Original Contributions

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Evolutionary implications of multiple SINE insertions in an intronic region from diverse mammals

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Abstract

An analysis of the nuclear β-fibrinogen intron 7 locus from 30 taxa representing 12 placental orders of mammals reveals the enriched occurrences of short interspersed element (SINE) insertion events. Mammalian-wide interspersed repeats (MIRs) are present at orthologous sites of all examined species except those in the order Rodentia. The higher substitution rate in mouse and a rare MIR deletion from rat account for the absence of MIR in the rodents. A minimum of five lineage-specific SINE sequences are also found to have independently inserted into this intron in Carnivora, Artiodactyla and Lagomorpha. In the case of Carnivora, the unique amplification pattern of order-specific CAN SINE provides important evidence for the ''pan-carnivore'' hypothesis of this repeat element and reveals that the CAN SINE family may still be active today. Particularly interesting is the finding that all identified lineage-specific SINE elements show a strong tendency to insert within or in very close proximity to the preexisting MIRs for their efficient integrations, suggesting that the MIR element is a hot spot for successive insertions of other SINEs. The unexpected MIR excision as a result of a random deletion in the rat intron locus and the non-random site targeting detected by this study indicate that SINEs actually have a greater insertional flexibility and regional specificity than had previously been recognized. Implications for SINE sequence evolution upon and following integration, as well as the fascinating interactions between retroposons and the host genomes are discussed.

Supplementary materials: The nucleotide sequence data newly reported in this paper have been submitted to the GenBank and have been assigned the accession numbers: AY726642–AY726655

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Introduction

Short interspersed elements (SINEs) are repetitive retroposons less than 500bp in length, thought to be propagated in eukaryotic genomes by retroposition via an RNA intermediate (Rogers 1985; Okada 1991). The enormous dispersion of SINE elements may have had a significant impact not only on genomic diversity, but also on regulation of gene expression and function (Deininger 1989; Maraia 1995; Shedlock and Okada 2000). A variety of SINEs classes, the majority of which are tRNA-like derivatives, have been found in mammalian species and identified as either lineage specific (e.g., primate Alu and rodent B1 repeats) or ubiquitously distributed (CORE-SINEs).

In general, a typical SINE is not a simple tRNA pseudogene but has a composite structure, consisting of an internal RNA polymerase III promoter, a tRNA-unrelated region and an A + T-rich tail (Okada 1991; Smit and Riggs 1995). Insertions of SINE elements at new genomic sites has often been considered to be irreversible and random (Nikaido et al. 2001). As a result, SINE insertion analysis is becoming a tool for the determination of phylogenetic relationships.

Here we initiated an analysis of both ancient and recent insertions of mammalian SINEs that occurred in a single intron region to better understand their evolutionary significance. Using representative taxa from twelve mammalian orders, we report the unusual presence of mammalian-wide

interspersed repeats (MIRs) and several distinct lineage-specific SINE sequences in association with intron 7 of the β-fibrinogen gene, which encodes a protein involved in blood clotting (Doolittle 1984). MIRs represent one of the oldest tRNA-derived SINE elements examined to date, as inferred from its integration into the host genomes before the radiation of mammalian orders (Jurka et al. 1995; Smit and Riggs 1995). Other SINE sequences were detected along with MIRs but apparently inserted into their host genomes at later evolutionary times, being characterized by more recent amplification histories and restricted taxonomic distributions. In this paper, detailed analyses of these independent SINE retropositions in the nuclear β-fibrinogen intron 7 locus from various mammals revealed cases of a MIR excision as a result of a random deletion and preferential site targeting, both of which have rarely been detected in previous SINEs studies. Hence, our results demonstrate that SINEs actually have a greater insertional flexibility and regional specificity than had previously been recognized. These two intriguing findings with regard to the insertional properties of SINES have, on one hand, important implications for SINE sequence evolution upon and following integration, and on the other hand, demonstrated the fascinating interactions between retroposons and host genomes.

Materials and methods

DNA preparation and amplification. Individual or multiple representatives from 12 orders of placental mammals used in this study and their geographic sources are listed in Table 1. Genomic DNA was isolated from blood or frozen tissues according to the method of Sambrook et al. (1989). PCR amplifications of β-fibrinogen intron 7 gene, including primers and reaction conditions, were performed as described in our previous study (Yu et al. 2004), wherein the phylogenetic relationships among a wide range of cat species belonging to feliform carnivores were examined using the same intron sequences (Table 1). Acquired sequences were put into GenBank for BLAST searching (Altschul et al. 1997) and data validity was ensured. Target DNA segments of order Primates (*Homo sapiens*, *Pan troglodytes*) and Rodentia (*Mus musculus*, *Rattus norvegicus*) were directly extracted from the public database.

Identification of repetitive elements. DNA sequences were screened for interspersed repeats known to exist in mammalian genomes through program RepeatMasker (Smit and Green, unpublished; http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). The detection of matching elements and their assignments to specific repeat classes were provided. To eliminate possibilities of false positives, we checked the obtained alignments and the Smith-Waterman scores (MIRs, 219–495; others, 519–1939) which were significantly higher than the cutoff values (MIRs, 175; others, 195). Target site duplications flanking putative SINEs were identified by program REPFIND (Betley et al. 2002) and eye.

Sequences alignment and data analyses. Alignments of β-fibrinogen intron sequences were generated by CLUSTAL X (Thompson et al. 1997) and manually refined by eye. The relative positions of different mammalian SINE families in the intron alignment and their characteristic tRNA-like structures were determined. We calculated pairwise sequence divergences from the Kimura's two-parameter model (K2P) and performed phylogenetic analyses under neighbor-joining (NJ) criterion to reconstruct interordinal relationships in mammals using program MEGA (Kumar et al. 2001), with all specific SINE elements removed (except for orthologous MIRs). Branch support was assessed by bootstrap analysis (Felsenstein 1985; 1000 replicates).

Results

Previous studies of mammalian β-fibrinogen intron 7 have only been performed on the family Felidae of order Carnivora, and species-specific insertion of the CAN SINE family in two feline taxa has been detected (Yu et al. 2004). In the present report, we extended SINEs analyses of the same intron by including additional taxa from other carnivoran families and also placental orders of mammals beyond the Carnivora. The results revealed that β-fibrinogen intron 7 sequences were 0.6–0.7 kb in length for most of the species examined, while less than 0.6kb for members of order Rodentia and 0.7–1.2kb for six taxa belonging to the order Carnivora, Artiodactyla, Lagomorpha and Insectivora. The presence/absence of specific SINE insertions can account for the remarkable length variation of this intron among diverse mammals. The RepeatMasker results indicate that the short intron in Rodentia contains no matching repeats while all the other species harbor an ancient MIR insertion. Each of the six taxa with longer sequence lengths was shown to include both a MIR and a SINE unit that were specifically amplified in that lineage. Figure 1 shows the location and diversity of all SINE elements found in β-fibrinogen intron 7 region.

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

					β-*Fibrinogen Intron 7 Gene*			*Presence of SINEs*	
Mammalian order	*Family*	*Common Name*	*Scientific Name*	*Sample Source*	*Accession Numbers*	*References*	*Length (bp)*	*MIR*	*lineage-specfic SINE*
Primate	Hominidae	human	*Homo sapiens*	–	AADC01045796	Genome Database	619	+	-
	Pongidae	chimpanzee	*Pan troglodytes*	–	AADA01127961	Genome Database	619	+	-
Rodentia	Muridae	mouse	*Mus musculus*	–	CAAA01040050	Genome Database	505	-	-
	Muridae	rat	*Rattus norvegicus*	–	AABR03012101	Genome Database	184	-	-
Pholidota	Manidae	pangolin	*Manis pentadactyla*	Yunnan Province, China	AY726645	this study	593	+	-
Cetaceans	Balaenopteridae	whale	*Balaenoptera physalus*	Shandong Province, China	AY726643	this study	604	+	-
Artiodactyla	Surdae	pig	*Sus scrofa*	Yunnan Province, China	AY726642	this study	830	+	+
	Bovidae	cow	*Bos taurus*	Yunnan Province, China	AY726644	this study	594	+	-
Perissodactyla	Equidae	horse	*Equus caballus*	Yunnan Province, China	AY726647	this study	608	+	-
Insectivora	Soricidae	mole	*Anourosorex squamipes*	Yunnan Province, China	AY726648	this study	1225	+	?
Chiroptera	Vespertilionidae	bat	*Myotis chinensis*	Yunnan Province, China	AY726646	this study	589	+	-
Proboscidea	Elephantidae	elephant	*Elephas maximus*	Yunnan Province, China	AY726650	this study	609	+	-
Lagomorpha	Leporidae	rabbit	*Lepus oiostolus*	Yunnan Province, China	AY726651	this study	881	+	+
Pinnipedia	Otariidae	sea lion	*Zalophus californianus*	San Diego Zoo, USA	AY726649	this study	626	+	-
Carnivora	Felidae	Asiatic golden cat	*Profelis temminckii*	South of Yunnan Province, China	AY634369	Yu et al. (2004)	849	+	+
		pallas's cat	*Otocolobus manul*	Xining Zoo, China	AY634375	Yu et al. (2004)	849	+	+
		panther	*Panthera pardus*	South of Yunnan Province, China	AY634371	Yu et al. (2004)	611	+	-
		lion	*Panthera leo*	Kunming Zoo, China	AY634374	Yu et al. (2004)	611	+	-
		tiger	*Panthera tigris*	Yunnan Province, China	AY634372	Yu et al. (2004)	611	+	-
		snow leopard	*Panthera uncia*	China	AY634370	Yu et al. (2004)	611	+	-
		clouded leopard	*Neofelis nebulosa*	Yunnan Province, China	AY634373	Yu et al. (2004)	611	+	-
		Asiatic leopard cat	*Prionailurus bengalensis*	Yunnan Province, China	AY634376	Yu et al. (2004)	611	+	-
		lynx	*Lynx lynx*	XinJiang Provine, China	AY634377	Yu et al. (2004)	611	+	-
		domestic cat	*Felis catus*	Guangxi Province, China	AY634379	Yu et al. (2004)	611	+	-
		Chinese desert cat	*Felis bieti*	Qinhai Province, China	AY634378	Yu et al. (2004)	611	+	-

(Continued)

Table 1. Continued

Mammalian order	*Family*	*Common Name*	*Scientific Name*	*Sample Source*	β-*Fibrinogen Intron 7 Gene*			*Presence of SINEs*	
					Accession Numbers	*References*	*Length (bp)*	*MIR*	*lineage-specfic SINE*
	Viverridae	Masked palm civet	*Paguma larvata*	Yunnan Province, China	AY634380	Yu et al. (2004)	613	+	-
	Canidae	gray wolf	*Canis lupus*	China		this study	772	+	+
	Procyonidae	Raccoon	*Procyon lotor*	San Diego Zoo, USA	AY726653	this study	603	+	-
	Mustelidae	Marten	*Martes flavigula*	Kunming Zoo, China	AY726654	this study	605	+	-
	Ursidae	Brown bear	*Ursus arctos*	Heilongiang Province, China	AY726655	this study	596	+	-

Distribution and characterization of the MIR element ubiquitous among mammals. In the β-fibrinogen intron sequences, the MIR element was shown to integrate into the orthologous locus of all examined mammals except Rodentia, confirming a retropositional activity prior to the placental radiation (Gilbert and Labuda 2000). Further, an inspection of the GenBank database for the same intron sequences of non-mammalian vertebrates indicated that orthologous MIRs insertions were lacking in fish, birds and reptiles, suggesting that this MIR copy began to amplify after the divergence of mammals from other vertebrates but before the placental mammals' separation.

Previous analyses have recognized that MIR element of about 260 bp in length is composed of a tRNA-like Pol III promoter region, a central ''core'' domain and a 3′ variable segment (Gilbert and Labuda 1999, 2000). The alignment shown in Figure 1 indicates that MIR sequences, the complement of which inserted into the β-fibrinogen intron, were substantially divergent from the consensus previously compiled by Smit and Riggs (1995) [K2P distances from 30.8 (Carnivora)–52.8% (Insectivora) and 40.65% on average] and lack direct repeats at their boundaries. The sequence length corresponding to a MIR element was about 190bp in most mammalian species. The average distance between MIR sequences within the family Felidae of Carnivora was 3.6% while those among carnivoran families and mammalian orders were 17.4% and 26.8%, respectively. Present analyses of MIR repeats reveal that the highest sequence divergences were present in the mole and rabbit lineages, confirming an earlier conclusion using GenBank searches that a faster decay of MIRs may have occurred in lagomorphs and insectivores (Jurka et al.1995).

Sequences analysis showed that all MIR sequences include a B box, ''core'' region and an intact 3′ variable segment with nearly identical truncation sites except for that of *Lepus oiostolus*, where the 3′ variable segment contained large stretch of deletions. The A box and adjacent portions of the tRNA-like promoter (about 60bp) are the fastest evolving and ultimately unidentifiable due to the large numbers of random mutations since integration, as previously noted for MIR insertions in the zfOC1 and IGF1 gene sequences from three mammalian species (Hugher 2000). Our results raise the possibility that an inserted MIR copy exhibits mutation rate heterogeneity and nonrandom degeneracy among its three major segments following integration.

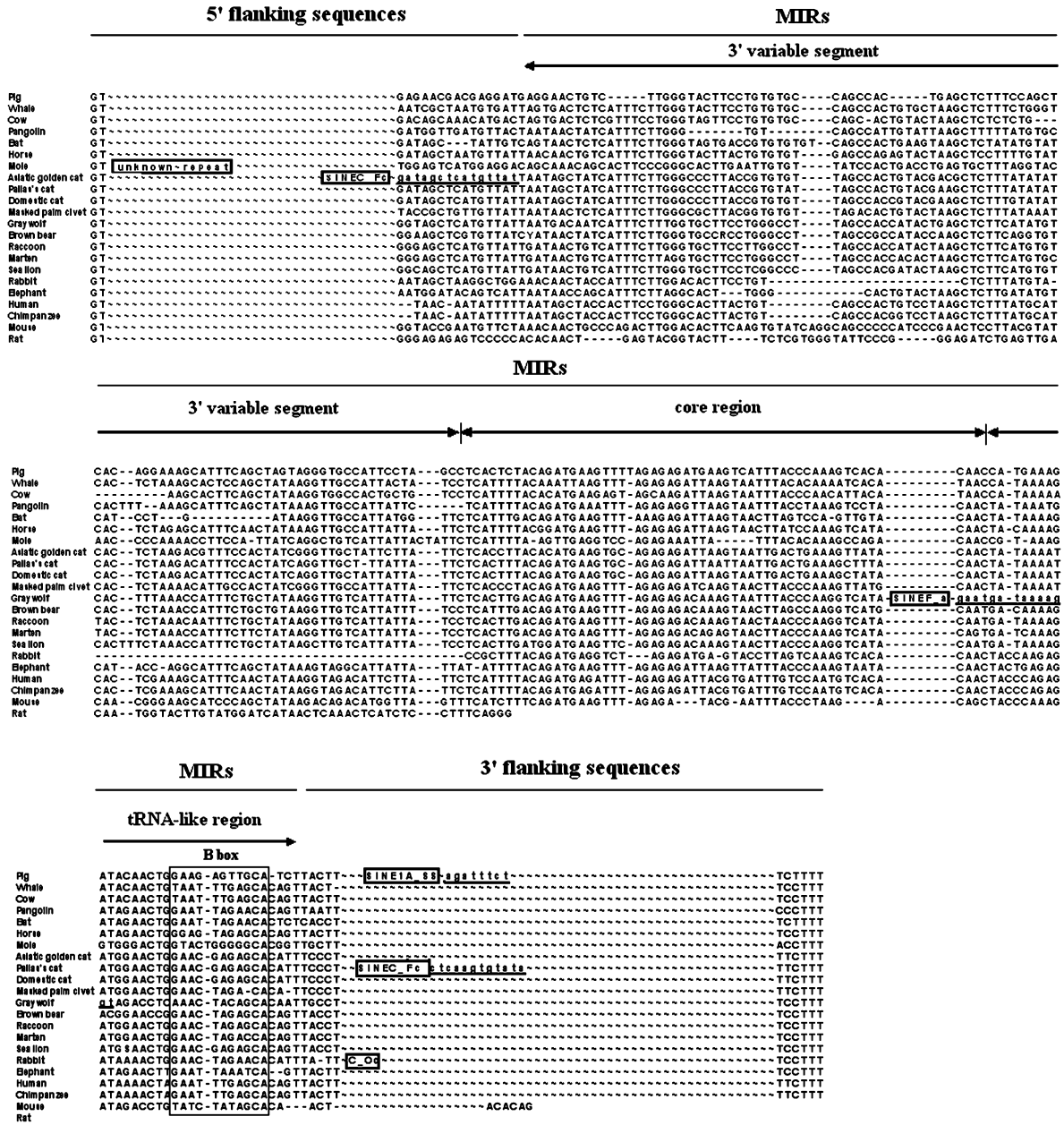

Fig. 1. The location and diversity of SINE sequences found in β-fibrinogen intron 7 from representatives of 12 mammalian orders. The family Felidae of Carnivora is represented here by three taxa, *P. temminckii*, *O. manul* and *F. catus*. Nucleotide sequences of MIRs and their immediate flanking sequences are shown. Shaded boxes indicated lineage-specific SINEs. The underlined lower case letters in the alignment indicate one side of the direct repeats flanking the SINEs.

Distributions and characterizations of several lineage-specific SINE elements. Of the 12 mammalian orders analyzed, four also displayed a lineage-specific amplification of a SINE family at different locations in the intron. The four orders were Carnivora, Artiodactyla, Lagomorpha and Insectivora (Figure 1).

In the order Carnivora, three new members of CAN SINE (Coltman and Wright 1994) were identified, each restricted in a single and distantly related species, *P. temminckii* (Asiatic golden cat), *O. manul* (Pallas's cat) and *C. lupus* (gray wolf), suggesting independent origin of these insertional events, especially in view of their different positions in the intron. On closer examination, sequence analyses revealed that the CAN SINEs in *P. temminckii* and *O. manul* were 225bp full-length sequences and belonged to subfamily SINEC_Fc

while that in *C. lupus* was 179bp of the intact subfamily SINEC_a. There were perfect 14 or 15 bp target-site duplications flanking them and these CAN SINE sequences differed from their respective consensus by low divergences (0.9 and 4.1% for SINEC_Fc in *P. temminckii* and *O. manul* respectively; 15.6% for SINEC_a), suggesting recent amplification of the CAN SINE family. These observations, together with the detection in other canines in family Canidae, such as *Canis familiaris*, *Canis niger*, *Vulpes vulpes*, etc., also of a SINEC_a at the identical intron location (data not shown), indicated that two species-specific insertions of SINEC_Fc in felid taxa and an SINEC_a insertion in a common ancestor of the dog family Canidae occurred in the evolution of β-fibrinogen intron.

Inserted within pig (order Artiodactyla) and rabbit (order Lagomorpha) lineages at different sites of the β-fibrinogen intron were an intact (131bp) porcine CHRS (SINE1A_SS; for SINE *Sus scrofa*; Frengen et al. 1991) and a fragment of the rabbit C repeat (C_Oc; for C repeat *Oryctolagus cuniculus*; Krane et al. 1991; 255 of full-length 339bp), respectively. There was 8bp target site duplication flanking the porcine CHRS and 17.9% sequence divergence for porcine CHRS compared to the consensus in the database. At the 5′ truncated rabbit C repeat that begins at the B box of the internal promoter, a large divergence of 29.2% was observed, corroborating its old age relative to SINE families in the other mammalian orders (Krane et al. 1991). Additionally, another type of repetitive element (≈600bp) also appears to have integrated into the mole lineage (order Insectivora) in the 5′ end of the β-fibrinogen intron and may represent a new class of repeat, given that it has no obvious homology to any known retroposons. Therefore, porcine CHRS, rabbit C repeat and the novel element represent three separate insertional events that occurred subsequent to the divergences of pig, rabbit and mole lineages, respectively. Regardless, they are all in the same orientation as the β-fibrinogen gene intron, possibly revealing a non-random insertion direction for SINE units.

In all, a minimum of six independent SINE insertions can explain the patterns observed in this small β-fibrinogen intron: During the Mesozoic era about 170-65 million years ago (MYA), a MIR element was presumably inserted, with the orthologous repeats now present in the mammalian species due to a pattern of common descent. Subsequently, on the lineages leading to *Lepus oiostolus* and *Sus scrofa*, order-specific families of SINEs, namely porcine CHRS and rabbit C repeat, were respectively amplified. Most recently, three sporadic insertions of CAN SINE took place in order Carnivora. Illustration of this series of SINEs integrations at this locus in the context of mammalian phylogeny is shown in Figure 2.

Discussion

Detailed characterization of multiple SINE insertions in β-fibrinogen intron 7 from diverse mammals are provided in the present study. This intron is shown to be remarkable in that both ubiquitous mammalian-wide interspersed repeats (MIRs) and distinct lineage-specific families of SINEs are evidenced in such a small region.

The classical tRNA-like MIR element was found at orthologous locations of all examined mammalian intron sequences except the Rodentia. Two possible scenarios can explain its absence in rodents: one is that a rare MIR deletion from the rodent lineage took place subsequent to the separation of Rodentia from the other mammalian groups; the other is that increased rates of sequence divergence in rodents have made identification of ancient MIR impossible (Deininger et al. 2003).

In all previous studies based on GenBank searches, the latter hypothesis had been proposed for the much lower genomic abundance of MIR element detected in rodents than in other mammalian groups (Jurka et al. 1995). Examination of mouse β-fibrinogen sequence supported this view, given that recognizable regions corresponding to the entire MIR length were detected in the alignment, albeit with low sequence similarity to the MIR consensus (Figure 1). However, analysis of the rat intron sequence clearly supports the former explanation instead, since the 184bp full-length rat sequence only contains the 3′ variable segment of the MIR element and the MIR 5′ flanking sequence, while the other parts of MIR and MIR 3′ flanking sequence were deleted from the intron. Thus, this rat intron sequence first supports the other interpretation for the missing of MIR element in Rodentia. To our knowledge, no cases of the precise excision of SINEs from the integrated genome has yet been reported (Batzer and Deininger 2002), and only a gross deletion of an entire gene locus and a partial deletion of an Alu element from the human genome have been described (Edward and Gibbs 1992; Salem et al. 2003). The present study thereby not only provides a new example of the imprecise excision of a SINE insertion, which apparently occurred after the divergence of the rat lineage and left minor ''footprints'' of the presence of MIR, but reveal two differential mechanisms for the lack of MIR se-

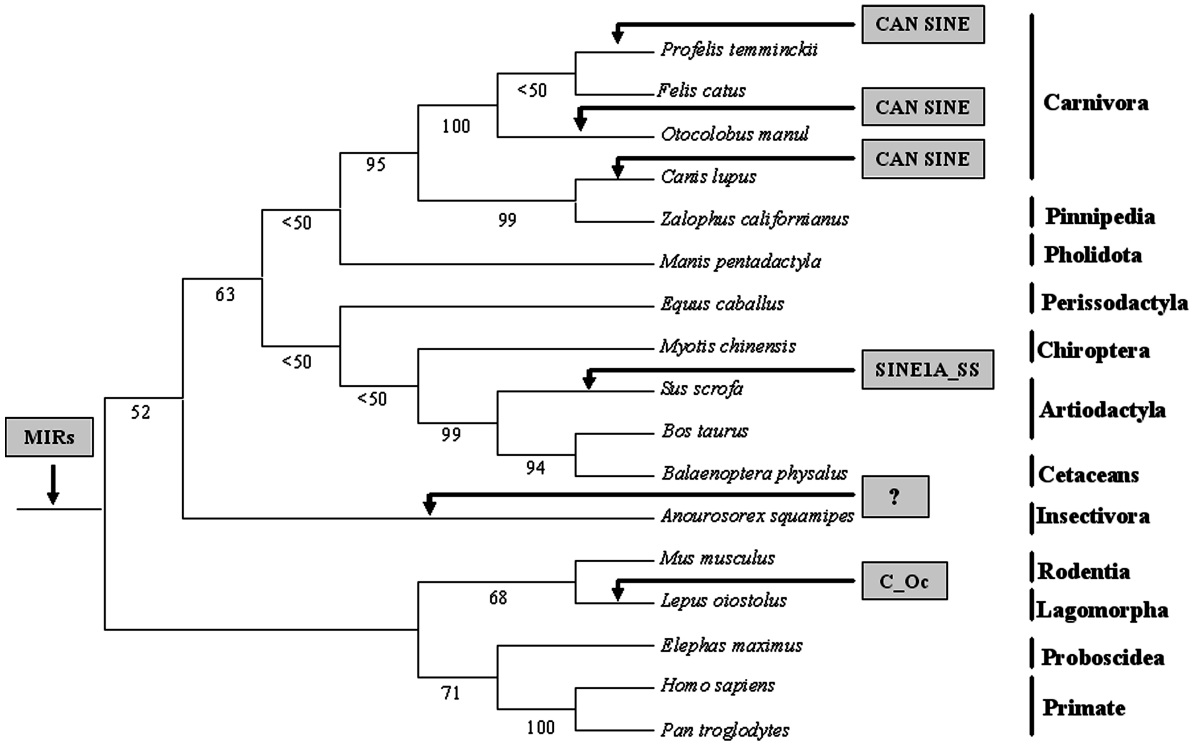

Fig. 2. Multiple SINE insertions occurred in nuclear β-fibrinogen intron 7 during mammalian evolution. The rat (*Rattus norvegicus*) intron sequence of Rodentia is not included because of its excessive deletions. Carnivora is only represented here by four taxa, *P. temminckii*, *O. manul*, *F. catus and C. lupus*. The estimated time of each insertion event is indicated by arrows and corresponding families of SINEs are shaded. Phylogenetic relationships among mammalian orders were constructed as described in Materials and methods.

quences in the rodents. It seems likely that either this MIR copy had been maintained over a long period of time for some functional role, whereas this selective conservation has not been required in the rat lineage, or that the removal of MIR from the rat lineage serves as a host defense mechanism to counteract the current high rate of retropositions in rodents, which would become fatal to the host if no efficient mechanism existed for removal of retroposons from the genome (Wichman et al. 1992). Moreover, this type of excision process of a mobile element, with simultaneous deletion of host sequences flanking the insertion site, has been found to play a role in the insertional mutagenesis and genetic variations (Wessler 1988; Schiefelbein et al. 1988; Kidwell and Lisch 2001). Clearly, such events contribute to our understanding of the evolution of MIRs as well as of their hosts.

Compared with the ancient highly divergent MIRs, a remarkable feature of the lineage-specific SINE families was their recent origins as demonstrated by the discernible target site duplications, narrow taxonomic distributions and low sequence divergences. In the order Carnivora, characterizations of three independently amplified CAN SINEs provided a confirmation of the ''pan-carnivore'' hypothesis for this repetitive element. Initial studies from genomic hybridization data restricted CAN SINE to the superfamily Caniformia (Minnick et al. 1992; Coltman and Wright 1994; Das et al 1998) because of its apparent absence in cat genomes. However, analyses by database searches and IRS PCR demonstrated that CAN SINE was present in all carnivore lineages (van der Vlugt and Lenstra 1995; Vassetzky and Kramerov 2002). Thus, prior to our report, the taxonomic distribution of this SINE family had been actively debated. The presence of the CAN SINEs in one caniform and two feliform carnivores here indicate that they are present in Caniformia, as well as in Feliformia genomes, similar to the distribution of SINES in intron regions of three Y-chromosome genes (Pecon Slattery et al. 2000) but contrasting with SINEs on the first intron of the transthyretin gene (Zehr et al. 2001), at which the CAN SINE was found to be specific for the caniform carnivores. In addition, the present amplification pattern of three independent CAN SINEs in the respective lineages of *P. temminckii*, *O. manul* and Canidae after their divergence, observed from the single β-fibrinogen intron locus, was unique

within the carnivore genomes compared to those exhibited in other intronic sequences (Pecon Slattery et al. 2000; Zehr et al. 2001), giving new insights into the evolution of this dynamic SINE family. We also conclude that CAN SINE may still be propagating and continue to impact carnivoran evolution and speciation.

We were surprised to detect a minimum of six independent SINE insertions from diverse, old MIRs to recent, active CAN SINEs, interrupting the same intronic region across 12 mammalian orders. This small locus may represent a ''favorable'' chromosomal region for high-frequency retroposition of SINE elements. Although noncoding DNA regions are intrinsically prone to accumulate repetitive families because of their high A+T content and lack of function, this may be only a partial explanation for the present observation. As shown in Figure 1, further examination of the integrated positions of these SINE sequences strikingly indicated that three of the five species-specific SINEs were located extremely close to the 5′ B box of their respective MIRs (rabbit C repeat, porcine CHRS and SINEC_Fc in *O. manul* within 12, 50 and 48bp, respectively), and possibly in the tRNA-like region of MIRs, if we extend the length of MIR element to include the divergent A box region. The SINEC_a in Canidae was found well inside of the MIR ''core'' region while the SINEC_Fc in *P. temminckii* was immediately upstream of the 3′ part of its cohabited MIR. This interesting observation suggests that there is a strong tendency of specific SINE families to insert within or in very close proximity to the preexisting MIRs, leading us to conclude that the MIR element may be a hot spot for successive integrations of other SINEs, driving the unusual collections of retroposons in this intron region. It also appears that additional mammalian SINE sequences have the tendency to insert into previously existing SINE element than have been previously recognized, examples of which have only documented for rabbit C repeats and human Alu-SINEs as far as we know (Slagel et al. 1987; Krane et al. 1991).

Previous studies have described various patterns of insertion site preferences for retroposons, from overall gene loci selectivity in rodents and humans (interleukin-6 and histone H3.3 genes; Qin et al. 1991; Wells and Bains 1991), to particular regional insertions within a gene locus in Sigmodontine rodents (two regions in mys-9 allele; Cantrell et al. 2001), to extreme sequence specificity in mammals and plants (5′ TT AAAA; TG, CA and TA dinucleotides; Jurka and Klonowski 1996; Jurka et al. 1998; Tatout et al. 1998). Ours is the first reported case of the MIR element being an insertional hot spot for other mammalian retroposons, not only casting new light on evolutionary and functional roles of this repetitive family, but also providing a second example supporting the attractive hypothesis that attrition of an earlier retroposition may provide a proper environment for successive retropositions, and function as a catalyst, as proposed by Wang et al. (2004) based on their analysis of SINEs in the third intron of interleukin-1β1 gene. Taking all the evidence together, we propose that SINE insertions may actually have a greater insertional flexibility and regional specificity than has previously been recognized and that consecutive retropositions of MIRs and integration of several distinct lineage-specific families of SINEs in this intron do not seem to be fortuitous. The β-fibrinogen intron is demonstrated to be among the few loci that have been subject to extensive invasions of SINE insertion events. We are thus convinced that additional SINE sequences would be discovered in this intron if more mammalian taxa are examined.

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