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Full Length Research Paper

Isolation and characterization of sixty sequences of cot-1 DNA from the Asiatic black bear, *Ursus thibetanus*

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The Asiatic black bear (*Ursus thibetanus*) is a class II protected species in China whose genome is not well characterized. All mammalian genomes have a high proportion of repetitive sequences; thus, studying the repetitive sequences of the Asiatic black bear can provide new insight into the organization of its genome. It is well known that cot-1 DNA is enriched for repetitive DNA elements with high and moderately high copy numbers within the genome. In this small-scale study, cot-1 DNA from *U. thibetanus* was first isolated and characterized using DNA reassociation kinetics, then a Southern blotting probe was used to indicate the presence of polymorphic repetitive DNA families in this species. Analysis of homologous sequences by online database searches revealed that the obtained fragments contained satellite sequences, mitochondrial DNA, retrotransposons, DNA transposons, and unclassified fragments whose deduced amino acid sequences has significant relationships to known proteins. The data produced by this study may lead to more research into repetitive DNAs.

Key words: Asiatic black bear, cot-1 DNA, repetitive elements, southern blotting.

INTRODUCTION

The Asiatic black bear (Ursus thibetanus) is a medium-sized bear that is distributed throughout southern and eastern Asia (Servheen, 1990). It is a class II protected species in China because of its declining population due to overhunting, extensive deforestation, and destruction of habitat (Ma and XU, 1998). To protect these populations and to clarify the phylogenetic relationships of Ursidae family members, this research is currently focused on the genetic structure of U. thibetanus. Recent studies have examined polymorphic microsatellite loci (Kitahara et al., 2000; Wu et al., 2010), the mitochondrial genome (Hwang et al., 2008; Yasukochi et al., 2009), Y chromosome gene divergence (Nakagome et al., 2008; Slattery et al., 2000), and nuclear genes (Pagès et al., 2008).

Repetitive genomic DNA sequences are a major component of many eukaryotic genomes. Approximately 46% of the human genome, 36% of the giant panda genome, and 31% of the dog genome consists of repetitive sequence (Kirkness et al., 2003; Li et al., 2010). However, no information on repetitive DNA in the Asiatic black bear genome is currently available. Thus, the isolation and characterization of the repetitive DNA may provide useful information for studying the U. thibetanus genome.

Repetitive sequences, which were also called low-cot DNA, reassociate much more rapidly than low-copy sequences due to differences in the complexity of the DNA sequence. Hřibová et al. (2007) and colleagues used DNA reassociation kinetics to isolate the highly repeated fraction of the banana (Musa) genome to significantly expand our knowledge of the organization of its chromosomes (Hřibová et al., 2007). Of the low-cot DNA, cot-1 DNA is known to be for repetitive DNA elements, high and moderate in copy numbers, and can therefore be used to pre-hybridize and competitively hybridize repetitive elements that would otherwise cause nonspecific hybridization in southern and in situ hybridization (Lichter et. al., 1988; Lichter et al., 1990b; Lichter et al., 1990a).

In recent years, effective medium have been used to

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comprehend the genome. Anamthawat-Jónsson generated a cot-1 DNA plasmid library from genomic DNA of the American tetraploid species Leymus triticoides, from which he obtained a family of highly repetitive satellite DNA sequences (Anamthawat-Jónsson et al., 2009). Falk Zakrzewski also analyzed a cot-1 library to identify targeted minisatellite and satellite families in Beta vulgaris (Zakrzewski et al., 2010). Combining the established principles of DNA reassociation kinetics with high-throughput sequencing also known as Cot-based cloning and sequencing (Peterson et al., 2002), is a powerful tool for isolating and characterizing the various repetitive components of any genome. This technique has been used to describe the repetitive DNA sequences in Panax ginseng (Ho and Leung, 2002) as well as the repetitive landscape of the chicken genome (Wicker et al., 2005).

Repbase Update is a comprehensive database of repetitive elements from diverse eukaryotic organisms. First developed in 1990, the most recent update contains >7,600 sequences of transposable elements and other repeats (Jurka et al., 2005; Kapitonov and Jurka, 2008). Repetitive sequences, such as cot-1 DNA, can be classified using a Censor server, which permits screening of homologous sequences in the cot-1 DNA with the elements in Repbase Update (Kohany et al., 2006). In addition to using Repbase Update, we can also search homologs using the National Center for Biotechnology Information (NCBI) database.

In this study, we report the first isolation of cot-1 DNA from the Asiatic black bear U. thibetanus using DNA reassociation kinetics. Portions of cot-1 DNA were classified into known repetitive families, and other portions had deduced amino acid sequences showing significant relationships with known proteins. We then, examined related reports and discussed the constitutive properties and phenomena that are similar to human studies.

MATERIALS AND METHODS

Animal materials

The Asiatic black bear is divided into five subspecies in China: U. t. thibetanus, U. t. laniger, U. t. mupinensis, U. t. formosanus, and U. t. ussuricus (Cowan 1970; Ma and Xu 1998). All samples used in this study were from U. t. ussuricus animals raised in the Beijing Zoo. DNA was obtained from fibroblast cell lines derived from the skin that were maintained under conventional culture conditions (37°C in medium 199 enriched with 15% fetal bovine serum (Gibco, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin).

Isolation and cloning of cot-1 DNA

Total genomic DNA was extracted from fibroblast cell cultured with phenol/chloroform extraction. The cot-1 fraction of the genomic DNA was prepared as described by Zwick et al. (1997), with some modifications. Briefly, the genomic DNA was sonicated into a fragment size of predominantly less than 1 kb, denatured in boiling water for 10 min, and then reassociated at 65°C for the calculated time to isolate cot-1 DNA. Cot is defined as the starting concentration of DNA fragments, and 't' is the reaction time. The time for cot reassociation was calculated using the formula cot = 1 = mol/L × T_s, where cot is calculated in moles of nucleotides per liter and time is in seconds. In this study, 490 µg/µL (0.49 g/L) of sonicated DNA was denatured in 0.3 M NaCl, using 1 M of dNTP equal to an average concentration of 339 g/mol, thus, the concentration of the sonicated DNA was (0.49 g/L)/ (339 g/L) = $1.445X10^{-3}$ mol/L and the reassociation time was 1/ ($1.445X10^{-3}$ mol/L) = 11 min 30 s. The remaining single-stranded DNA was digested with 1 U/µg S1 endonuclease for 1 h at 37°C, and the double-stranded DNA fraction was purified by phenol/chloroform extraction and ethanol precipitation.

An A-overhang was added to cot-1 DNA using *Taq* polymerase (Takara, Japan), and the resulting products were ligated into the pMD19-T vector (Takara, Japan). Competent *Escherichia coli* DH5α cells were transformed with the ligation reactions, and then the cloned DNA was extracted from the recombinant bacteria and sequenced.

Sequence analysis of positive clones

Cloned sequences were screened for known transposable elements using the online Censor server (http://www.girinst.org/censor/index.php) and searched for homology using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Micro-satellite repeats were detected using the online microsatellite repeat finder tool available (http://www.biophp.org/minitools/ microsatellite_repeats_finder/demo.php) (Anistoroaei et al., 2006).

Details of the analysis were as follows: first, clones were excluded if no reliable genome fragments were found by homology searches to the NCBI whole-genome shotgun read database using Mega BLAST and BLASTN. The remaining clones were searched for homology using Censor. Clones that produced no hits were searched for homology again in NCBI nucleotide and non-redundant protein database using the criteria described byWicker et al. (2005). When there was no information regarding any repetitive sequence characteristics using either Censor or NCBI tools, we defined the clone as an unclassified fragment.

Southern blotting

Total genomic DNA (12 µg) was individually digested overnight with the restriction enzymes *Bam*H I, *Hae*III, *Hifi* I, or *Alu* I. The digested samples were electrophoresed on a 1% agarose gel and transferred to Hybond-N+ membrane. Cot-1 DNA was labeled with digoxygenin (DIG) using random primers (Roche, USA) for use as a probe to hybridize to the digested DNA. The lumogenic substrate CSPD (Roche, USA) was used to visualize the hybridization signals.

RESULTS

Southern blotting

We isolated cot-1 DNA from the total genomic DNA of the Asiatic black bear and labeled it with DIG for use as a Southern blotting probe. The cot-1 DNA was hybridized to restriction digests of genomic DNA from the Asiatic black bear and from the giant panda *(Ailuropoda melanoleuca*), which was used as a comparative species. A distinct pattern of bands was observed when Asiatic black bear genomic DNA was digested with *Bam*H I, *Hifi* I, or *Alu* I, but no distinct pattern was found after digesting with *Hae*III (Figure 1). Furthermore, there were no distinct

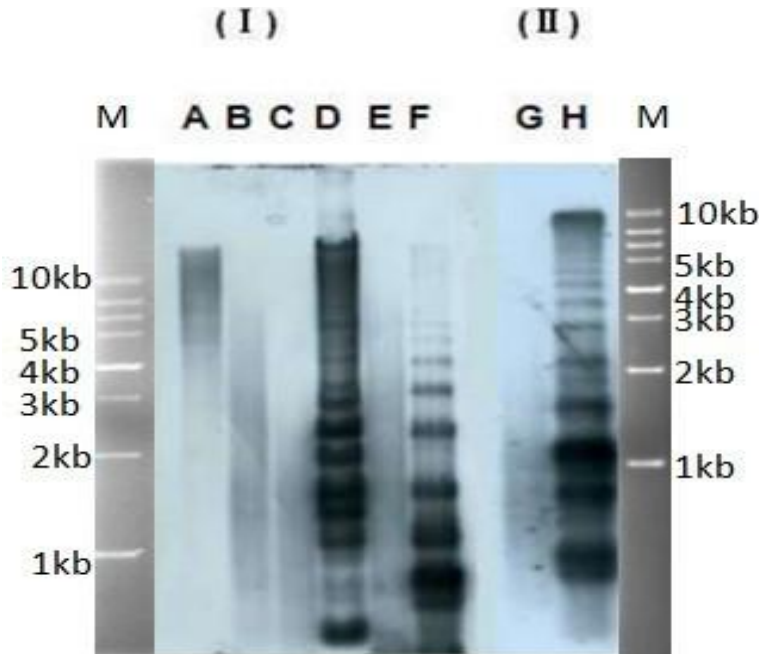

Figure 1. Southern blotting. (I), Lanes A, B, and C are genomic DNA from the giant panda digested with *Bam*H I, *Hae*III, and *Hifi* I, respectively; Lanes D, E, and F are genomic DNA from the Asiatic black bear digested with *Bam*H I, *Hae*III, and *Hifi* I, respectively; (II), lanes G and H are genomic DNA digested with *Alu* I from the giant panda and the Asiatic black bear, respectively. Digested DNA was probed with cot-1 DNA from the Asiatic black bear.

patterns of bands when Asiatic black bear cot-1 DNA was cross-hybridized to panda genomic DNA.

Classification using censor

63 positive clones were isolated from the Asiatic black bear cot-1 DNA and sequenced. Three clones were removed, and 44 clones were classified into different known transposable element families using Censor, which generated 58 corresponding homologous fragments. The final results from Censor analysis are shown in Table 1. One of these fragments was homologous to the *Felis catus* repetitive satellite DNA monomer FA-SAT, which is 483 bp in size, contains 64% G + C, and represents about 1 to 2% of the cat genome. FA-SAT-related sequences are found in many mammals (Fanning, 1987). Satellite sequences in the Asiatic black bear appeared to be scattered throughout the genome and were not tandemly arranged as in the cat.

45 fragments were homologous to retrotransposons, including nine fragments that were homologous to NonLTR/SINE, eight that were homologous to pseudogenes, nine that were homologous to NonLTR /L1, two that were homologous to NonLTR /CR1, one that was homologous to LTR/DIRS, seven that were homologous to LTR/Gypsy, three that were homologous to LTR/Copia, six that were homologous to ERV, and ten that were homologous to DNA transposons. Four fragments (from clones 11, 60, 70, and 99) were homologous to pseudogenes from the Diplomonadida *Giardia intestinalis* small subunit ribosomal RNA gene and may have come from various regions of this pseudogene as shown by the detailed locations of the homologous positions (Figure 2).

12 clones had two corresponding homologous fragments (not overlapped). For two of the 12 clones the, two homologous fragments were from a common class (for clone 15, both fragments were LTR/Gypsy homologs and for clone 46, both were non-LTR/L1 homologs). While for the remaining ten clones (clones 2, 7, 9, 10, 23, 34, 37, 66, 74, and 84), the two homologous fragments were each from different classes.

Classification using BLAST

The remaining 16 clones for which no hits were found using Censor, were searched against the NCBI nucleotide sequence database using Mega BLAST and BLASTN and against the NCBI non-redundant protein sequence database using BLASTX (Table 2). Clone 67 was not detected by running Mega BLAST or BLASTN in the whole-genome shotgun reads database, but was detected as satellite repeats consisting of (TTCC) 13

Table 1. Analysis of Asiatic black bear genomic clones using the Censor server.

Clone	Length (bp)[a]	Name	Class	Source
13	32	SINEC1B_AMe	NonLTR/SINE/SINE2	Giant panda
7-2	200	SINEC1_AMe	NonLTR/SINE/SINE2	Giant panda
32	100	tSINE_Fc	NonLTR/SINE/SINE2	Cat
48	100	tSINE_Fc	NonLTR/SINE/SINE2	Cat
69	176	MIRb	NonLTR/SINE/SINE2	Mammalia
68	135	TUBE1	NonLTR/SINE	Tree shrew
65,94	36	SINEC1D_CF	NonLTR/SINE	Dog
2-1	184	SINEC1D_CF	NonLTR/SINE	Dog
87	74	TRNA_ASN	Pseudogene/tRNA	Primate
11	255	SSU-rRNA_Giardia	Pseudogene/rRNA	*G. intestinalis*
60	255	SSU-rRNA_Giardia	Pseudogene/rRNA	*G. intestinalis*
70	102	SSU-rRNA_Giardia	Pseudogene/rRNA	*G. intestinalis*
99	87	SSU-rRNA_Giardia	Pseudogene/rRNA	*G. intestinalis*
45	96	LSU-rRNA_Ath	Pseudogene/rRNA	Viridiplantae
66-2	153	LSU-rRNA_Ath	Pseudogene/rRNA	Viridiplantae
56	48	LSU-rRNA_Ldo	Pseudogene/rRNA	Euglenozoa
17	108	L1-2_Ttr	NonLTR/L1	Dolphin
2-2	162	L1_Carn5_3end	NonLTR/L1	Carnivore
46-1	406	L1-1_AMe	NonLTR/L1	Giant panda
46-2	322	L1-1_AMe	NonLTR/L1	Giant panda
24	185	L1MAB_EC	NonLTR/L1	Horse
59	196	L1ME1	NonLTR/L1	Primate
23-2	57	L1ME_ORF2	NonLTR/L1	Primate
7-1	158	L1MC1_EC	NonLTR/L1	Horse
84-1	32	L1-65_XT	NonLTR/L1	Frog
73	105	L2_Plat1e	NonLTR/CR1	*Ornithorhynchus*
74-1	47	L2A	NonLTR/CR1	Human
37-1	342	DIRS-8_DR	LTR/DIRS	Zebrafish
43	156	Gypsy-38_NVi-I	LTR/Gypsy	*Nasonia vitripennis*
14	411	Gypsy-6-I_CR	LTR/Gypsy	Green algae
21	360	Gypsy-124N_SBi-LTR	LTR/Gypsy	Sorghum
15-1	276	Gypsy9-I_VC	LTR/Gypsy	Sorghum
15-2	438	ERIKA1_TM_LTR	LTR/Gypsy	Diploid wheat
37-2	120	Gypsy-106_SB-I	LTR/Gypsy	Sorghum
66-1	153	RETRO2_I	LTR/Gypsy	Rice
49	243	Copia-6-I_DR	LTR/Copia	Zebrafish
93	60	Copia-32_SB-I	LTR/Copia	Sorghum
10-2	846	ZMCOPIA1_I	LTR/Copia	maize
52	324	TguERVK7_I	ERV/ERV2	Estrildidae
89	161	MLT1E2	ERV/ERV3	Mammalia
23-1	181	LTR50	ERV/ERV3	Primate
97	96	ERV15_MD_I	ERV/ERV1	Mammalia
9-1	83	LTR18F_ML	ERV/ERV1	Bat
34-1	101	CarERV2b2_LTR	ERV/ERV1	Carnivore
20	312	MER45C	DNA/hAT	Primate
63	134	MER5A	DNA/hAT	Human
74-2	62	hAT-N22_DR	DNA/hAT	Zebra fish
30	66	HARB2_ZM	DNA/Sola	maize
3	81	Sola3-1_BF	DNA/Sola	*Branchiostoma floridae*
9-2	145	MuDR3_OS	DNA/MuDR	Rice

Table 1. Contd.

41	87	ENSPM7_OS	DNA/EnSpm	Rice
84-2	91	EnSpm-16_OS	DNA/EnSpm	Rice
34-2	47	EnSpm-22_SBi	DNA/EnSpm	Sorghum
10-1	481	MERMITE18D	DNA	Rice
72	52	FA-SAT	Simple/Sat/SAT	Cat

[a]Length of the homologous region. Name denotes locus name of Repbase library sequences; class denotes class/subclass of the repeat as specified in the repeat annotation; source denotes source of the repetitive sequence; nonLTR, non-long terminal repeat; SINE, short interspersed nuclear element; LTR, long terminal repeat; ERV, endogenous retroviruses; DIRS, dictyostelium intermediate repeat sequence. The version of the Repbase Update we used was released May, 2010.

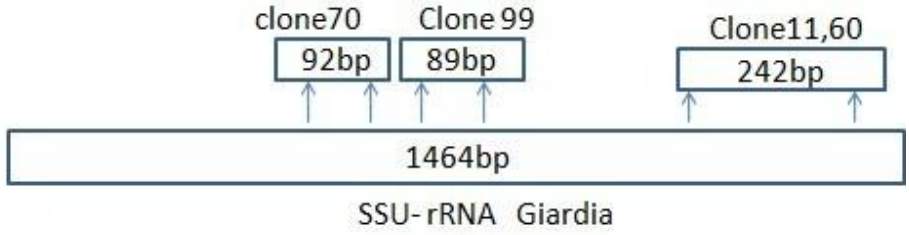

Figure 2. Schematic representation of the homology pattern between clones 11, 60, 70, and 99 and the SSU-rRNA from *Giardia.* Clones 11 and 60 have identical sequences.

perfect tandem repeats using the microsatellite repeat finder, thus suggesting that this clone may be part of a satellite sequence. Another satellite repeat was isolated from clone 55 and was homologous to microsatellite markers from the American mink. Clones 71 and 86 contained fragments homologous to the *U. thibetanus* clone UT36 microsatellite sequence. In addition, clone 83 was homologous to the SINE element in the *Zfy* gene, clone 33 was highly homologous to mitochondrial DNA from *U. thibetanus*, and some unclassified fragments encoded amino acids related to known proteins.

In total, we obtained 26 fragments that were homologous to non-LTR retrotransposons and belonged to the SINE (pseudogene), L1, and CR1 clades. 16 fragments were homologous to LTR retrotransposons and belonged to the Gypsy, Copia, ERV1, ERV2, ERV3, and DIRS clades. Seven fragments were homologous to DNA transposons and belonged to the Sola, MuDR, hAT, Harbinger, and EnSpm clades. We also identified clones with homologies to five satellite sequences, one mitochondrial DNA sequence, and seven unclassified fragments encoding amino acids related to known proteins.

DISCUSSION

We observed clearly distinct band patterns for Asiatic black bear genomic DNA that was digested by the restriction enzymes *Bam*H I, *Hifi* I, and *Alu* I. Our results indicate the presence of polymorphic repetitive DNA families found when the genomic DNA was digested by HaeIII, a pattern may be detectable after subcloning, as discussed by Leung (Leung, 1999). There were also no distinct band patterns when Asiatic black bear cot-1 DNA was cross-hybridized with giant panda genomic DNA, demonstrating that the two species have no common dominant repetitive sequences ,except faint bands detected when the giant panda genome was digested by *Bam*H I. Zheng'an et al. (1990) reported a tandemly repeated DNA which designated satellite 1 ,when the giant panda genome was digested by *Bam*H I, suggesting that similar tandemly repeated DNA exists in the cot-1DNA from the Asiatic black bear. Although the low similarity between the two species is surprising since they both belong to the family Ursidae, the use of cot-1 DNA as a fingerprinting probe in the Asiatic black bear maybe feasible.

This study therefore provides a small-scale analysis of the repetitive components in Asiatic black bear genomic DNA in which a few positive clones were sequenced and characterized. Although we obtained partial repetitive sequence families in the Asiatic black bear, we can infer from our results that the repetitive DNA isolated mainly contains retrotransposons, which is similar to human repetitive DNA. In the human genome, retrotransposons represent the majority of mobile elements, and they are predominant even when the DNA transposons are not taken into account (Deininger and Batzer, 2002; Gonçalves et al., 2000; Lander et al., 2001). Most homologous fragments belong to mammalians, and four of the clones were homologous to three non-LTR retrotransposons from the giant panda. Only four reports from the giant panda (L1-1 _ Ame, SINEC1B _ Ame

Table 2. Analysis of Asiatic black bear genomic clones using BLAST.

Clone	Length (bp)	BLASTN or BLASTX
1	624	ref|XP_001882980.1| predicted protein [Laccaria bicolor S238N-H82]
4	105	ref|XP_002952519.1| hypothetical protein VOLCADRAFT_93234 [*Volvox carteri f. nagariensis*]
19[a]	280	
25	413	ref|NM_001003315.1| *Canis lupus familiaris* Sec61 alpha 1 subunit (*S. cerevisiae*) (SEC61A1), mRNA
26[b]	149	ref|XM_845244.1| PREDICTED: *Canis familiaris* similar to Copine-6 (Copine VI) (neuronal copine) (N-copine) (LOC608275), mRNA
28	624	ref|XP_001882980.1| predicted protein [Laccaria bicolor S238N-H82]
33	278	gb|EF587265.1| *Ursus thibetanus thibetanus* mitochondrion, complete genome
47	220	ref|ZP_06280727.1| hypothetical protein SACT1DRAFT_4752 [*Streptomyces* sp. ACT-1]
55[b]	98	gb|DQ272092.1| Mustela vison clone I268 genomic sequence (microsatellite markers from American mink)
67[a]	54	(TTCC)13
71, 86	455	EU883636.1 *Ailuropoda melanoleuca* ribosomal protein S25 gene, complete cds and FJ640084.1 *Ursus thibetanus* clone UT36 microsatellite sequence
75	355	ref|XP_002591009.1| hypothetical protein BRAFLDRAFT_69438 [*Branchiostoma floridae*]
83[b]	58	dbj|AB261810.1| *Ursus thibetanus Zfy* gene, final intron, complete sequence SINE element in *Zfy* gene
92	1252	ref|YP_003343896.1| Serine/threonine protein kinase–like protein [*Streptosporangium roseum* DSM 43021]
95[b]	183	emb|AL359390.14| Human DNA sequence from clone RP11-139E24 on chromosome 1, complete sequence

[a]No effective hit with BLASTN and BLASTX; [b]no effective hit with BLASTX.

in this species. Although no distinct band patterns were SINEC1_Ame, and SINEC2_Ame) existed in Repbase Update as of October, 2010 (http://www.girinst.org/repbase/update/search.php?query=panda&querytype=Taxonomy). This is however not surprising because the two species belong to the same family.

Four clones were homologous to a pseudogene from the Diplomonadida *Giardia intestinalis* small subunit ribosomal RNA gene, which is consistent with a report that *Giardia lamblia* 16S-like rRNA has retained many of the features that may have been present in the common ancestor of eukaryotes and prokaryotes (Sogin et al., 1989). According to a previous study (Smit et al. 1995), the L1MA family is the youngest of the mammalian-wide long interspersed nuclear elements (LINE1), followed approximately by L1MB, L1MC, L1MD, and L1ME. We obtained fragments homologous to L1MAB_EC, L1ME1, and L1MC1_EC, suggesting that there are both young and old L1 sequences in the Asiatic black bear genome. Moreover, we identified two fragments homologous to L1-1_Ame, which are probably still undergoing active transposition (Jurka, 2010).

Six fragments were homologous to ERV, with clone 52 being a non-autonomous derivative (Smit, 2009). The mammalian genome contains various classes of ERVs, including human ERVs (HERVs), which comprised approximately 8% of the human genome. The Rhesus Macaque genome contains over a half-million recogni-zable copies of ERVs and their non-autonomous derivatives (Griffiths, 2001; Han et al., 2007). ERVs played an important role in the evolution of mammalian genomes. Like other transposable elements, in future studies, we may be able to find evidence that ERVs played a significant role during the evolution of the Asiatic black bear genome and the speciation of this animal.

Clones with two corresponding homologous fragments may have resulted following more than one transposition event, and fragments with partial corresponding elements suggest that the event may have been ancient and then interrupted. Clone 15 has two corresponding homologous fragments from the same LTR/Gypsy. Recombination between the LTRs of single or multiple elements occurs frequently in many families of retrotransposons (Shirasu et al., 2000), suggesting that clone 15 is likely to be the

product of elements of recombination events.

We also identified a few unclassified fragments that were homologous to known proteins. A similar phenomenon was reported in humans by Britten (2006), who described many distant but significant relationships between proteins and transposable elements. Britten concluded that many of these relationships were the result of past duplications of genes or gene regions, rather than a direct result of transposable element insertion. This may also have occurred in the Asiatic black bear genome. Alternatively, a specific class of repetitive fragments may exist in the Asiatic black bear genome, or the fragments may not be long enough to contain the characteristic motifs used for classification.

In conclusion, this study represents a small-scale use of Cot-based cloning and sequencing to study cot-1 DNA from the Asiatic black bear. Future work should carefully and thoroughly expound the information on these repetitive DNAs, thus leading to more research regarding repetitive DNA. Such studies should identify additional repetitive sequences in the genome that may provide mobile elements for use as markers in evolution and population biology (Deininger and Batzer, 2002).

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