Exploring Massive Incomplete Lineage Sorting in Arctoids (Laurasiatheria, Carnivora)

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Abstract

Freed from the competition of large raptors, Paleocene carnivores could expand their newly acquired habitats in search of prey. Such changing conditions might have led to their successful distribution and rapid radiation. Today, molecular evolutionary biologists are faced, however, with the consequences of such accelerated adaptive radiations, because they led to sequential speciation more rapidly than phylogenetic markers could be fixed. The repercussions being that current genealogies based on such markers are incongruent with species trees.

Our aim was to explore such conflicting phylogenetic zones of evolution during the early arctoid radiation, especially to distinguish diagnostic from misleading phylogenetic signals, and to examine other carnivore-related speciation events. We applied a combination of high-throughput computational strategies to screen carnivore and related genomes in silico for randomly inserted retroposed elements that we then used to identify inconsistent phylogenetic patterns in the Arctoidea group, which is well known for phylogenetic discordances.

Our combined retrophylogenomic and in vitro wet lab approach detected hundreds of carnivore-specific insertions, many of them confirming well-established splits or identifying and solving conflicting species distributions. Our systematic genome-wide screens for Long INterspersed Elements detected homoplasy-free markers with insertion-specific truncation points that we used to distinguish phylogenetically informative markers from conflicting signals. The results were independently confirmed by phylogenetic diagnostic Short INterspersed Elements. As statistical analysis ruled out ancestral hybridization, these doubly verified but still conflicting patterns were statistically determined to be genomic remnants from a time of ancestral incomplete lineage sorting that especially accompanied large parts of Arctoidea evolution.

Key words: **Pholidota, Carnivora, Arctoidea, phylogeny, truncated LINEs, retroposition, retrophylogenomics, retrogenomics**

Article

Introduction

Some mammalian evolutionary relationships persistently resist all attempts at phylogenetic resolution probably because their speciation events occurred during an era of dramatic environmental changes that occasionally included extreme reductions in populations and explosive radiations when new potential habitats were available or competitors disappeared. Such was the case for the radiation of marsupials after their migration to Australia, a continent without competitors, some 50 Ma (Nilsson et al. 2010).

Similar conditions may have prevailed for mammalian carnivores about 63 Ma after the major dinosaur groups disappeared during the Cretaceous–Paleogene mass extinction ~66 Ma (see Rose 2006 for review). In the meantime, there is accumulating evidence from morphological (Shoshani and McKenna 1998) and molecular (Murphy, Eizirik, Johnson et al. 2001; Murphy, Eizirik, O'Brien et al. 2001; Arnason et al. 2002) data suggesting that carnivores shared a common ancestor with pangolines (Pholidota). Although some of the reported bootstrap values for the position of pangolin are relatively low (Murphy, Eizirik, Johnson et al. 2001; Matthee et al. 2007; Arnason et al. 2008), several studies based on nuclear data do show stronger support for this relationship (Murphy, Eizirik, O'Brien et al. 2001; Amrine-Madsen et al. 2003; Meredith et al. 2011).

One of the early Eocene carnivoraformes (crown group Carnivora plus the stem family "Miacidae") (Flynn et al. 2010) was *Dormaalocyon latouri*, an ~1-kg, tree-dwelling hunter of small mammals or insects representing a 56-million-year-old ancestor of our extant carnivore species (Sole et al. 2014). The first bifurcation within the monophyletic carnivores occurred between Caniformia (dog-like carnivores) and Feliformia (cat-like carnivores) about 59 Ma (for dating see Eizirik et al. 2010). The split of Caniformia, separating Canidae and the clade Arctoidea about 49 Ma, is also robustly supported (Flynn et al. 2005, 2010; Eizirik and Murphy 2009).

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It is uncertain how the common ancestor of Arctoidea might have looked. A small carnivoran Amphicticeps was proposed as a stem taxon (Schmidt-Kittler 1981) and the last common ancestor of bears, pinnipeds, and musteloids (Finarelli 2008), although this remains controversial (Wang et al. 2005). However, for the time of the Arctoidea radiation, about 43 MYA, phylogenetic signals are scarce. This can be explained by an extensive diversification of arctoids from a Dormaalocyon- or Amphicticeps-like ancestor forming three clades—Ursoidea (comprising the only extant family Ursidae), Pinnipedia (including the families Phocidae, Otariidae, and Odobenidae), and Musteloidea (containing the families Mephitidae, Procyonidae, Mustelidae, and Ailuridae)—that was exposed to incomplete lineage sorting and/or ancestral hybridization.

Although the monophylies of the three superfamilies are now undisputed (for review see Flynn et al. 2010), the relationships reported among them have been contradictory. While the Ursoidea/Musteloidea sister group relationship (to the exclusion of Pinnipedia) was only weak supported from multiple sets of mitochondrial, nuclear, and morphological data (Flynn and Nedbal 1998), the sister group relationship between Ursoidea and Pinnipedia was more supported by morphological and paleozoological data (Wyss and Flynn 1993; Berta and Wyss 1994; Wang et al. 2005; Rybczynski et al. 2009), evidence from mitochondrial studies (Vrana et al. 1994; Agnarsson et al. 2010), nuclear sequences (Yu et al. 2011), indel markers (Luan et al. 2013), and from supermatrix applications (Meredith et al. 2011). The third variant—Pinnipedia/Musteloidea excluding Ursoidea—was supported by mitogenomic analyses (Arnason et al. 2007), analysis of combined mitochondrial and nuclear data (Flynn et al. 2005), an increasing amount of nuclear sequence data (Yu et al. 2004; Fulton and Strobeck 2006; Sato et al. 2006, 2009; Schröder et al. 2009; Eizirik et al. 2010), and evidence from a supermatrix application (Nyakatura and Bininda-Emonds 2012). Other studies showed that the Arctoidea tree changes from molecular locus to molecular locus (Yu and Zhang 2006) or from one analytic method to another (Peng et al. 2007). Accordingly, Delisle and Strobeck (2005) proposed a trichotomy to explain the problematic relationship among ursids, pinnipeds, and musteloids. Currently, the most favored scenario in arctoid relationships, supported by nuclear data reported in the last decade, is the sister group relationship between Pinnipedia and Musteloidea (Sato et al. 2006; Eizirik et al. 2010).

The interfamilial relationships within Musteloidea are also rather complex. Opposing phylogenetic signals indicate another zone of rapid radiation and incomplete lineage sorting or ancestral hybridization. Although the Mustelidae–Procyonidae sister group relationship finds some support (Flynn et al. 2005; Fulton and Strobeck 2006; Sato et al. 2009, 2012), it still remains an open question whether Mephitidae or Ailuridae is the most basal group in Musteloidea (Flynn et al. 2005; Fulton and Strobeck 2006; Sato et al. 2009; Eizirik et al. 2010).

Ancient rapid radiations challenge our attempts to find diagnostic signals of speciation. What often remain are marker conflicts designated as phylogenetic hemiplasy (Avise and Robinson 2008). Analyses of DNA sequences often fail to recognize hemiplasious signals because they are inseparable from frequent homoplasious transitions and transversions (phylogenetic noise) due to the low number of character states. In contrast, the phylogenetic signals of retroposon insertion data are more suitable for distilling ambiguous phylogenetic signals to a quantifiable account of the real noise-free portion of the data. The insertion of retroposed elements is largely neutral and random (Shedlock and Okada 2000), and the number of character states nearly infinite. The probability of the same element being inserted at a specific genomic location independently in two species (parallel insertions) is very low (Shedlock et al. 2004; Ray et al. 2006), as is the probability of the precise excision of homologous elements (van de Lagemaat et al. 2005; Walters-Conte et al. 2014). While finding and evaluating noise-free insertion patterns requires careful bioinformatics screening, experimental verification, and statistical interpretation of phylogenetic signals and hemiplasious interferences, this molecular approach is ideal for re-examining these controversies and decidedly confirming stronger tendencies. It is also ideally suited to determining the sources of these discordances.

There are three main categories of retrogenomic elements: 1) Short INterspersed Elements (SINEs), 2) Long INterspersed Elements (LINEs), and 3) Long Terminal Repeats (LTRs), and hundreds of their subfamilies and subtypes in all mammalian genomes. Choosing the appropriate phylogenetically informative element families might reveal hundreds of thousands of signals, all of which present independent individual character states (millions of unique insertion sites) whose character homologies among species are possible to analyze. This motivated Dettai and Volff (2006) to call retroposed elements "morphological characters of the genome." Retroposed elements were successfully applied to reconstruct the complicated phylogenies of birds (Suh et al. 2011 2015), marsupials (Nilsson et al. 2010), and many groups of placentals (Nikaido et al. 2001; Nishihara et al. 2005; Möller-Krull et al. 2007; Churakov et al. 2009, 2010; Chen et al. 2011; Hallström et al. 2011; Nilsson et al. 2012; Hartig et al. 2013). Although there were previous attempts to use retroposed elements as markers in Carnivora (Pecon-Slattery et al. 2000, 2004; Yu and Zhang 2005; Walters-Conte et al. 2014), and specifically in the Arctoidea group (Schröder et al. 2009; Yu et al. 2011), the lack of genome-sequence data impeded these studies from identifying sufficient numbers of markers to sequence-independently resolve the most relevant phylogenetic questions in arctoid evolution. Now, with the growing amount of available genome data from carnivores, the application of high-throughput computational screening in combination with experimental Zoo-PCR techniques should enable us to more unambiguously re-examine or decidedly confirm contradictory phylogenetic signals. The main goal of our study was to investigate problematic phylogenetic zones of Carnivora, with a particular focus on the disputed splitting events of early arctoids, by applying genome-wide screens and analyses of retroposon markers to examine conflicting tree topologies, distinguish and quantify phylogenetically

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informative signals from the hemiplasious signals, and determine the sources of these conflicts.

Results

We focused our genome-wide screening for phylogenetically informative retroposon insertions on carnivore-specific SINE (Can-SINE) and LINE1 (L1_Carn and L1MA9) families active during the critical phases of carnivoran rapid radiations. Because LTR elements exhibit a comparably low activity pattern in internal branches in Carnivora, they were not included in our survey.

During an initial multidirectional screening of 3 dog-associated 2-way genome alignments (dog/giant panda, dog/cat, and dog/horse) in addition to an individual genome screening for ferret (see Materials and Methods), we obtained 816 potential phylogenetically informative LINE and 3,853 SINE loci. All the LINE and about 700 randomly chosen SINE loci underwent manually constructed alignments, which were supplemented with PCR-derived sequences from additional key species (252 loci) that revealed a total of 67 loci containing 77 informative markers (supplementary tables S1a and b, Supplementary Material online). Furthermore, to extract potentially informative loci from nonreference species (those without genome sequence information), we applied the inverse PCR strategy (Wang and Kirkness 2005; see Materials and Methods). We sequenced 417 inverse PCR loci for coati (*Nasua* sp.), selected those containing potentially informative retroposon insertions (77 loci) for further experimental investigations in additional key species, and found 19 loci containing 25 informative markers (24 SINEs, 1 LINE), including 10 markers that, in addition to the targeted elements, had inserted randomly into the inspected sequences. Additionally, by sequencing 297 inverse PCR loci for red panda (*Ailurus fulgens*), we found one informative SINE marker (supplementary table S1a, Supplementary Material online).

Phylogenetic Relationships of Early Carnivores and Their Sister Group

Our screen of the 3 dog-associated 2-way genome alignments yielded 10 phylogenetically informative LINE markers that are shared among Pholidota and Carnivora species and absent in other laurasiatherians providing significant evidence supporting the monophyly of Ferae (Carnivora plus Pholidota) (10:0:0; KKSC insertion significance test $P < 1.7 \times 10^{-5}$; see Materials and Methods). Combining screens of the dog-associated 2-way genome alignments, individual annotated genome screenings, and the inverse PCR approach, we obtained 1 SINE and 14 LINE markers corroborating the monophyly of extant carnivores (15:0:0; KKSC [Kuritzin-Kischka-Schmitz-Churakov] insertion significance test $P < 7.0 \times 10^{-8}$). Significant support was also found for the monophyly of both Caniformia (12 SINE markers) (12:0:0; KKSC insertion significance test $P < 1.9 \times 10^{-6}$) and Arctoidea (22 SINE and 2 LINE markers) (24:0:0; KKSC insertion significance test $P < 3.6 \times 10^{-12}$) (fig. 1).

Resolving the Ursoidea–Pinnipedia–Musteloidea Relationship

A preliminary screen of the 3 dog-associated 2-way genome alignments and the ferret genome combined with inverse PCR approach confirmed the inconsistent relationships within arctoids. Therefore, we performed an additional high-throughput screen using the custom-built 2-way genome alignments to test the three potential hypotheses: 1) An Ursoidea/Pinnipedia clade with Musteloidea as the sister group, 2) an Ursoidea/Musteloidea clade with Pinnipedia outside, and 3) a Pinnipedia/Musteloidea clade with Ursoidea outside (see Materials and Methods).

In the first of these screens of intronic SINE markers, we obtained 585 preliminary phylogenetic markers. From manually constructed alignments we extracted 34 informative markers supporting an Ursoidea/Pinnipedia clade, 40 markers for the Ursoidea/Musteloidea clade, and 106 informative markers supporting a Pinnipedia/Musteloidea clade (supplementary table S1a, Supplementary Material online). The KKSC insertion significance test favored the third hypothesis ($P < 2.0 \times 10^{-12}$) (fig. 2). To avoid being misdirected by possible parallel insertions of SINEs, we performed a second screen for specifically truncated (incomplete reverse transcription) LINE elements. Random insertion together with a locus-specific truncation provide a homoplasy-free picture of evolutionary history. This screen revealed 146 diagnostic LINE loci in the proportion 26:34:86 (Ursoidea/Pinnipedia:Ursoidea/Musteloidea:Pinnipedia/Musteloidea with the KKSC insertion significance test again favoring the third hypothesis, $P < 2.3 \times 10^{-10}$; supplementary table S1a, Supplementary Material online). From this we randomly selected six loci from each direction for expanded experimental sampling, which confirmed the signals for all three hypotheses (supplementary table S1a, Supplementary Material online). Analysis of the combined SINE and LINE data set (60:74:192; corresponding to 18%, 23%, and 59%) provides highly significant support for the Pinnipedia/Musteloidea clade ($P < 3.3 \times 10^{-21}$).

Because we received similar results for precisely truncated LINEs (26:34:86 Ursoidea/Pinnipedia:Ursoidea/Musteloidea:Pinnipedia/Musteloidea) and full-length SINEs (34:40:106), we assume that parallel insertions or exact deletions are not relevant for either data set. Rather, we assume that, as the combined data give no significant signal for hybridization (KKSC insertion significance test $P > 0.2$), the conflicting phylogenetic patterns of retroposon insertions directly reflect the impact of ancient incomplete lineage sorting at the time of ancestral arctoid diversification.

Resolving Phylogenetic Relationships in Terminal Branches of Arctoidea

The genome-wide screen of the 3 dog-associated 2-way alignments and the ferret genome information combined with the inverse PCR approach yielded retroposon markers that provide strong, statistically significant support for various intraclade relationships. Based on comparative presence/absence insertion patterns of retroposons in polar bear (*Ursus*

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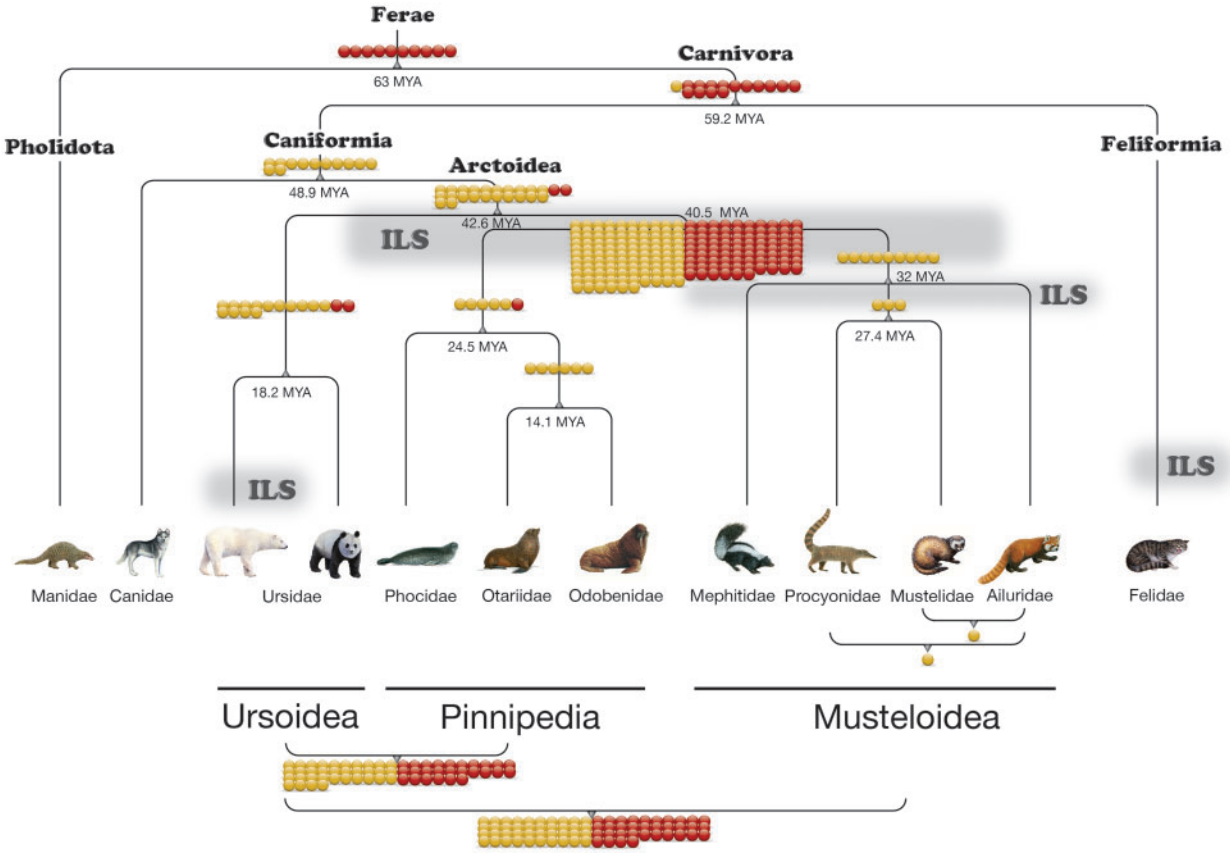

FIG. 1. Retroposon-based phylogenetic tree of carnivores according to the most statistically favored data. SINEs and LINEs are presented as yellow and red balls, respectively. Divergence times in millions of years ago (MYA) were taken from Eizirik et al. (2010). Alternative relationships are presented at the bottom of the figure (Ursoidea plus Pinnipedia supported by 34 SINEs and 26 LINEs; Ursoidea plus Musteloidea supported by 40 SINEs and 34 LINEs). Zones of possible ILS are indicated as diffuse gray areas. The tree topology was derived by PAUP based on the presence/absence data. The same tree topology was obtained using the Bayesian reconstruction method. ILS, incomplete lineage sorting.

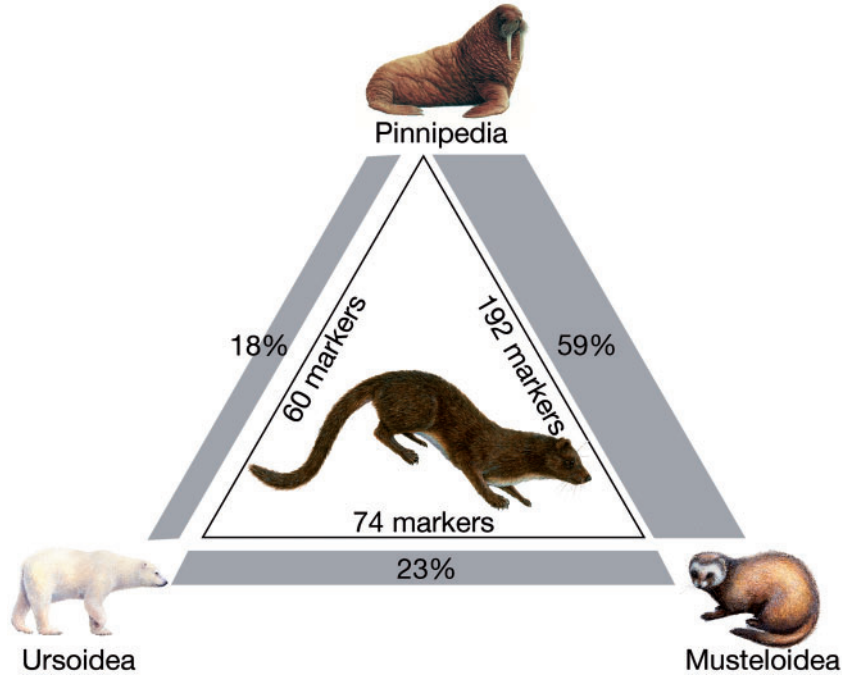

FIG. 2. Triangular illustration representing the Arctoidea relationships derived by retroposed element presence/absence information. Eighteen percent of all investigated markers (60 markers) indicate a shared relationship of Ursoidea plus Pinnipedia, 23% of Ursoidea and Musteloidea (74 markers), and 59% of Pinnipedia plus Musteloidea (192 markers). An imaginary reconstruction of the early common ancestor of Ursoidea, Pinnipedia, and Musteloidea, based on descriptions of Flynn (1998) and Sole et al. (2014), is presented in the center of the triangle.

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maritimus) and giant panda (*Ailuropoda melanoleuca*) compared with other arctoids, 16 markers (14 SINEs, 2 LINEs) show the monophyly of Ursoidea (16:0:0; KKSC insertion significance test $P < 2.4 \times 10^{-8}$). Our data also indicate the monophyly of Pinnipedia (5 SINEs, 1 LINE) (6:0:0; KKSC significance test $P < 0.0015$) and support the broadly accepted Otariidae–Odobenidae sister group relationship (6 SINEs) (6:0:0; KKSC significance test $P < 0.0015$). We found strong support for the monophyletic superfamily Musteloidea (9 SINEs) (9:0:0; KKSC significance test $P < 5.1 \times 10^{-5}$). Yet, only five individual diagnostic markers were found within this group; three SINEs were shared among Mustelidae and Procyonidae, but one SINE was shared by Mustelidae and Ailuridae and one SINE was shared by Procyonidae and Ailuridae (fig. 1). Although we cannot completely exclude the improbable occurrence of precise parallel insertions or exact deletions, incomplete lineage sorting in terminal Arctoidea splits is a valid alternative hypothesis that awaits further testing.

Evidence for SINE Homoplasy

We found three SINE insertions with discordant phylogenetic patterns (supplementary table S2 and supplementary material S1, Supplementary Material online). In all three cases our RepeatMasker screen identified the same type of SINE in representatives of unrelated species, the orientations of the elements were the same in all species, and the target-site duplications were perfect, indicating exactly the same position of insertion. In "locus 61," a SINE insertion was present in two species of Mustelidae and in Ailuridae, but was absent in all other analyzed carnivoran species, including six species of Mustelidae, two species of Procyonidae, and one Mephitidae species. In "locus 68," a SINE insertion was present in all families of Musteloidea (nine analyzed species) and Otariidae (three analyzed species), but was absent in other pinnipeds. In "locus IPc20," a SINE insertion was present in representatives of Mustelidae and Procyonidae but not in Mephitidae. In Ailuridae, the SINE was not recognizable but there was a target site duplication indicating a partial deletion of the inserted orthologous SINE. Interestingly, the orthologous locus in the polar bear contained the same SINE type in the same orientation with the same target site duplication.

Discussion

Despite the recent increase in data and improved methodologies for investigating the relationships between species, the evidence for many major phylogenetic patterns is sometimes contradictory. Various methods of phylogenetic reconstruction work well when splits between lineages are separated by relatively long time intervals so that the species have time to accumulate substantial numbers of derived characters providing strong phylogenetic signals. However, elucidating the evolutionary history of some relationships is difficult, and rapid evolutionary radiations have been proposed to explain poorly resolved phylogenies in many groups of organisms (for review see Whitfield and Lockhart 2007). In cases when internodes are short and the terminal branches long, the insertion patterns of genome-level characters, such as retroposed elements, are especially powerful in resolving phylogenetic relationships (for review see Boore 2006). Carefully investigated, they provide an almost homoplasy-free source of information.

In mammals, the sister group relationship of Pholidota and Carnivora is broadly accepted (Rose 2006; Flynn et al. 2010) but many support values are rather low (Cabria et al. 2006). Even in large-scale nuclear data sets with high support values for Ferae, the support may drop significantly after adding particular species to the analysis (Zhou et al. 2012). Supporting the most recent sequence-based analyses (Meredith et al. 2011; Zhou et al. 2012), we show that the Carnivora/Pholidota relationship is significantly confirmed by retroposon data, markers that are not influenced by naturally occuring sequence phenomena such as long-branch attraction. The connection between Pholidota and Carnivora is supported by two types of phylogenetically informative LINEs—L1MA9 and L1_Carn7—active during early Ferae evolution. However, we did not find any diagnostic SINEs merging the two clades, indicating a low or lack of activity of such elements in these ancestral lineages. van der Vlugt and Lenstra (1995) reported that Can_SINEs are present in both Caniformia and Feliformia clades, so we suggest that Can_SINEs emerged about 59 MYA, after the Pholidota–Carnivora split (for dating see Eizirik et al. 2010) and before Caniformia and Feliformia diverged. Our data also support the monophyly of the order Carnivora and their internal splits—Caniformia and Arctoidea reported by other studies (Flynn et al. 2005; Nyakatura and Bininda-Emonds 2012). The absence of conflicting retroposon insertions suggests a long and efficacious fixation period in the common ancestral population.

The first hint of rapid radiation and its associated phylogenetic conflicts in carnivores appear during arctoids' split into the three superfamilies—Ursoidea, Pinnipedia, and Musteloidea. We analyzed all possible scenarios of superfamily phylogeny using a whole-genome screening for retroposon presence/absence patterns and multidirectional analysis of custom-made alignments yielding an unbiased exhaustive marker extraction and found markers supporting all three possible tree topologies: Ursoidea/Pinnipedia sister relationships excluding Musteloidea, Ursoidea/Musteloidea sister relationships excluding Pinnipedia, and Pinnipedia/Musteloidea sister relationships excluding Ursoidea. The Pinnipedia/Musteloidea clade received a significantly higher support (192 markers) than the other two. This decidedly confirms other studies indicating Ursoidea as the first divergence in arctoids (for review see Eizirik and Murphy 2009). However, we also found markers supporting Ursoidea/Pinnipedia (60 markers) and Ursoidea/Musteloidea (74 markers) clades, although in significantly lower numbers.

Interestingly, the number of markers supporting Ursoidea plus Musteloidea was slightly higher than the support for Ursoidea plus Pinnipedia, which disagrees with previous results (Meredith et al. 2011; Yu et al. 2011), but currently reflects only a slight tendency. There are three possible explanations for the presence of diagnostic markers supporting

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mutually exclusive tree topologies: 1) Independent parallel insertion or precise excision of the same element in different species, 2) retroposon homoplasy caused by incomplete lineage sorting, or 3) ancestral hybridization. We cannot fully exclude that a few of our retroposon insertions are the result of parallel insertion or precise excision. The probability of parallel SINE insertion in primates is calculated to be about 0.05% (Ray et al. 2006) and SINE precise excision less than 0.5% (van de Lagemaat et al. 2005). Therefore, the 41% of Arcotoidea markers supporting alternative tree topologies are far outside the probability for parallel insertions or precise excisions. On the other hand, both incomplete lineage sorting or ancestral hybridization scenarios may well have occurred during successive radiations within short periods, such as the estimated 2 My of the early arctoid superfamily radiation (for dating see Eizirik et al. 2010). This period is assumed to be very short for complete marker fixation (Schmitz and Zischler 2004), leaving behind polymorphic signals that are today visible as conflicting markers. Although we found a slightly stronger support for Ursoidea/Musteloidea than for Ursoidea/Pinnipedia clades, the KKSC significance test indicates incomplete lineage sorting rather than hybridization (see above). We used two types of retroposed elements for our phylogenetic reconstruction—SINEs and LINEs. Because of their abundance and short length, SINEs are proposed as ideal retroposon markers in phylogenetic reconstructions (Shedlock and Okada 2000). However, Pecon-Slattery et al. (2000, 2004) and Walters-Conte et al. (2014) reported conflicting SINE data in Felidae. Compared with SINEs, LINEs provide one additional critical level of complexity, diagnostic truncation points generated by stochastic interruption of the elongated retroposition process, rendering any truncated insertion a verifiable unique event (Kriegs et al. 2006). To avoid being confounded by potential, albeit highly unlikely, parallel insertions, we analyzed data from SINEs and truncated LINEs independently and obtained similar results demonstrating that the possible interference of parallel insertions or precise excisions was not critical for this quantitative analyses of high-throughput genome-wide screenings for retroposon presence/absence markers.

The arctoid groups—Ursoidea, Pinnipedia, and Musteloidea—evolved independently long enough for each to collect and fix individual retroposed markers supporting their respective monophylies. Another period of rapid radiation in Carnivora evolution appeared 32 Ma in Musteloidea. In agreement with findings of other studies (Flynn et al. 2005; Sato et al. 2012), we found some indication for a Procyonidae plus Mustelidae grouping to the exclusion of Ailuridae and Mephitidae. However, one marker provided incongruent evidence for a Procyonidae–Ailuridae sister group relationship and one for a Mustelidae–Ailuridae sister group. Due to a limited amount of available genomic data (only the ferret genome in the clade Musteloidea), the inverse PCR strategy should be useful for marker screening (Wang and Kirkness 2005; Suh et al. 2012), but is less efficient and does not provide a sufficient number of markers necessary for identifying splits in a rapidly radiating group.

Our analyses of SINEs did detect a few of what appear to be parallel insertions of the same type of elements in orthologous genomic positions of unrelated carnivore clades, which may have resulted from the slight target site preference of LINE1 mobilized elements (Jurka 1997). In general, in carnivores it is difficult to distinguish different Can-SINEs because of their high similarity and the few lineage-specific subtypes.

Our use of retroposon markers and similar results from other studies enabled us to conclude that several carnivore splits were most likely exposed to polymorphic characters leading to incomplete lineage sorting and conflicting phylogenetic patterns. Simple bifurcations were likely not the case for the Arctoidea divergence 42 Ma, the Musteloidea divergence 32 Ma, during the 10 My of Felidae divergence (Walters-Conte et al. 2014), or the only 2 My of Ursinae divergence (Kutschera et al. 2014).

Conclusions

Arctoids prove to be a key example of nonlinear character evolution. Characters change continuously, but especially changes that appear shortly before speciation events have little chance of being genomically fixed, a process that usually requires several million years. Unfixed characters distribute into the new population, species, or lineage randomly and produce conflicting phylogenetic signals. The shorter the period between speciation events, the higher the probability of unfixed characters that might lead, in extreme cases, e.g., to the equal polytomy exhibited at the root of the placental mammals (Churakov et al. 2009). Filtering such noise out of pure DNA sequence data is less successful because of the low complexity of the characters exposed to reversals and parallelisms. The highly complex and unbiasedly selected retroposon insertions, however, provide a reliable source to distinguish between the fixed and unfixed markers at any speciation point, which affords a clear perception of signal and noise and should be a significant supplementation to any sequence-based phylogenetic reconstruction in critical speciation zones. We applied this strategy to elucidate the evolutionary processes involved in the Arctoidea speciation pattern that evolved about 42 Ma, combining two strong criteria to extract homoplasy-free markers: 1) Selecting randomly inserted elements (SINEs and LINEs), with 2) insertion-specific truncations (LINEs). This revealed that 59% of all extracted insertions reached fixation and support a Pinnipedia plus Musteloidea clade to the exclusion of Ursoidea; the remaining elements analyzed were relatively equally distributed, 18% support Ursoidea plus Pinnipedia and 23% Ursoidea plus Musteloidea. This explains why many sequence-based data sets generated discordant reconstructions, and why even the same dataset, in silico translated and untranslated, may lead to conflicting trees (Meredith et al. 2011). Combining high-throughput analysis applied on large data sets of homoplasy-free retroposon markers with a statistical analysis capable of dealing with such a large database now makes it possible to shed more light on such conflicts and to go one step further in determining their probable source.

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Materials and Methods

We combined the generation of pairwise whole genome alignments (2-way) with high-throughput bioinformatics screening techniques and experimental verification of retroposon presence/absence patterns in carnivores and their pangolin sister group. In an initial application, we specifically screened three dog-associated 2-way genome alignments (dog/giant panda, dog/cat, dog/horse) and the ferret reference genome for retroposons located in short intronic regions flanked by conserved exons, where we placed conserved PCR primer pairs for caniform Zoo-PCR whenever possible. In a second approach, we performed a genome-wide screening of the custom-built 2-way genome alignments for intronic SINE and truncated LINE1 elements with subsequent amplification of some orthologous sequence regions in representative caniform species. In clades without available sequenced genomes, we applied an inverse PCR approach for selected Can-SINEs and extracted potentially informative loci for PCR primer design and Zoo-PCR corresponding to reference genomes (ferret, giant panda, Weddell seal, walrus) (Wang and Kirkness 2005).

Genome Screening

For initial screening, we used the available ferret genome sequence (http://hgdownload.soe.ucsc.edu/goldenPath/musFur1/bigZips/musFur1.fa.masked.gz, last accessed September 14, 2015). We used a local version of RepeatMasker (http://www.repeatmasker.org, last accessed September 14, 2015) and available genome annotation (http://genome.ucsc.edu/cgi-bin/hgTables, last accessed September 14, 2015) for extracting ferret introns containing carnivore-specific SINEs with their conserved exonic flanks. We used National Center for Biotechnology Information (NCBI) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed September 14, 2015) for BLAST of related carnivore sequences, constructed manual alignments, and chose potentially informative loci for PCR primer design and Zoo-PCR.

Two-Way Genome Alignments

We performed in silico screening for potentially informative retroelements and flanking regions based on the 2-way alignments described in Kent et al. (2003) and first applied for systematic phylogenetic screenings in Hartig et al. (2013). We used the following sources: *Canis lupus familiaris* (domestic dog) versus *Equus caballus* (horse; http://hgdownload.soe.ucsc.edu/goldenPath/canFam2/vsEquCab2/axtNet/, last accessed September 14, 2015), *C. l. familiaris* (domestic dog) versus *Felis catus* (domestic cat; http://hgdownload.soe.ucsc.edu/goldenPath/canFam2/vsFelCat3/axtNet/, last accessed September 14, 2015), and *C. l. familiaris* (domestic dog) versus *A. melanoleuca* (giant panda; http://hgdownload.soe.ucsc.edu/goldenPath/canFam2/vsAilMel1/axtNet/, last accessed September 14, 2015).

Additionally, we custom built 2-way genome alignments for the following: *A. melanoleuca* (giant panda) versus *Leptonychotes weddellii* (Weddell seal; http://hgdownload-test.cse.ucsc.edu/goldenPath/ailMel1/vsLepWed1/ailMel1.lepWed1.net.axt.gz, last accessed September 14, 2015), *A. melanoleuca* (giant panda) versus *Mustela putorius furo* (ferret; http://hgdownload-test.cse.ucsc.edu/goldenPath/ailMel1/vsMusFur1/ailMel1.musFur1.net.axt.gz, last accessed September 14, 2015), *A. melanoleuca* (giant panda) versus *Odobenus rosmarus* (walrus; http://hgdownload-test.cse.ucsc.edu/goldenPath/ailMel1/vsOdoRosDiv1/ailMel1.odoRosDiv1.net.axt.gz, last accessed September 14, 2015), *Mu. p. furo* (ferret) versus *L. weddellii* (Weddell seal; http://hgdownload-test.cse.ucsc.edu/goldenPath/musFur1/vsLepWed1/musFur1.lepWed1.net.axt.gz, last accessed September 14, 2015), and *Mu. p. furo* (ferret) versus *O. rosmarus* (walrus; http://hgdownload-test.cse.ucsc.edu/goldenPath/musFur1/vsOdoRosDiv1/musFur1.odoRosDiv1.net.axt.gz, last accessed September 14, 2015).

For analyzing 2-way alignments, we used RepeatMasker reports. The dog, giant panda, and ferret genomic reports were downloaded from http://hgdownload.soe.ucsc.edu/goldenPath/canFam2/bigZips/chromOut.tar.gz, last accessed September 14, 2015, http://hgdownload.soe.ucsc.edu/goldenPath/ailMel1/bigZips/ailMel1.fa.out.gz, last accessed September 14, 2015, and http://hgdownload.soe.ucsc.edu/goldenPath/musFur1/bigZips/musFur1.fa.out.gz, last accessed September 14, 2015, respectively.

For preanalysis of diagnostic presence/absence patterns, we selected gaps between blocks of axt pairwise alignments with contrasting sizes in both species ($<$11 nt for the absence state and $>$ 100 nt for potential presence state). If 70% of the respective gap was occupied by a solitary retroposon (Can_SINE or LINE1), we assumed a potentially diagnostic presence/absence pattern. Coordinates of presence/absence states were projected onto different combinations of 2-way alignments involving diverse species arranged with one of the preanalyzed species from the initial 2-way alignments. Coordinates of diagnostic insertions and their projection onto other species via 2-way alignments were then used to extract genomic loci for further alignments and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed September 14, 2015) and BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat, last accessed September 14, 2015) screening. We projected coordinates of potential markers close to exonic coordinates extracted from respective genome annotations (dog, giant panda, or ferret), and for SINEs we defined the distances to the closest flanking exons (selecting regions 1,700 nt or 2,200 nt, see below).

Searching for Informative Retroposed Elements

We screened for the following presence (+)/absence (−) patterns (reference genomes in italics): 1) +*Dog*+cat−horse, 2) −*dog*+giant panda, and 3) −*dog*+ferret. Potential diagnostic presence/absence patterns from (1) were computationally tested for their status in the Chinese pangolin genome and at least one representative of the following outgroups: Perissodactyla, Cetartiodactyla, and Chiroptera. To achieve successful PCRs for (2) and (3), we selected retroposon markers located in short intronic regions flanked by conserved exons ($\leq$ 1,700 nt, based on dog-genome annotations) for PCR primer design. To expand alignments we added

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Table 1. List of Investigated Species and Sources of Genome Information from NCBI and UCSC Genome Bioinformatics.

Carnivora and Outgroups	Common Name	NCBI (UCSC)
Mustelidae		
Mustela putorius furo	Domestic ferret	(musFur1)
Mu. nivalis	Least weasel	
Mu. sibirica	Siberian weasel	
Neovison vison	American mink	
Martes foina	Beech marten	
Ma. flavigula	Yellow-throated marten	
Ma. zibellina	Sable	
Meles leucurus	Asian badger	
Procyonidae		
Nasua sp.	Coati	
Procyon lotor	Raccoon	
Ailuridae		
Ailurus fulgens	Red panda	
Mephitidae		
Mephitis mephitis	Striped skunk	
Phocidae		
Leptonychotes weddellii	Weddell seal	APMU00000000.1 (vsLepWed1)
Pagophilus groenlandicus	Harp seal	
Phoca vitulina	Harbor seal	
Odobenidae		
Odobenus rosmarus	Walrus	ANOP00000000.1
Otariidae		
Arctocephalus pusillus	Brown fur seal	
Arctocephalus australis	South American fur seal	
Callorhinus ursinus	Northern fur seal	
Eumetopias jubatus	Steller sea lion	
Ursidae		
Ailuropoda melanoleuca	Giant panda	(ailMel1)
Ursus maritimus	Polar bear	AVOR00000000.1
U. arctos	Brown bear	
U. thibetanus	Asian black bear	
U. americanus	American black bear	
Helarctos malayanus	Sun bear	
Canidae		
Canis lupus familiaris	Domestic dog	AAEX00000000.3 (canFam3; canFam2)
Felidae		
Felis catus	Domestic cat	AANG00000000.3(felCat3; felCat5)
Panthera tigris	Tiger	ATCQ00000000.1
Outgroup species		
Pholidota		
Manis pentadactyla	Chinese pangolin	JPTV00000000.1
Perissodactyla		
Equus caballus	Horse	(equCab2)
Cetartiodactyla		
Bos taurus	Domestic cow	(bosTau8)
Balaenoptera acutorostrata	Common minke whale	(balAcu1)
Chiroptera		
Pteropus vampyrus	Large flying fox	(pteVam1)
Myotis lucifugus	Little brown myotis	(myoLuc2)

The UCSC genomes are given in parentheses. All other sources are from the NCBI GenBank.

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sequences from orthologous genomic loci of carnivores and representative outgroups (ferret, giant panda, dog, cat, horse, cow, common minke whale, large flying fox, and little brown myotis from University of Santa Cruz (UCSC); polar bear, Weddell seal, walrus, cat or tiger, and Chinese pangolin from NCBI) (table 1).

To investigate the problematic relationships of the three superfamilies Ursoidea, Pinnipedia, and Musteloidea, we built 2-way genome alignments involving the giant panda, Weddell seal, walrus, and ferret genomes to detect the presence/absence of orthologous retroposons. The combination of species was chosen to unbiasedly investigate all possible phylogenetic scenarios to find the closest relationships or conflicting patterns. We screened for the following presence/absence patterns (reference genome in italics): 1) +*Giant panda*+seal+walrus−ferret, 2) +*giant panda*+ferret−seal−walrus, and 3) +*ferret*+seal+walrus−giant panda, and then extracted sequences from all four tested genomes (giant panda, Weddell seal, walrus, ferret) based on coordinates of flanking regions of the insertions and their projections onto other genomes. Loci with inserted LINEs distributed randomly and SINEs located in intronic regions flanked by conserved exons ($\leq$ 2,200 nt, based on dog-genome annotation) were extracted and analyzed by constructing manual alignments. Only full length SINEs and truncated LINEs with clear 3′ ends (maximum 25 missing nucleotides) were selected.

Experimental Work

Samples, Amplification, and Sequencing Strategy

Twenty-one samples representing seven families of Carnivora were included in our study and are listed together with the sources of published data from the NCBI and the UCSC Genome Bioinformatics in table 1. Total genomic DNA was isolated from tissue or blood samples using the DNeasy Blood and Tissue Kit (QIAGEN). The PCR amplification of DNA from red panda was preceded by whole genome amplification with REPLI-g Midi Kit (QIAGEN).

To gain a largely complete presence/absence pattern, loci of species without corresponding in silico sequence representations were PCR amplified. Conserved PCR primers were constructed on the basis of genomes of the reference species ferret, Weddell seal, walrus, giant panda, and polar bear (as far as available) (supplementary table S3, Supplementary Material online).

PCR reactions were conducted in 30 µl containing ~50 ng template DNA, 0.5 U ThermoPrime Taq DNA polymerase (Thermo scientific), 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.3 µM of each primer. PCR reactions were performed using the touchdown PCR strategy: 5 min at 95 °C was followed by 10 cycles of 30 s at 95 °C, 40 s at 55 °C, and 120 s at 72 °C with a decrease in the annealing temperature at a rate of 1 °C per cycle. The final 40 cycles of 30 s at 95 °C, 40 s at 45 °C, and 120 s at 72 °C were followed by 5 min at 72 °C. A total of 5 µl of PCR products were visualized on a 1.5% agarose gel containing ethidium bromide to detect presence/absence patterns via size shifts of fragments and were excised and purified (High Pure PCR Product Purification Kit; Roche). After ligation into the pDrive Cloning Vector (QIAGEN) and chemical transformation, colonies were PCR screened using standard M13 primers. For each positive PCR product, at least two colonies were sequenced.

Inverse PCR Approach

Genomic DNA (200 ng) of coati and red panda was digested with *Blp*I in a 20 µl restriction assay, subsequently heat inactivated (80 °C, 20 min), and ligated overnight at 16 °C in 500 µl with 10,000 U of DNA ligase (T4 DNA Ligase; New England Biolabs). After phenol–chloroform extraction and ethanol precipitation, ~50 ng was PCR amplified in 30 µl (see the protocol above) using the following primers: 5′—TCGAGTCCCACRTCRGGCTCCYTG (T_m = 68.7 °C) and 5′—GACCTGAGCCGAAGGCAG (T_m = 60.5 °C) designed consensus PCR primers from Can-SINEs.

PCR conditions were 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 40 s at 59 °C, and 90 s at 72 °C with a final elongation step of 5 min at 72 °C. Purification of PCR products was conducted as described above. After transformation (electroporation into TOP10 cells; Invitrogen), plasmid templates were prepared from white colonies and sequenced using the M13F primer.

We used inverse PCR-derived sequences of coati and red panda containing retroposons for making manual alignments with reference species (ferret, Weddell seal, walrus, giant panda, and polar bear) and identified those containing potentially informative markers. For those loci, we designed PCR primers (supplementary table S3, Supplementary Material online) and performed the procedures described above. All derived sequences have been deposited at GenBank (accession numbers KT265345–KT265682).

Sequence Alignment and Phylogenetic Analyses

All amplified sequences were manually aligned. For detection and classification of additional random insertions, we used the RepeatMasker Server (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker, last accessed September 14, 2015) and the CENSOR software tool (http://www.girinst.org/censor/index.php, last accessed September 14, 2015). All alignments are provided as supplementary material S1, Supplementary Material online.

We build a presence/absence (1/0) data matrix for retroposons of merged carnivore families (supplementary table S1b, Supplementary Material online) and reconstructed a strict consensus, most parsimonious tree in PAUP*4.0b10 (Swofford 2002) to derive the tree topology shown in fig. 1. Tree reconstruction was done using the Dollo parsimony irrev.up character transformation in a heuristic search with 1,000 random sequence additions and tree bisection and reconnection branch swapping and also using MrBayes v3.2.5 for a Bayesian inference (Standard Discrete Model [binary]; ctype irreversible) (Ronquist et al. 2012). For outgroup we used Perissodactyla.

The KKSC insertion significance test was designed by our group to reliably evaluate the statistical significance of large-

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scale presence/absence data in phylogenetic studies. The KKSC statistics is imbedded in a freely available R application located at http://retrogenomics.uni-muenster.de:3838/KKSC_significance_test/, last accessed September 14, 2015. and is described in Kuritzin et al. (in review). The underlying mathematical model presumes a polynomial distribution of elements and differentiates among binary branching, ancestral hybridization, and polytomy. For binary branching a significant dominance of markers supporting a single tree topology is necessary. Testing for hybridization the two remaining alternative tree topologies are analyzed for asymmetric (indicating hybridization) or symmetric (no hybridization) distribution of markers (signals for a binary tree and hybridization can coexist in the same data set). A more or less symmetric distribution of markers for all three possible tree topologies indicates polytomy.

Supplementary Material

Supplementary material S1 and tables S1–S3 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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