not sampled (see text for details). Only point estimates are shown; see Table 6 for Credibility Intervals.

be performed across the order, especially in the case of terrestrial carnivoran families, each of which had its most basal node sampled in this study, and whose estimated age was mostly congruent between the two dating methods (Fig. 3B). The youngest family was Hyaenidae (crown age of 7.2 MYA with Divtime and 5.1 with BEAST), with results remarkably concordant with those observed for Canidae (7.8 MYA with Divtime and 5.5 MYA with BEAST). In contrast, some families presented very old crown-groups, with Viverridae being the most extreme example (28.6 MYA with Divtime and 24 MYA with BEAST – see Table 6 and Fig. 3B). Overall, the youngest family was Otariidae, with a crown age of 3.4 MYA in Divtime and 1.4 MYA in BEAST; however, recent studies (e.g. Arnason et al., 2006; Flynn et al., 2005) have indicated that *Zalophus* and *Arctocephalus* likely do not represent the most divergent extant lineages in this clade, so that the age estimated here may not be that of the family-level crown group in this case. In addition to Canidae and Hyaenidae, the crown base of three other terrestrial lineages was dated at the Late Miocene (see Table 6): Felidae, Herpestidae, and the New World component of the Mephitidae (9.2 MYA with Divtime and 10.5 MYA with BEAST – see Supplementary Information).

Above the family-level, divergence dates could be established for all nodes in both the feliform and caniform components of the order Carnivora (Table 6). The base of Feliformia was dated at 44.5 MYA with Divtime and 39.8 MYA with BEAST, highlighting the depth of divergence between *Nandinia* and the remaining extant feliforms. In the suborder Caniformia, the divergence between the superfamilies Cynoidea (family Canidae) and Arctoidea (all other caniforms) was dated at 48.9 MYA with Divtime and 47.6 with BEAST. Overall, the divergence between the two suborders (i.e. the base of crown Carnivora) was estimated to have occurred 58–59 MYA (see Table 6).

An intriguing pattern emerged when supra-familial divergence dates were compared on both sides of the carnivoran tree. Some nodes appear to have occurred in rather quick succession, suggesting the existence of periods with increased cladogenesis in the order Carnivora (Fig. 2). One such period is the Late Eocene, when two caniform nodes (45 and 47 – see Fig. 1 for node identification) occurred between 42.6 and 39.6 MYA (considering both dating methods). The Divtime results also place two closely spaced feliform nodes (19 and 20) in the same time period (see Fig. 2), whereas their dates are younger with BEAST (32.7 and 33.9 MYA), albeit still very close to each other. The Divtime dates also suggest a similar pattern for the Early Oligocene, when two caniform nodes (39 and 40) and two feliform nodes (5 and 16) occur between 33.3 and 30 MYA. The BEAST dates are very similar for

the caniform nodes, but again younger for the feliform pair (see Table 6). A third instance of multiple nodes concentrated on a time period is the Late Miocene (5–11 MYA), when several divergences were inferred to have occurred (see Table 6 and Fig. 2), including the basal diversification of the families Felidae, Hyaenidae, Herpestidae and Canidae.

4. Discussion

The evolutionary relationships among carnivoran families have been extensively investigated in the last three decades (see Flynn and Wesley-Hunt (2005), Wozencraft (1989, 2005), Eizirik and Murphy (2009) for reviews), and a growing consensus is currently emerging with respect to the supra-familial structure of this mammalian order. Some classical hypotheses have been corroborated by large molecular data sets, while new ones have recently emerged (e.g. Gaubert and Veron, 2003; Yoder et al., 2003). The last few years have seen a surge in studies employing DNA sequences to resolve these relationships, most of which focused on a single suborder (Feliformia or Caniformia). In addition, most of these studies have not attempted to date the divergences among the major carnivoran lineages, which is required to allow comprehensive comparative inferences with respect to the pattern and timing of carnivoran diversification. In this study, we have attempted to address all outstanding issues in supra-familial carnivoran systematics by generating and analyzing a large data set of nuclear genes including all extant families and major lineages within each of them. By simultaneously analyzing Feliformia and Caniformia, we were able to generate an integrated framework for consolidating the family-level taxonomy of the order Carnivora, as well as to provide an evolutionary timescale of the diversification of living lineages. We discuss below the implications of our results and inferences to different aspects of carnivoran systematics and evolutionary history.

4.1. Family-level systematics of the living Carnivora

In the last several years, many studies have been dedicated to the resolution of the carnivoran tree employing various types of morphological and molecular data sets. Some of them have revealed evidence that challenged the monophyly of traditional families, initiating a revision that has led to a major reorganization of the carnivoran phylogeny. The Viverridae has been the most problematic family, as morphological and/or molecular evidence have indicated that *Nandinia*, *Prionodon* and Malagasy species are not closely related to the rest of the family or to each other (Flynn, 1996; Gaubert and Veron, 2003; Hunt, 1987; Yoder et al., 2003). As recently as 2003, two major feliform lineages were proposed, fragmenting the classically recognized Viverridae. Gaubert and Veron (2003) showed that Asian linsangs (genus *Prionodon*), traditionally placed in the Viverridae, were the sister-group to the Felidae, a finding which was subsequently corroborated by two other papers (Gaubert and Cordeiro-Estrela, 2006; Johnson et al., 2006) using both extant species of the genus *Prionodon*. This has led to the recognition that this genus should be placed in its own family, Prionodontidae (Gaubert et al., 2005). A separate line of investigation led to a different assault on the monophyly of traditional Viverridae (and also Herpestidae) when Yoder et al. (2003) showed that Malagasy carnivorans comprised a previously unrecognized monophyletic lineage, which was corroborated by two subsequent papers (Flynn et al., 2005; Gaubert and Cordeiro-Estrela, 2006) and awarded family rank as Eupleridae (Wozencraft, 2005). In the present study we have tested and corroborated the monophyly of these two new families, employing a molecular data set that is fully independent from those originally used to propose Prionodontidae and Eupleridae.

The relationships among feliform families have also been progressively established by some of the same studies (e.g. Flynn et al., 2005; Gaubert and Cordeiro-Estrela, 2006; Gaubert and Veron, 2003; Johnson et al., 2006; Yoder et al., 2003), gradually converging on trees that are concordant with our results shown in Figs. 1 and 2. The only node that remained difficult to resolve was the one defining the placement of Viverridae (*stricto sensu*) with respect to two other clades: (Felidae + Prionodontidae) and (Hyaenidae + Herpestidae + Eupleridae). Flynn et al. (2005) concluded that this node could not be confidently resolved with their molecular data set (comprising three nuclear and three mtDNA genes), leaving it as a polytomy. On the other hand, Gaubert and Cordeiro-Estrela (2006), using a subset of the same genes (two nuclear and one mitochondrial) but a different taxon-sampling scheme (that included Prionodontidae, which had not been sampled by Flynn et al. (2005)), observed moderate to strong support for a node uniting Viverridae and (Hyaenidae + Herpestidae + Eupleridae). This hypothesis had been previously reported (e.g. Flynn and Nedbal, 1998; Gaubert and Veron, 2003; Yoder et al., 2003), but not strongly supported in those initial studies. Our data set, which is independent from (and contains more characters than) those previously employed to address this question, consistently supports the placement of viverrids as the sister-group to (Hyaenidae + Herpestidae + Eupleridae), consolidating this phylogenetic hypothesis (node 19 in Fig. 1). As suspected by Flynn et al. (2005), the branch immediately preceding this node is very short, spanning only 1.2 million years (see Fig. 2 and Table 6), which explains the difficulty in resolving this portion of the feliform tree based on previous data sets.

Concerning the caniforms, monophyly of the traditionally recognized Mustelidae has been questioned by the proposition that skunks and stink badgers comprise a separate lineage (the Mephitidae), on the basis of molecular data published in the 1980s and 1990s (Dragoo and Honeycutt, 1997; Wayne et al., 1989). In addition, the historically controversial placement of the giant panda (*Ailuropoda melanoleuca*) and red panda (*Ailurus fulgens*) also led to instability in the number of recognized caniform families. The placement of the giant panda as a basal ursid is now solidly established (e.g. O'Brien et al., 1985) as is the validity of Mephitidae (e.g. Flynn et al., 2005) and the conclusion that *Ailurus* is a monotypic lineage that warrants family-level recognition as Ailuridae (e.g. Flynn et al., 2000; Fulton and Strobeck, 2006). As the familial composition of Caniformia now appears to be consolidated, only two major nodes in the caniform phylogeny have remained resilient to conclusive resolution in the recent literature: the position of Pinnipedia within Arctoidea and the relationships among Procyonidae, Mustelidae, *Ailurus* and Mephitidae (e.g. Delisle and Strobeck, 2005; Flynn et al., 2005; Fulton and Strobeck, 2006; Sato et al., 2006). Our study strongly supports the monophyly of Pinnipedia, its placement as the sister-group to Mustelida (i.e. leaving Ursidae as the most basal arctoid lineage), and an internal relationship connecting Otariidae and Odobenidae (see Fig. 2). In addition, we tested the position of Pinnipedia within Arctoidea via an outgroup jackknifing approach, which also strongly supported the topology shown in Figs. 1 and 2 (see Section 3). The same topological pattern has been reported in other recent studies using different data sets (e.g. Flynn et al., 2005; Fulton and Strobeck, 2006; Sato et al., 2006; Yu and Zhang, 2006), indicating that this phylogenetic problem has now been settled.

While the pinniped position has been congruently resolved by multiple papers, the placement of *Ailurus* has been less clear. Three recent papers have tackled this issue by including Mustelidae, Procyonidae, Ailuridae and Mephitidae in phylogenetic studies, leading to different results. Flynn et al. (2005) supported the placement of Ailuridae as the most basal lineage in this group, while Fulton and Strobeck (2006) placed it as the sister-group of

(Mustelidae + Procyonidae). A third study (Sato et al., 2006) presented a set of analyses that mostly agreed with Fulton and Strobeck's (2006) results, but in some cases also suggested different positions, i.e. Ailuridae grouping with Mephitidae or Procyonidae. In addition, support for these alternative resolutions was moderate in these three papers, highlighting the need for additional sampling of characters to resolve this question. Our present data set, which is larger and mostly non-overlapping with those of these three earlier studies, strongly supports the placement of Ailuridae as the sister-group of (Mustelidae + Procyonidae), based on congruent resolution by multiple methods (Table 4) and robust stability regardless of the outgroups employed (Table 5). Given these results, we conclude that this node has also reached stability, thus consolidating the overall topology of the caniform tree. The same conclusion has also been reached by Sato et al. (2009), in a parallel study that was recently published while this paper was under review.

4.2. Classification of the order Carnivora

The overall resolution of the relationships among extant carnivoran lineages allows for an updated taxonomic framework for this mammalian order. Instead of the traditional division into 11 families, there is now robust support for the recognition of 16 family-level clades (see Table 1), which should be incorporated into standard reference sources. In addition, several supra-familial clades (e.g. Caniformia, Feliformia, Arctoidea, Pinnipedia) are supported by our results and other recent analyses, warranting their continued usage in taxonomic studies.

One aspect of carnivoran classification that could be further clarified and standardized pertains to the usage of the names "Musteloidea" and "Mustelida". We consider it important to distinguish more effectively, in terms of taxonomic designation, two arctoid clades that have been called "Musteloidea" in the recent literature (e.g. Flynn et al., 2005; Flynn and Nedbal, 1998). One is a more internal clade composed of the sister-groups Mustelidae and Procyonidae (defined by node 38 here), referred to as "Musteloidea *stricto sensu*" by Flynn and Nedbal (1998). The second one is a more inclusive clade that also encompasses Mephitidae and Ailuridae (node 40 here), and was referred to as "Musteloidea *lato sensu*" by Flynn and Nedbal (1998). Some recent papers addressing carnivoran relationships (e.g. Sato et al., 2006) have been employing the name "Musteloidea" mostly for the inclusive clade (node 40), and we currently lack an effective name for the inner group, and thus a concise nomenclatural strategy to distinguish between them. We here propose to apply the name "Musteloidea" to the inner clade (Mustelidae + Procyonidae), which seems appropriate given its super-family suffix and the placement of this node immediately above the family-level groups. Furthermore, we propose to apply the name "Mustelida" to the more inclusive clade (Mustelidae + Procyonidae + Mephitidae + Ailuridae), an assemblage that has already received this designation in the compilation by McKenna and Bell (1997). Even though the taxonomic structure within this group in that reference work was not congruent with the present understanding of the implicated phylogenetic relationships, the full composition of the Mustelida in that compilation nevertheless does serve as precedent for this proposed usage. The name "Mustelida" has also been used in other taxonomic studies of arctoid lineages, but not necessarily with this same composition. For example, Wolsan (1993) employed this name to define a clade that comprised several extant and extinct arctoid lineages, including pinnipeds. This same idea (inclusion of pinnipeds in Mustelida) was also conveyed in the parallel study by Sato et al. (2009) that has recently been published. However, given the morphological cohesion of the group proposed here (all being small-bodied terrestrial carnivores, in contrast to pinnipeds being marine and much larger), we argue that the name is more applicable to the lineage defined by node 40 in this study (see Fig. 1). Mustelida would therefore include Musteloidea, Ailuridae and Mephitidae, and would be the sister-group of Pinnipedia.

4.3. Within-family relationships

Although our study focused mostly on the inter-familial relationships within the order Carnivora, taxon sampling within some families allows us to compare inferred phylogenetic relationships with those based on recent studies that targeted these specific groups. For example, the inferred inter-generic relationships within Hyaenidae and Procyonidae are perfectly congruent with the topologies found in the studies by Koepfli et al. (2006) [Hyaenidae], Fulton and Strobeck (2006) [Procyonidae] and Koepfli et al. (2007) [Procyonidae] even though our sampling of loci here overlapped with these studies by only three, six, and two loci, respectively. This indicates that the additional loci sampled in this study robustly support the same topological structure within Hyaenidae and Procyonidae reconstructed by these parallel intra-familial analyses, highlighting the conclusion that these inter-generic nodes of the carnivoran tree have been resolved with high confidence. Our inferred relationships among genera within Mephitidae are also entirely concordant with previous studies based either exclusively on mitochondrial sequences (Dragoo and Honeycutt, 1997) or a combination of mitochondrial and nuclear sequences (Flynn et al., 2005, 2000).

For families in which sampling of genera was less complete here, phylogenetic relationships inferred in this study are nonetheless largely consistent with previous molecular analyses. For example, within the Canidae, each of the three genera sampled here represented a major evolutionary lineage in this family. Our results (node 22) support quite strongly the view that *Urocyon* is a basal divergence relative to *Nyctereutes* + *Canis*, as seen in a recent study focusing on this family using a large molecular data set (Lindblad-Toh et al., 2005). With respect to the Mustelidae, the relationships among the eight genera sampled in this study are congruent with all nodes reconstructed by Koepfli et al. (2008) except one. The sole exception concerns the sister taxon of the Lutrinae (otters), which in our study was *Ictonyx* (node 29), in agreement with an earlier paper on the Mustelidae by Koepfli and Wayne (2003) that included fewer loci and taxa, but which disagrees with the more recent study by Koepfli et al. (2008), where *Mustela* was inferred as the closest relative of the Lutrinae. The node implicated in this relationship (node 29) was one of the few in our tree that consistently received low to moderate support in MP, ME and most ML analyses (Table 4). Interestingly, however, the placement of either Galictinae or Mustelinae *stricto sensu* as sister to the Lutrinae in Koepfli and Wayne (2003) and Koepfli et al. (2008), also received low to moderate support in those studies, suggesting that the relationships among these taxa are highly sensitive to character and taxon sampling. The estimated short branch separating these subfamilies and thus the potential for gene tree discordance (e.g. see Degnan and Rosenberg, 2006) may be one reason for the difficulty in robustly resolving this relationship. The same reasoning may apply to node 31 as well (see Koepfli et al., 2008 for comparison).

Another relevant difference in intra-familial topology relative to other recent studies was observed in the Felidae. Although the basal placement of the genus *Panthera* was strongly supported (see node 3 in Table 4), in full agreement with our recent study focusing on the Felidae (Johnson et al., 2006), the two other internal nodes (1 and 2) were not congruent with those results. Importantly, these two nodes did not receive high support with this data set, but were more robustly supported in that intra-familial study. Since our previous study employed a much larger data set (18.7 kb of nuclear sequences) to resolve the relationships among felid lineages, and

showed that these and some other nodes were extremely difficult to resolve, it is not surprising that this present data set did not fully retrieve those rapid intra-familial divergences.

Finally, a study focusing on the phylogeny of Herpestidae has recently been published, which employed a fully independent set of molecular markers relative to those used here (Patou et al., 2009). This allows the opportunity for a comparison with the herpestid topology observed in this study. All herpestid nodes reconstructed here but one were congruent with those inferred by Patou et al. (2009). The only exception was node 11, which received the lowest support within Herpestidae in this study. Patou et al. (2009) inferred a sister-group relationship between *Suricata* and *Helogale*, a finding which was only observed in this study with the BEAST relaxed phylogenetics approach (albeit weakly supported: posterior probability = 0.59).

4.4. Pattern and timing of Carnivoran divergences

Molecular divergence dating analyses allowed the construction of a timescale of carnivoran evolution, whose results were considerably robust when different analyses were compared (see Table 6 and Fig. 3A). This observation indicates that our Divtime and BEAST analyses have converged in most cases onto rather stable estimates of divergence times for carnivoran nodes.

Previous studies have provided age estimates for some of the nodes investigated here, in many cases producing congruent results with those we obtained. For example, dates for several supra-familial nodes in Caniformia have been estimated by Arnason et al. (2007) using mitogenomic data, and by Sato et al. (2009) using five nuclear segments (only one of which [APOB] overlapped with our data set). For every supra-familial node, the estimates from these two studies were contained in at least one of our Credibility Intervals (Table 6), and in some cases the point estimates were very similar (e.g. 27–29 MYA for the base of Musteloidea [node 38 in our Fig. 1]). An interesting discrepancy was observed in Pinnipedia, whose basal node exhibited low congruence between our two analyses (16 vs. 24.5 MYA), and whose age was also variable in these two studies (30 MYA in Arnason et al. (2007) and 22 MYA in Sato et al. (2009)). Considering the results from these two studies, more congruence with respect to Pinnipedia is observed with our Divtime analysis than with BEAST, suggesting that the latter may have underestimated the age of this node (see Table 6). On the feliform side, dates have been provided for some supra-familial nodes in the studies of Gaubert and Veron (2003), Gaubert and Cordeiro-Estrela (2006) and Koepfli et al. (2006). Our dates were mostly concordant with those reported by Koepfli et al. (2006), while those estimated by Gaubert and Cordeiro-Estrela (2006) tended to be older. An interesting example of congruence was the age our node 5 (Felidae + Prionodontidae), whose point estimate was identical between our Divtime result (33.3 MYA) and that reported by Gaubert and Veron (2003) on the basis of an independent data set. On the other hand, a relevant discrepancy was observed for the age of the basal node in Herpestidae, which was estimated to be quite young in this study (CIs ranging from 6.1 to 11.6 MYA), and much older (21.8 ± 3.6 MYA) in the analysis reported by Patou et al. (2009), possibly due to differences in the fossil calibrations employed.

The timescale produced here allowed the comparison of the relative ages for the crown-group of each of the carnivoran families (except for Otariidae – see Section 3), revealing some interesting patterns. From a temporal perspective, we observe that the definition of family-level clades in the order Carnivora is rather variable (see Fig. 3B). The three oldest families consist of small- to medium-sized carnivores with mostly generalist habits (Viverridae, Mephitidae and Procyonidae). In each of these families, the basal divergence occurred at least 20 MYA, indicating that considerably old lineages have been able to persist in these clades. In contrast, families that have a documented history of trends towards large body size and hypercarnivory (Felidae, Hyaenidae, Canidae) show a very recent crown origin, all of which occur in the Late Miocene. This is likely due to more intense turnover rates in terms of species (and lineage) composition in these clades.

Supra-familial nodes could also be dated with confidence, allowing comparisons of temporal diversification patterns between Caniformia and Feliformia. Although the timescale denotes a gradual diversification of both carnivoran lineages throughout the Cenozoic, some time periods are marked by rapid diversification, suggesting higher rates of cladogenesis at those moments. An interesting observation is the temporal clustering of two caniform and two feliform nodes in the Early Oligocene, between 33.3 and 30 MYA (based on the Divtime results – Fig. 2 and Table 6). This period is coincident with a documented paleontological event known as the "Grand Coupère", a major faunal turnover in Eurasia in which many groups of mammals went extinct, while others diversified (Agustí and Antón, 2002; Prothero, 2006). It may thus be hypothesized that this faunal turnover was accompanied by diversification of surviving groups, including rapid phylogenetic divergence events that can still be detected in present-day lineages.

A more recent process of faunal turnover that transformed carnivore communities in the fossil record occurred in the Late Miocene, when several lineages went extinct and others seem to have diversified (Agustí and Antón, 2002). Our timescale suggests that there was high cladogenesis at that time (7–11 MYA), with multiple carnivoran lineages diversifying in parallel (see Fig. 2). This concordant pattern suggests a simultaneous process of adaptive radiation spurred by environmental changes, in this case likely induced by the extinction of competing carnivoran lineages. As in this case, many examples of evolutionary processes may be investigated in the future by combining fossil evidence with a molecular timescale. In the case of Carnivora, its relatively rich fossil record, now combined to this molecular-based timescale of lineage divergences, should allow in the future for a detailed reconstruction of the evolutionary history of this mammalian order, integrating in-depth assessments of the biogeographic and ecological processes that have shaped its morphological, physiological and genomic diversity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.033.

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