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Original article

Title

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Running title

HERV-K14C drove Y chromosome evolution

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Abstract

The male-specific region of Y chromosome (MSY) has accumulated a higher density of human endogenous retroviruses (HERVs) and related sequences when compared with other regions of the human genome. Here, we focused on one HERV family, HERV-K14C that seemed to integrate preferentially into the Y chromosome in humans. To identify every copies of HERV-K14C in the human genome, we applied computational screening to map precisely the locus of individual HERV-K14C copies. Interestingly, 29 of all 146 copies were located in Y chromosome, and these 29 copies were mostly dispersed in the palindromic region. Three distinct HERV-K14C-related transcripts were found and were exclusively expressed in human testis tissue. Based on our phylogenetic analysis of the solitary LTRs derived from HERV-K14C on the Y chromosome we suggested that these sequences were generated as pairs of identical sequences. Specifically, analysis of HERV-K14C-related sequences in the palindromic region demonstrated that the Y chromosomal amplicons existed in our common ancestors and the duplicated pairs arose after divergence of great apes approximately 8-10 million years ago. Taken together, our observation suggested that HERV-K14C-related sequences contributed to genomic diversification of Y chromosome during speciation of great ape lineage.

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25 Keywords:

great ape/ hominoid/ long terminal repeat/ old world monkey/ palindrome/ solitary LTR/ testis-specific transcripts

Introduction

The human Y chromosome is believed to have evolved from an autosomal ancestor. The pseudoautosomal regions (PAR) at the termini of the X and Y chromosome recombine during meiosis, but most of parts of the X and Y chromosome do not undergo X-Y genetic exchanges. The male-specific region of Y chromosome (MSY) is directly inherited from father to son and is, therefore, highly informative regarding the evolutionary history of male lineages. The MSY contains at least 156 transcript units that seem to be expressed abundantly and exclusively in testis suggesting that these male-specific transcripts are associated with spermatogenesis.¹

Roughly, 41 mega bases (Mb) of MSY is heterochromatic sequences, and most of the heterochromatin comprises the distal long arm of the Y chromosome. The euchromatic region of MSY, which spans 23 Mb, has three structurally distinct features, X-degenerated, X-transposed, and ampliconic sequences. The ampliconic region make up 25% of the euchromatic portion of the human MSY, which includes eight palindromes with pairs of duplicated amplicon that ranges from 9 Kb (kilo bases) to 1.45 Mb (Figure 1). There is approximately 99.97% sequence identity between the repeats within each palindrome, implying that the palindromes arose through duplication events approximately 10 million years ago.² Based on sequence comparisons of the inner boundaries of each palindrome (P), six out of eight palindromes (P1, P2, P4, P6, P7, and P8) seem to have arisen after divergence of the great apes lineage suggesting that structures within the MSY has evolved recently. In particular, these palindromic sequences contained a high frequency (11.8%) of human endogenous retroviral sequences (HERVs) when compared to the average from the human whole genome (8%).³⁻⁵

Several distinct families of HERVs exist in the human genome.^{6,7} The majority of HERVs inserted into primate genome after the divergence of New World and Old World monkeys and were subjected to several amplification events during primate evolution.^{8,9} When HERVs integrate into a host genome, they generate a form of the viral genome, including a 5' LTR (long terminal repeats) - *gag* (capsid protein) - *pol* (viral enzyme) - *env* (envelope protein) - 3' LTR,

that is for replication and infection.¹⁰ Most HERVs are thought to be remnants of ancient germ
55 line infection because they are defective and have accumulated multiple stop codons and
deleterious mutations during evolution. Nevertheless, some HERV-related sequences are
actively expressed and able to influence the host genome. For instance, the envelope protein of
one of the most abundant families, HERV-W, serves an important function in the morphogenesis
of the placenta in human.¹¹ Not only the coding regions of HERVs contribute to transcriptome
60 diversity; the LTRs contain transcription factor binding sites, hormone response elements, and
polyadenylation signals that influence the host genome. Many reports demonstrate that HERV
sequences regulate expression of functional genes near the site of integration.¹²⁻¹⁴

An ancient member of HERV-K family entered the genome in the Old World monkey lineage
but HERV-K recurrently amplified and expanded during primate evolution.¹⁵ Some of HERV-K
65 members are present only in chimpanzees and humans, indicating a relatively recent integration
event within in the last five to eight million years.¹⁶ The solitary HERV-K LTR sequences,
rather than the full-length retrovirus, give rise to the genetic variants found in some human
individuals.^{17,18} In addition, the amplification of HERV-K elements within the human lineage
causes insertion polymorphism in the human genome.¹⁹

70 High sequence similarities between different HERV copies at different loci mediate non-
reciprocal homologous recombination causing the accumulation of deleterious mutations in the
human genome. For instance, recombination between HERV15 proviruses resulted in the
deletion of the azoospermia factor a (*AZF_a*) region on MSY and caused spermatogenic
impairment.²⁰ These previously published reports suggest that HERVs are a potential source of
75 genetic diversity due not only to integration events but also to providing transcriptome
variations.

The HERV-K14C element entered the primate germ line after the divergence between the
Old World and New World monkey lineages approximately 39 million years ago.²¹ Flockerzi *et*
al. surmised that 12 out of 23 copies of HERV-K14C copies were located on Y chromosome.

80 Based on this knowledge, we hypothesized that the HERV-K14C seemed relevant to Y
chromosomal evolution and we aimed to investigate this possibility with a high-resolution
analysis of HERV-K14C elements on the Y chromosome. Indeed, our computational analysis
demonstrates that copies of HERV-K14C were heavily dispersed in palindromic regions. We
investigated a multilateral effect of HERV-K14C during Y chromosome evolution.

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Materials and Methods

Bioinformatic analysis

All human sequences were obtained from the NCBI database, Build 36.1. HERV-K14C
90 elements were identified in the Repbase database (<http://www.girinst.org/rebase/>) and in the
genomic sequences using RepeatMasker ([http://ftp.genome.washington.edu/cgi-
bin/RepeatMasker](http://ftp.genome.washington.edu/cgi-bin/RepeatMasker)), which uses a cross_match program to perform perfect sequence
alignments.^{5,22} As a consensus sequences, LTR14C and HERV-K14C consensus sequences
were joined. Finally, LTR14C-HERV-K14C-LTR14C sequences were consensus sequences for
95 our study. The internal retroviral sequences of the HERV-K14C were constructed by comparing
conserved residues as potential coding region (gag, pro, pol, env) using the Blastx program.²³
The human expressed sequence tag (EST), RefSeq mRNA, and non-redundant databases were
screened using BLAST, version 2.2.11 with the “-q -e -e 0.01 -F T” options, and HERV-K14C
consensus sequences to identify hybrid transcripts.²⁴ HERV-K14C-related transcripts (more
100 than 95% sequence identity) were identified by comparing HERV-K14C elements with each
database.

Phylogenetic analysis

Multiple alignment analysis and phylogenetic trees were constructed using loci containing
105 solitary LTRs.²⁵ The solitary LTRs from the human genome were selected to align with
consensus sequence of LTR14C and these were matched over 80% of sequence identity. There
were 93 loci identified in this study. To improve the accuracy of the comparisons, we limited
our analysis to sequences greater or equal to roughly 500 – 600 bp. In addition, solitary LTRs
were deleterious sequences or disturbed by insertions of other genomic sequences that 17 of 93
110 loci were eliminated. In all, 76 LTRs were analyzed to construct for phylogenetic tree. For a
smooth phylogenetic analysis, incomplete elements and elements containing many gaps
were manually excluded. The neighbor-joining trees were obtained with the MEGA4 program.²⁶
Bootstrap values for branches were calculated based on 1,000 replications. Distances were
estimated by the Kimura two-parameter method in the MEGA4 program to estimated sequence
115 divergence.

Preparation of Genomic and RNA samples

Total RNA from Japanese monkey tissues was extracted by RNeasy Mini Kit (Qiagen,
Hilden, Germany), and total RNA from human tissue (brain, liver, lung, testis, heart, stomach,
120 spleen, prostate, bladder, and ovary) was purchased from Clontech (Clontech Laboratories, Inc,
Mountain View, CA, U.S.A). Pure mRNA was isolated using PolyA Tract mRNA isolation
systems (Promega Corporation, Madison, WI, USA). Genomic DNA was isolated from blood
samples by a standard protocol from the following species: (1) hominoids: chimpanzee (*Pan
troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), and gibbons (*Hylobates
125 agilis*); (2) Old World monkeys: Japanese monkey (*Macaca fuscata*), rhesus monkey (*Macaca
mulatta*); (3) New World monkeys: night monkey (*Aotus trivirgatus*), and common marmoset
(*Callithrix jacchus*) using a standard protocol.

Genomic and RT-PCR amplification

130 cDNA samples from human and Japanese monkey RNA were to be synthesized by MMLV-
derived reverse transcriptase with oligo (dT) and random hexamer primers (Promega
Corporation). Genomic PCR and RT-PCR reactions were carried out using the standard PCR kit
supplied by Takara. Primer information and accession numbers are documented in Table 1. *SRY*
and *GAPDH* were used as positive controls.

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Sequencing

PCR products amplified from genomic DNA were isolated using a PCR purification kit
(Qiagen, Hilden, Germany) and were sequenced with the BigDye Terminator version 3.1 Cycle
Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI wi337 DNA sequencer
140 using the same primer pairs that had been used for amplification.

Statistical analysis

The difference between expected and observed copy number of HERV-K14C in proportions
were contrasted by computing odds ratios (OR) and 95% CIs. All analyses were performed
145 using Statistical Package for Social Sciences, version 11.0 (SPSS, Chicago, IL, USA) and the *P*
value was corrected using Fisher's exact test.

Results and Discussion

We first identified the genomic locations of all HERV-K14C-related sequences using
150 computational approaches to search the entire human genome. In total, 146 loci dispersed across
the human chromosomes. Only 14 of these loci had comparatively intact copies of HERV-K14,
and 93 loci had LTR-related sequences (Suppl. Table 1). The other 39 loci were deleted and
mutated their internal sequences that mostly lost a 5' or 3' LTR. Presumably, the high frequency
of solitary LTR copies implied that the majority of the HERV-K14C inserts underwent intra-

155 homologous recombination between 5' and 3' LTR and then recurrently amplified in human
genome. In addition, eight transcripts with HERV-K14C-related sequences were found
suggesting that HERV-K14C elements provided promoters, exons, and polyadenylation signals
for endogenous genes near the HERV insertion sites (Suppl. Table 2). Of these eight HERV-K-
related transcripts, three resided in the MSY (Figure 1d).

160 The chromosomal distribution of HERV-K14C loci suggested that these sequences were not
evenly dispersed in the human genome. HERV-K14C-related sequences were absent from
chromosomes 9 and 22, whereas a high frequency of all HERV-K14C-related sequences (up to
20% or 29 copies) were found on the Y chromosome (Figure 2). To determine the statistical
significance of high frequency of HERV-K14C copies on Y chromosome, we calculated the
165 expected copy numbers based on the size of each chromosome. As shown in Figure 2,
chromosome 8, 19 and Y seemed to present higher density of HERV-K14C copies when
compared with expected copy numbers. Specifically, based on its size of Y chromosome was
expected, theoretically, to have 3.09 copies of HERV-K14C element. However only Y
chromosome had a statistical significance between expected and observed copy numbers ($P <$
170 0.001 , OR = 11.8, CI: 3.5 – 39.8). Indeed, HERV-K14C sequences were more likely to insert
onto the Y chromosome than onto any of autosomes or the X chromosome.

To understand the evolutionary relevance of HERV-K14C sequences, we chose to study
solitary LTRs for phylogenetic analysis because other fragment of HERV-K14C-related
sequences were too truncated and mutated for useful analysis. Having eliminated 17 incomplete
175 LTR copies, we used 76 copies of solitary LTR for this study (Suppl. Table 1). The neighbor-
joining method was used to construct a phylogenetic tree with all 76 intact solitary LTR loci
(Figure 3). Of the 76 LTR loci in the genome, 15 were located on the Y chromosome (Table 2).
These 15 loci are marked with starred numbers in Figure 3, and the other loci are named by their
chromosomal location.

180 As shown in Figure 3, the 76 solitary LTRs clustered into distinct phylogenetic groups that

suggest these sequences were amplified at least four times after the initial genome integration event. Members of group I were scattered on autosome and both sex chromosomes. In contrast, no members of group II were found on the Y chromosome. One interesting features of this tree was that group I members derived from the Y chromosome clustered into same nodes. For instance, LTRs 7&14, 27&28, 9&12, and 10&11 formed five distinct pairs and LTRs 24, 25,26, and 29 clustered into a single node. These observations implied that each pair of LTRs was duplicated from a single copy of LTR during Y chromosomal evolutionary past. As we described above, HERV-K14C-related sequences were enriched on Y chromosome, and this observation explained, in part this feature of the phylogenetic analysis of the solitary LTRs. The insertion of initial HERV-K14C element predated to integrate into the Y chromosome, and HERV-K14C element underwent subsequent genomic amplification events producing identical pairs.

The copies of HERV-K14C-related sequences on the Y chromosome are listed in Table 2 and Figure 1a,b, and c. As we mentioned above, 29 different HERV-K14C-related loci were identified on the Y chromosome; of these 17 loci were solitary LTRs and eight copies preserved both the 5' and 3' LTR. Three of loci only had a side of LTRs and its internal sequences; 5'LTR (8 and 13) and 3'LTR (3). In all, 18 copies were part of palindromes, meaning that these 18 copies actually represented 9 pairs, each with one member of the part on an arm of the respective palindrome. For example, 7-10 resided on the proximal arm of P5 and 11-14 resided on the distal arm of P5, the pairs were in reverse complementary direction. In a similar manner, two pairs — 15/19 and 16/18 — were identical sequences in P4. The b amplicons had four of copies (24/25/26/29) scattered along P3 (b1 and b2) and P1 (b3 and b4) and these four duplicated copies were generated from a single HERV-K14C element. The duplicated pair (27/28) was found on P1.1 and P1.2. In each case, the sequence alignment between duplicated copies within a palindrome showed perfect identity between the duplicated pair. The solitary LTRs derived from the b amplicons were the only exceptions, there were single nucleotide

difference (C → A) between the pairs 24/29 and 25/26. Four comparatively long HERV-K14C-related sequences — 8, 13, 16, and 18 — also revealed perfect matched pairs (8&13 and 16&18) that did not have a single nucleotide gap or difference.

210 Although, 24, 25, 26 and 29 loci were all located in the b amplicons, they arose from different two duplication lineages. As shown in the topology of the tree of solitary LTRs on Y chromosome in Figure 4, duplicated partners clustered together. Each LTR copy on an arm of a palindrome was clearly clustered with one LTR copy on the other arm of the same palindrome. For example copy 24 formed a pair with 29 and 25 formed a pair with 26 on the b amplicons. As
215 illustrated in Figure 1c, P1 contains the b3/b4 amplicon as an inner segment and b1/b2 amplicon is dispersed across P3. According to our results, the 24 (b1)/29 (b4) and 25 (b2)/26 (b3) pairs were mingled indicating that genomic structures of P1 and P3 were generated in concert.

To investigate, the integration history of each HERV-K14C copy on the Y chromosome, we looked for the orthologs of the boundaries each of HERV-K14C locus on Y chromosome to
220 estimate an ancestral structure of the Y chromosome. Unlike autosomal chromosomes, the Y chromosome contains massive palindromes and many repeated sequences making it very difficult to amplify specific loci. Therefore, we chose fifteen HERV-K14C-related copies for amplification of genomic DNA from male primate genomes. The chosen loci are denoted in Figure 1a,b, and c with vertical red arrows and in Table 2 with highlighted numbers.

225 The amplification boundaries covered the individual HERV-K14C sequences and their flanking regions. When a HERV-K14C locus was not observed in the orthologous region of primate genome, we can find a pre-integration site or intact structure of HERV-K14C that enabled us to estimate generation timing of Y chromosome. For each species tested, PCR products were not observed when DNA from female primates was used as template (data not
230 shown).

Overall, the integration of HERV-K14C loci occurred in Old World monkey and hominoids approximately 25 million years ago (Figure 5). Most of HERV-K14C copies (5, 9/12, 15/19, 20,

21, 22, 24/25/26/29, and 27/28) resulted from insertion events that occurred after divergence of the hominoids from Old World monkeys. Specifically, copies 5, 15/19, 21, and 27/28 were only
235 observed in humans and the samples from the great apes excluding orangutan suggesting that these insertions were occurred 10 million years ago.

In all copies, we only observed that 1 and 24/25/26/29 were an intact structure and pre-integration sites, respectively. The solitary LTR locus 1 (Table 2 no. 1) was present in not only the hominoid lineage, but an enlarged form of it was also found in the Old World monkey
240 samples. Based on our sequencing data, the large product amplified from the Old World monkey samples had both LTRs and internal HERV sequences, and the sequence identity between the 5' and 3' LTR was quite high at approximately 82% (data not shown). Many HERV sequences have been lost their functional domains by internal recombination between the 5' and 3' LTRs leading to solitary LTR formation. In addition, a recombination between the 5' and 3' LTRs
245 continues to occur in individuals of human population that has caused recent genetic variation in humans.²⁷ In case of LTR locus 1, an HERV-K14C had integrated into the primate genome and then underwent a subsequent deletion event owing to homologous recombination between 5' and 3' LTRs.

Conversely, copies 24/25/26/29 on the b amplicon were unexpectedly small at
250 approximately 384 bp with a gap of 7 bp) in Japanese and rhesus monkey genomes. In contrast, hominoids were revealed exact target size (976 bp); therefore, the LTR sequences copies 24/25/26/29 in the Old World monkey lineage seemed to have lost 585 bp. We attempted to confirm the sequences of unexpected product in the Old World monkeys. The sequencing results showed that a high degree of identity (86%) was found between flanking region of 24/25/26/29
255 and the small product. This observation suggested that 384 bp of small product was from a pre-integration site that existed before the integration of 24/25/26/29 LTRs. This data implied that the ancestral b amplicon has existed since the Old World monkey lineage. At least, the duplicated pairs that span P1 to P3 of the b amplicons were amplified after the divergence of

hominoids and Old World monkey. Because the b amplicons reside in palindromic regions, it
260 was quite difficult to amplify specific a locus from these regions. Thus, we could not clarify
which of the b amplicons predated the insertion event and existed in the ancestral Y
chromosome. Nevertheless, this investigation allowed us to analyze the complex evolutionary
past of the Y chromosome.

The pair 15/19 was dispersed in P4, and this region was present only in human and gorilla
265 consistent with an amplification of the inner boundaries of P4 from Page's study.² In addition,
an identical pair of 9/12 on P5 was observed in the great apes, and the 27/28 locus on P1
identified an insertion site in gorilla, chimpanzee and human.

Obviously, orthologs of the HERV-K14C loci found in humans were detected in our closest
evolutionary relatives, but we could not determine whether the integration time was consistent
270 with the complete formation of the palindromic structure. In spite of that, our results
demonstrated that at least an arm of P4 and of P5 already existed in the great ape lineage
approximately 8-15 million years ago. In our result, most of loci were incapable of revealing a
pre-integration site except locus 1 and loci 24, 25, 26, and 29. The majority of orthologs was not
detected in samples from lesser apes, indicating that the genomic structure of human Y
275 chromosome arose recently.

HERV-K14C was associated not only with palindromic structures but also with Y
chromosomal transcripts. Using a computational approach, we screened human EST and mRNA
databases with HERV-K14C consensus sequences to identify hybrid transcripts. As denoted in
Figure 1d and Table 2, HERV-K14C provided gene regulatory regions to three transcripts. The Y
280 chromosomal transcripts—*AW966153*, *AK026367*, and *TTY13*—were dispersed throughout the
P4 and inverted repeat 2 (IR2) (Figure 1d).

Two identical copies of *AW966153* were due to the duplication of P4 and solitary HERV-
K14C LTRs from loci 15 and 19 provided the polyadenylation signals for these transcripts.
Based on the integration lineage of LTRs on 15/19 locus, *AW966153* was only expressed in the

285 human and gorilla genomes. The 5' LTR of locus 21 provided the promoter region for
AK026367, and the first exon of this transcript was totally donated by HERV-K14C-related
sequences. Using TRANSFAC® Professional version 10.1 with a strict threshold (core match: 1
and matrix match: 0.95~1), we found five binding sites for multiple transcription factors
(including GATA box, TFE, TGIF, and others) distributed on 5' LTR of locus 21 which provided
290 the transcription starting sites for *AK026367*.²⁸ In addition, HERV-K14C sequences entirely
provided the last two exons on *TTY13* locus.

We used RT-PCR to examine the expression profiles of HERV-K14C-related transcripts in
various human tissues (Figure 6). All of these HERV-K14C-related transcripts were exclusively
expressed in human testis tissue. Based on genomic PCR result of loci 15/19, 21, and 22, these
295 transcripts arose in the genome after divergence of the orangutan and gibbon lineages.

Many researchers who study male infertility have investigated deletions of ampliconic
sequences.²⁹⁻³¹ For example, some studies reporting deletion between the distal arm of P1 to the
proximal arm of P4 explained only those deletions that resulted from micro-deletions between
direct repeat sequences that cause spermatogenic failure due to removal of Y chromosomal
300 specific transcripts³². In our investigation, three of HERV-K14C related transcripts spanned on
between P4 and P1. The removal of HERV-K14C related transcripts has a potential possibility
to be related with spermatogenic failure due to the deletion between P1 and P4 region. In this
investigation, we could not fully elucidate the molecular role of HERV-K14C-related transcripts,
but they were only detected in testis. In other words, HERV-related transcripts were not
305 expressed in human testis when HERV-K14C was not present. Namely, the acquisition of testis-
specificity could be attributed to the integration of HERV-K14C elements meaning that HERV-
K14C gave a rise to transcriptome diversity in the Y chromosome during primate speciation.

In summary, we have estimated the evolutionary timing of the emergence of palindromic
region of the Y chromosome. The orthologs of sequences flanking the HERV-K14C inserts were
310 detected mostly after divergence of hominid lineage around 18-20 million years ago (Figure 7).

Moreover, ancestral region of b amplicon existed in the Old World monkey lineage. We could not estimate the emergence date of both arms of each palindrome but one arm of P1.1/2, P3 and P4 was existed since gorilla lineage. Subsequently, they underwent the duplication event at least 10 million years ago. In our study, the integration timing of HERV-K14C presented orthologues of Y chromosome in primate genome that enabled to conjecture emergence timing of Y chromosomal sequences during primate evolution. Not only HERV-related sequences but also other transposable elements (TEs) should be useful tool to estimate evolutionary past of modern human Y chromosome as molecular clock. Moreover, these TEs have a possibility of different integration lineage that potentially have an effect on genomic differences between human and our closet relatives, implying that further studies of TEs lead us to understand a puzzled history of Y chromosome evolution.

Here, we suggested that the ancestral palindromic structure was constructed gradually as a pair of palindromes emerged during the evolutionary past and subsequently built up to its current structure of multiple palindromes due to duplication event that occurred in the great ape lineages. Analysis of Y chromosome sequences from the chimpanzee suggests that repeat sequences expanded and accumulated on the Y chromosome. In addition, this report suggested that the variation in sequence identity between repeat elements in human and chimpanzee was higher on the Y chromosome than it is on average across the genome implying that these repeated sequences changed more quickly.³³

In our study, we investigated the orthologs of HERV-K14C integration sites that reflected the evolutionary past of the human Y chromosome. Because most of HERV family members emerged in the primate genome after the divergence of Old World and New World monkey lineages and some HERV-K family members still exhibit variability in human populations,³⁴ HERVs may function as molecular clocks in the investigation of human evolution.

We aimed to verify the association between HERV-K14C and Y chromosome evolution. HERV-K14C-related sequences have been amplified during the evolution of the Y chromosome

and contributed to genomic diversification of Y chromosome during great ape lineages.

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455

Figure legends

Figure 1. The overview of the structure of the Y chromosome.

The male-specific region of Y chromosome (MSY). Black signifies the pseudoautosomal region, pale grey signifies euchromatin and dark grey is depicted as heterochromatic region of Y chromosome, respectively. Red triangles are depicted as locations of HERV-K14C locus on Table 2. (a). Enlarged schematic representation of the palindromic region, paralleled blue arrows represent each arm of eight palindromes. (b). Individual amplicons span from b1 on P3 to b4 on P1. Identical pairs of amplicons are colored the same color and arrows denote the direction of amplicons (c). The genomic structure of HERV-K14C-related transcripts (d). Exons are drawn as pale grey boxes and open reading frames are in black. The numbers above the boxes indicate exon number. Dark grey arrow depicts the HERV-K14C-related sequences, and the direction of the arrows illustrates the direction of transcription. Small black arrows indicate primer sequences (d). Vertical red arrows indicate the locations are used for genomic amplification in Figure 5 and represent a numbers in highlighted numbers on Table 2.

Figure 2. The chromosomal distribution of HERV-K14C-related sequences. Pale grey bar depicts the expected copy number of HERV-K14C-related sequences based on the genomic size of each chromosome. Dark grey bar presents observed copy number of HERV-K14C within each chromosome. The P – value was calculated and corrected between expected copy number and observed copy number. The value of $P > 0.05$ was not described.

Figure 3. The phylogenetic analysis of solitary LTRs from the entire genome.

Bootstrap values derived from 1,000 bootstrap replicates measured by the posterior probabilities are shown at the nodes. We also constructed a neighbor-joining tree using the MEGA4 program and the HERV-K14C consensus sequence. Each taxon name indicates the chromosomal location of a LTR locus except for those loci on the Y chromosome. Starred numbers detailed in Table 2

indicate the solitary LTRs on the Y chromosome.

485 Figure 4. Fine scale phylogeny of the solitary LTRs on the Y chromosome.

Only the gross topology of the tree is shown in this image. Bootstrap replicates support from 1,000 is indicated for each node. Each taxon is described in Table 2.

Figure 5. The integration lineage of HERV-K14C copies in Y chromosome.

490 The numbers correspond to the vertical red arrows and highlighted numbers in Figure 1 and Table 2, respectively. Hominoids: HU (human), CH (chimpanzee), GO (gorilla), OR (orangutan), and GI (gibbons); OWM (Old World monkeys): JM (Japanese monkey), RM (rhesus monkey); NWM (New World monkeys): NM (night monkey) and CM (common marmoset); ddH₂O was used as a negative control.

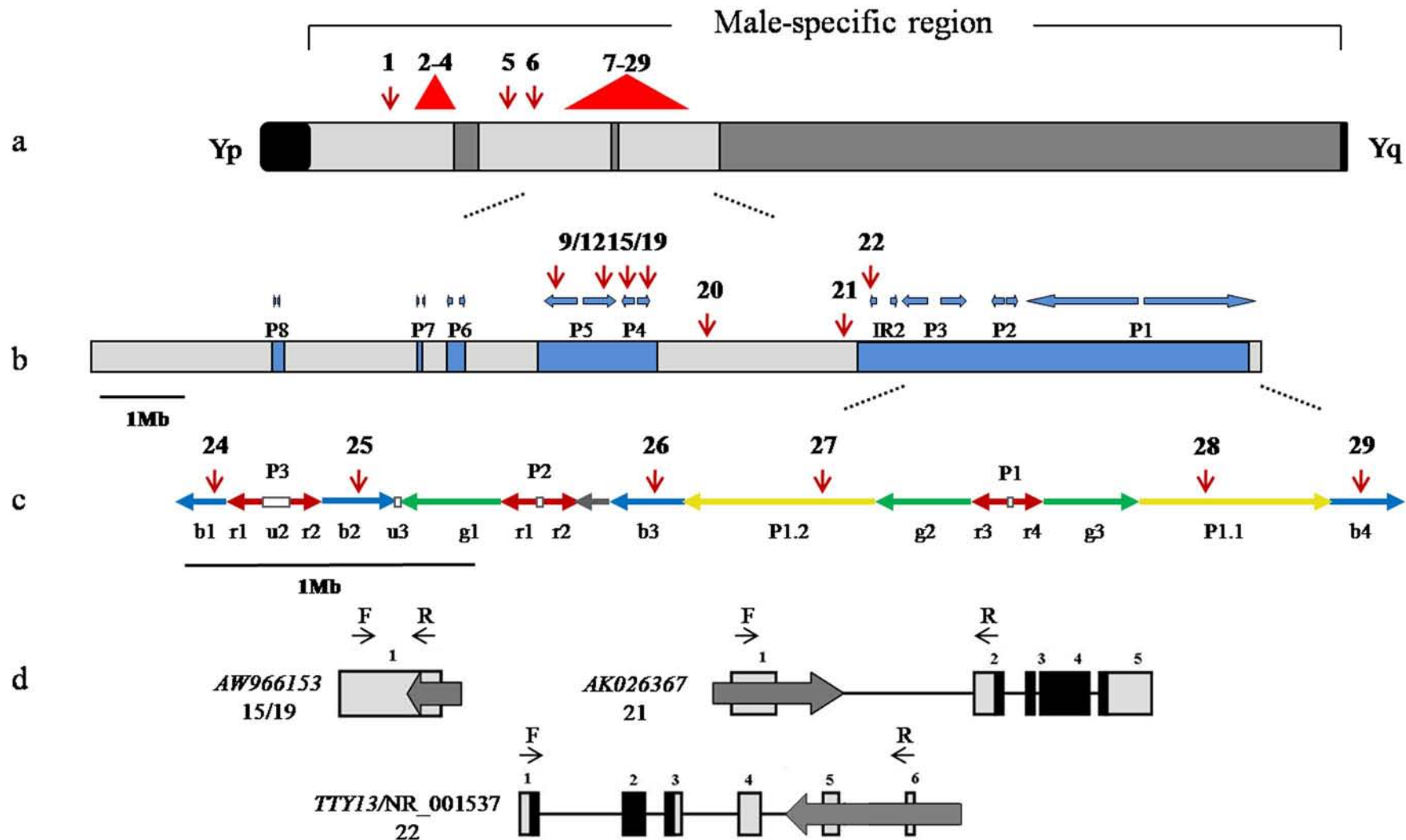
495

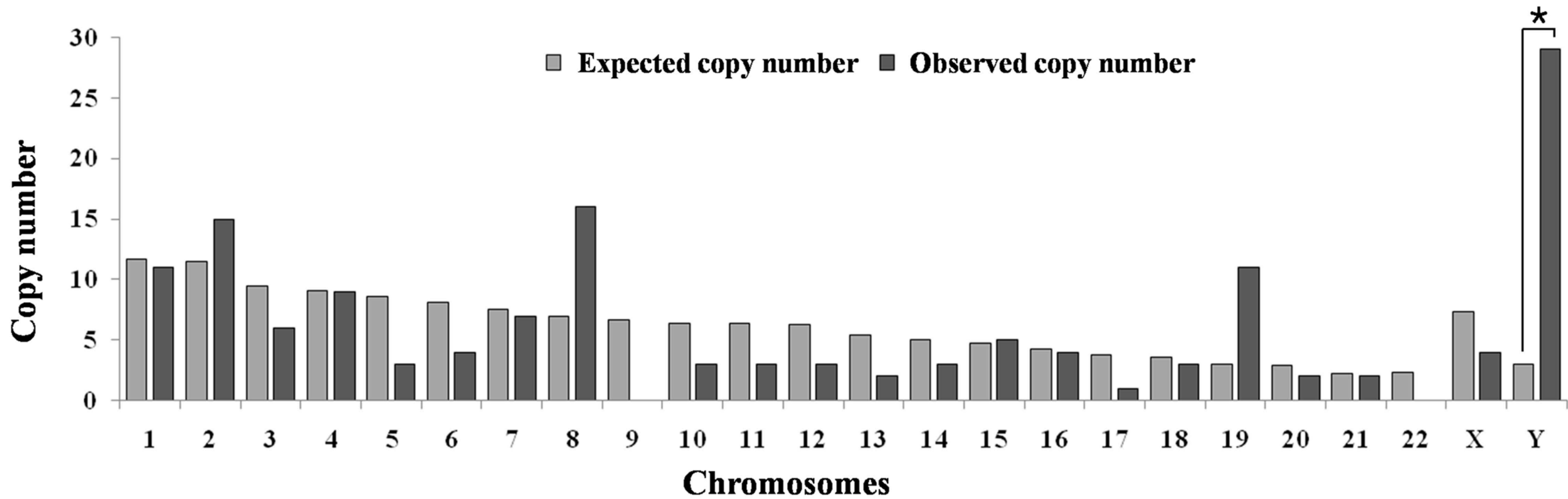
Figure 6. Expression profiles of HERV-K14C-related transcripts.

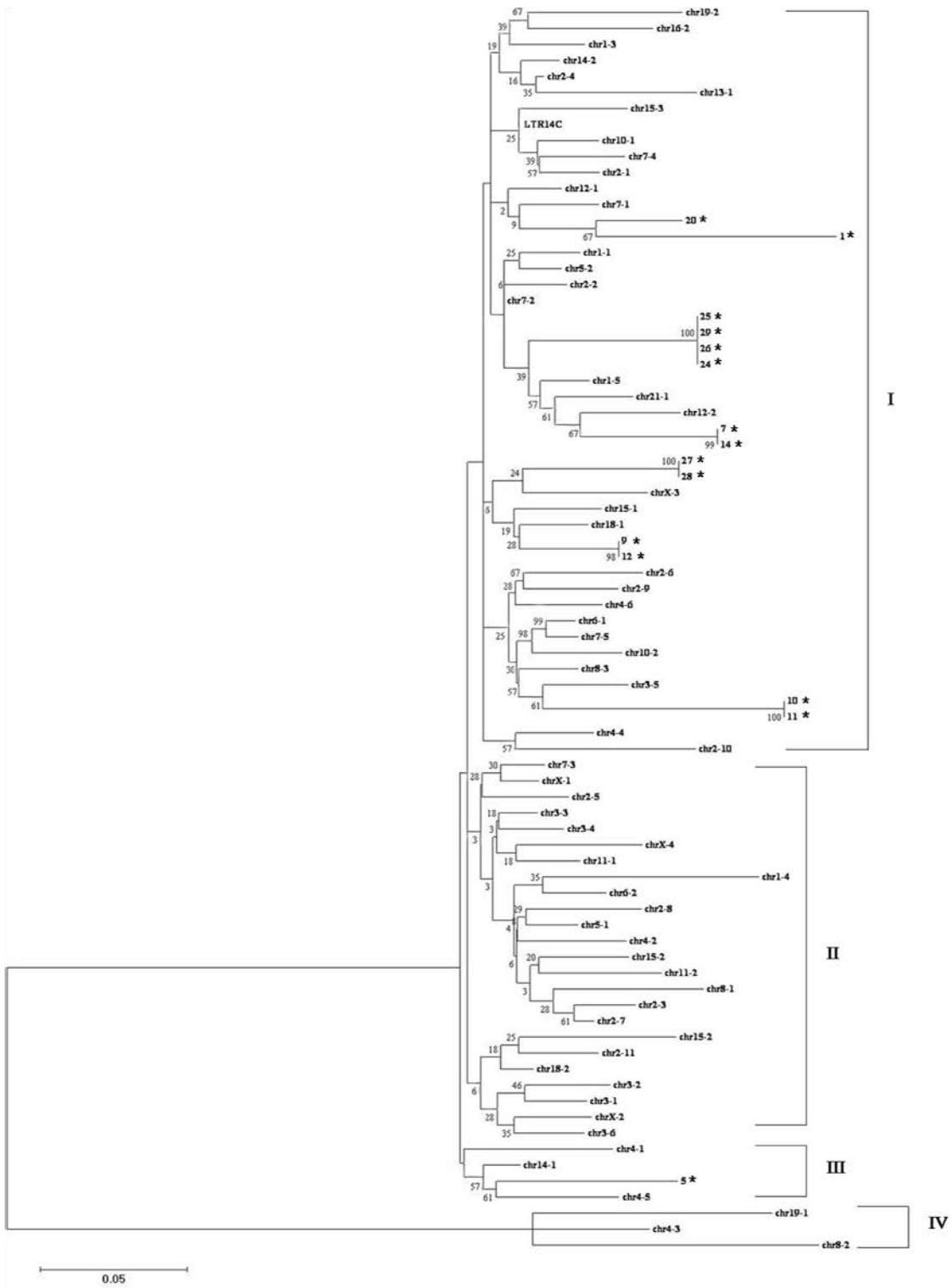
GAPDH was used as internal marker, and ddH₂O was used as a negative control.

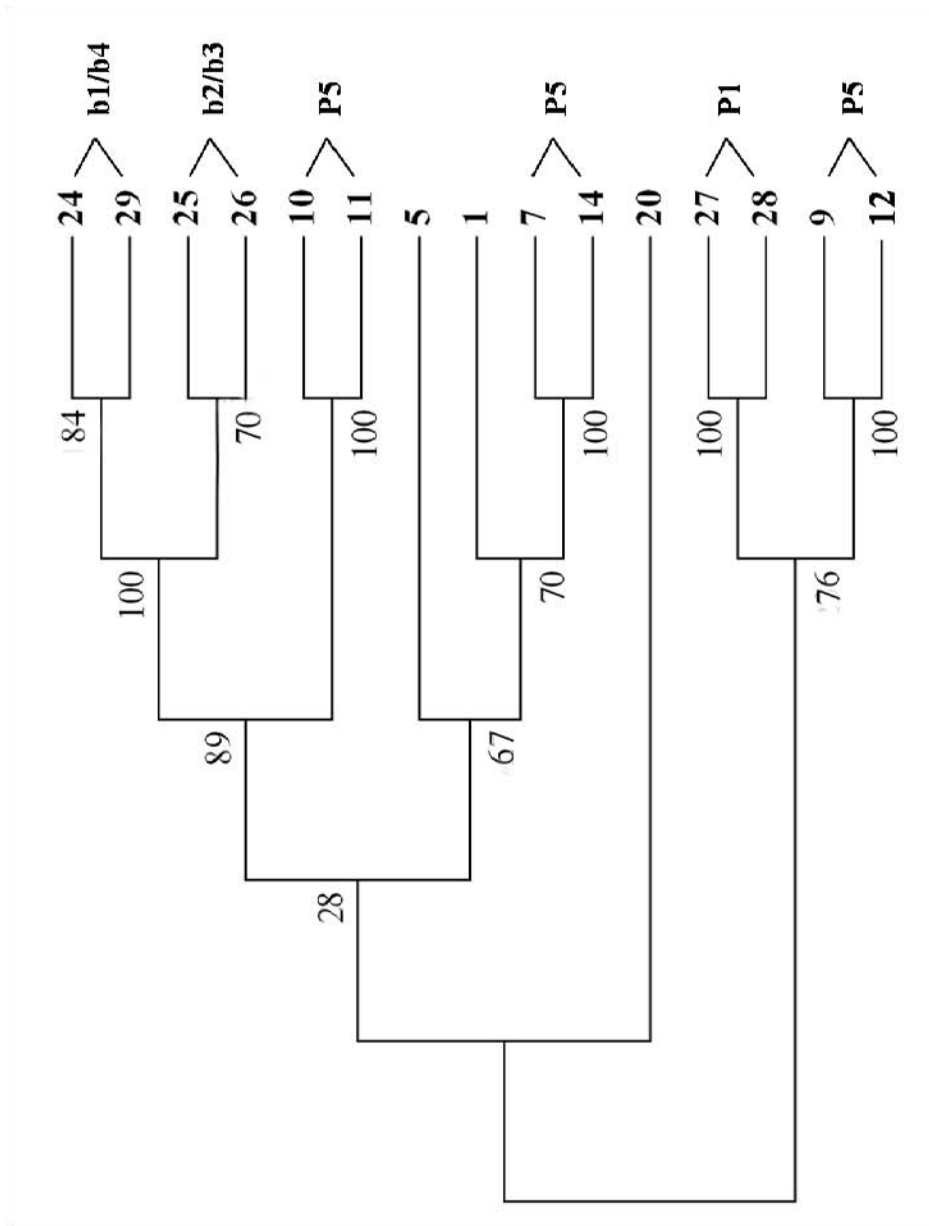
Figure 7. The putative time of amplicon formation based on the integration lineage of HERV-

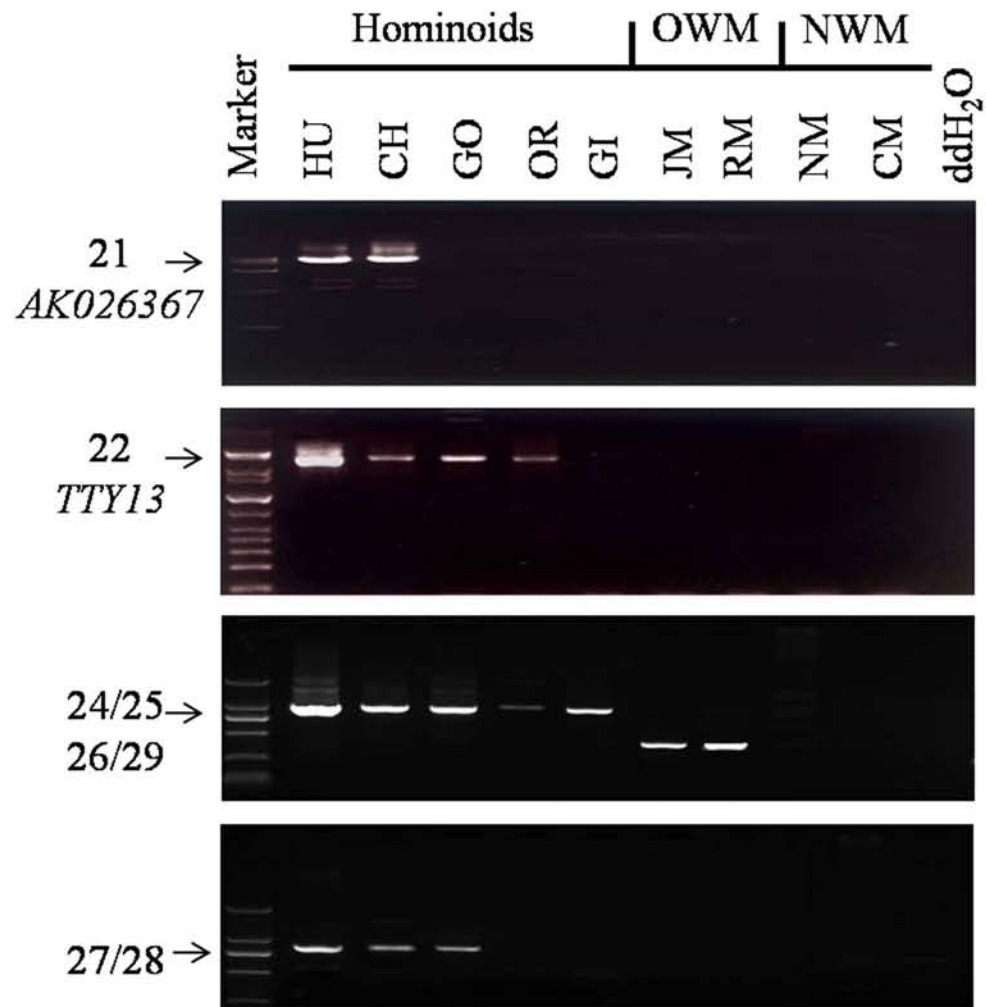
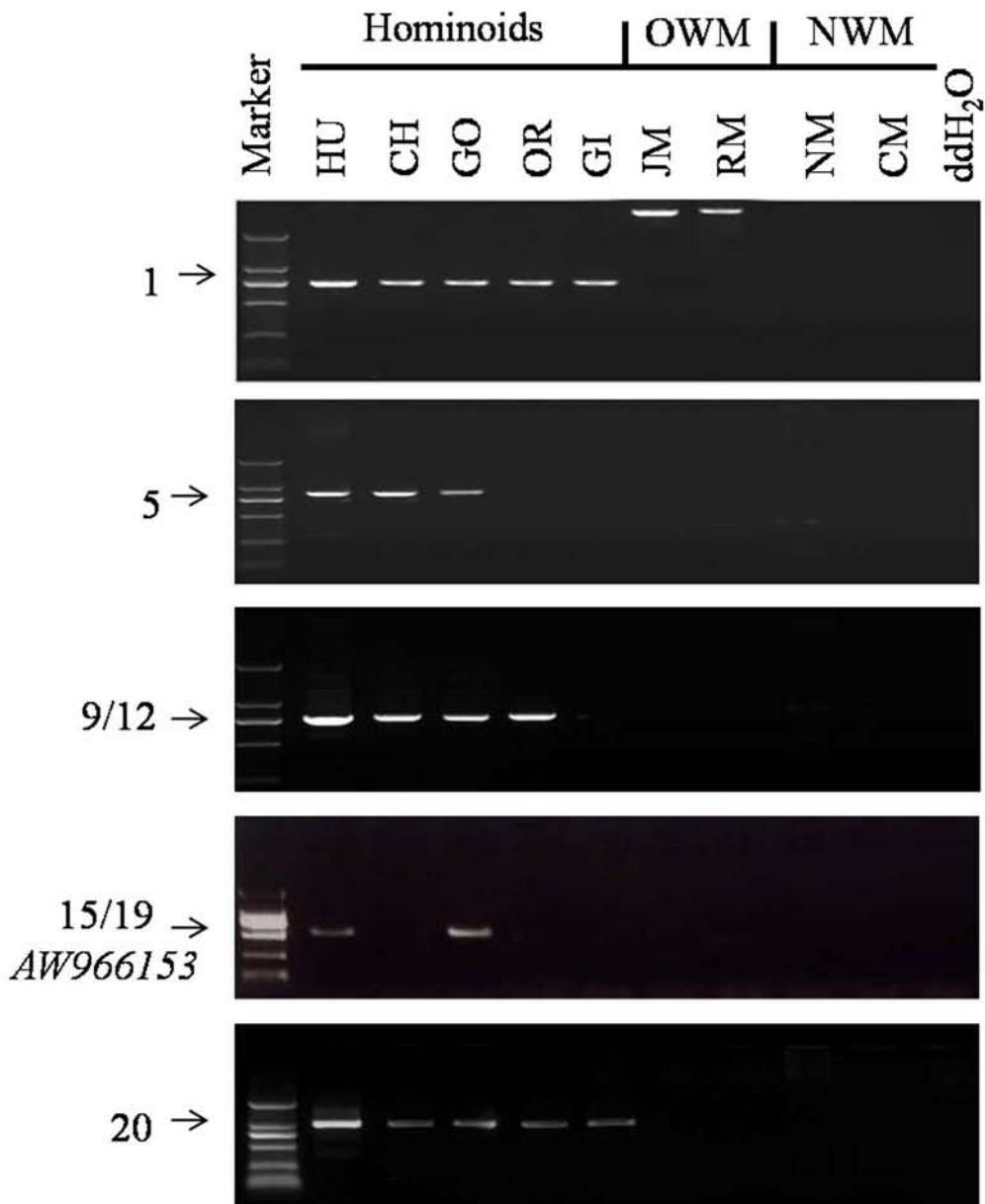
500 K14C-related elements in Figure 4. The evolutionary tree is based on and adapted from reported data.^{31 32}



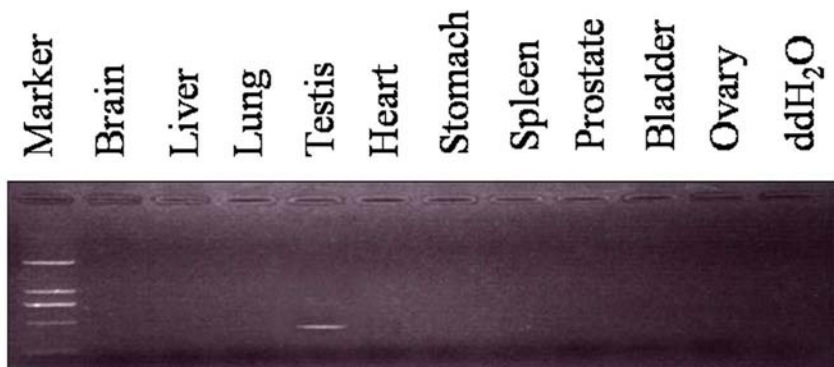








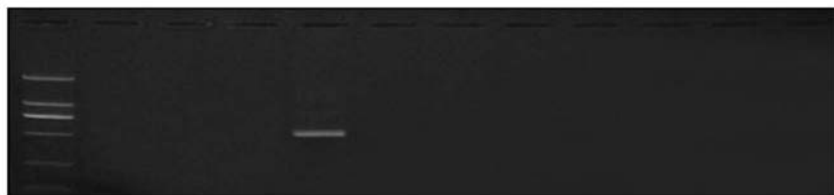
15/19
AW966153



21
AK026367



22
TTY13



GAPDH



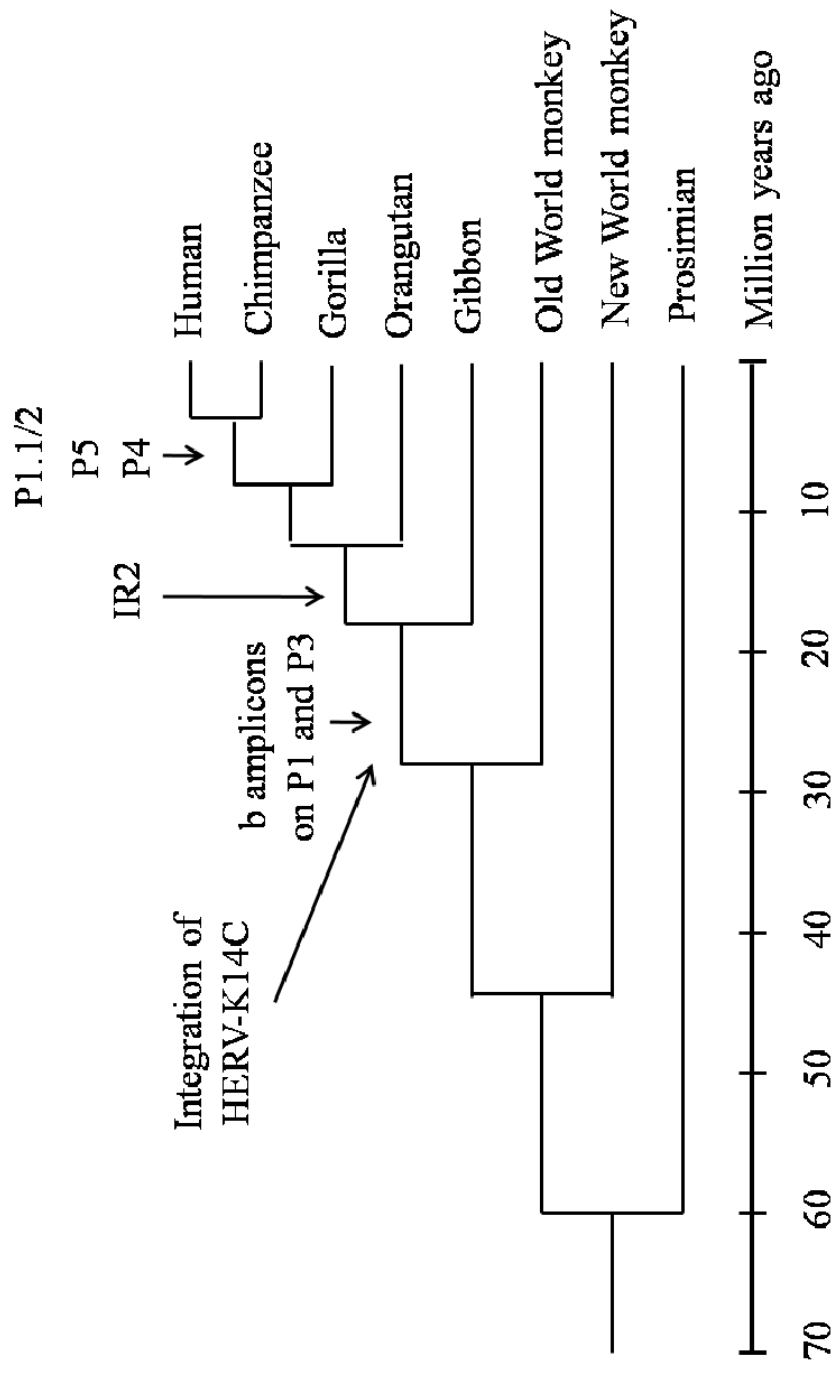


Table 1. Primers used in this study

Name	Forward 5' - 3'	Reverse 5' - 3'	Product	Reference
Genomic DNA				
1	TGTGGCACAGCTATTTGTCA	TGCAGGATGCTGATGCTTAT	730 bp	NT_011896.9
5	AAGAACTCCCTTTGTCTCCAA	GAGCAGTTCATGGGGAATG	859 bp	NT_011878.9
9/12	AGGAGGGCTTGTTGAGTTGA	CTTCCCAGCAGGTAAAGCAG	773 bp	NT_011875
15/19 (<i>AW966153</i>)	GTAAATACCACCTCTTGGTG	TCGCCGCTAGACTCAGGGT	517 bp	NT_011875
20	CCAGATCAGGAATCAATATAGCC	ACCTGCCGAAACTACAAGA	965 bp	NT_011875
21 (<i>AK026367</i>)	CCTGCCATAAATCTCTGGAAAC	GCCAATGGTGTCACTCTATCAA	6,395 bp	NT_011875
22 (<i>TTY13</i>)	CAGAGGCTGTCTGTGGCAATTC	GCAGCTTGTTTCACTCCTTTCTC	6,073 bp	NT_011875
24/25/26/29	TGGGAGAAGAGGTTTTGTGG	CACTTCCCTGTCCCTTTCAA	976 bp	NT_011903
27/28	CCAGTGGTTCTGATGGTCCT	CTTTGGCTGGAGAAAAGTGC	867 bp	NT_011903
<i>SRY</i>	GAATATTCCCCTCTCCGGA	GCTGGTGCTCCATTCTTGAG	400-472 bp	NM_003140.1
mRNA/EST				
<i>AW966153</i>	CTTCACATCCAGGCTCAACA	TGGTTTCCCAACAGTCTTC	226 bp	AW966153
<i>AK026367</i>	GGTCCCCATAATCTCTACAA	GGTGGTTGGTTTTCTGTCCTT	722 bp	AK026367
<i>TTY13</i>	CAAGCAGAGCCAAACAGACA	GACCACCAGTAATCTAATGGT	611 bp	NR_001537
<i>GAPDH</i>	CAAAGTTGTCATGGATGACC	CCATGGAGAAGGCTGGGG	195 bp	NM_002046

Table 2. The location of HERV-K14C-related sequences in the Y chromosome

Number	location	5' and 3' LTR	direction	length	mRNA/EST	Palindrome
1	7,934,660-7,935,245	Solitary	C	585 bp		
2	8,049,863-8,054,809	Both	C	4946 bp		
3	8,924,104-8,928,826	3'LTR	+	4722 bp		
4	9,171,317-9,177,286	Both	C	5969 bp		
5	10,502,263-10,502,845	Solitary	+	582 bp		
6	14,234,539-14,239,889	Internal	C	5350 bp		
7	18,194,256-18,194,814	Solitary	C	558 bp		Proximal-P5
8	18,291,100-18,293,064	5'LTR	C	1964 bp		Proximal-P5
9	18,366,095-18,366,679	Solitary	+	584 bp		Proximal-P5
10	18,374,133-18,374,713	Solitary	C	580 bp		Proximal-P5
11	18,773,845-18,774,425	Solitary	+	580 bp		Distal-P5
12	18,781,880-18,782,464	Solitary	C	584 bp		Distal-P5
13	18,855,500-18,857,464	5'LTR	+	1964 bp		Distal-P5
14	18,953,747-18,954,305	Solitary	+	558 bp		Distal-P5
15	19,233,969-19,234,110	Solitary	C	141 bp	AW966153	Proxiaml-P4
16	19,243,801-19,248,243	Both	C	4442 bp		Proxiaml-P4
17	19,287,744-19,293,649	Both	+	5905 bp		
18	19,314,707-19,319,149	Both	+	4442 bp		Distal-P4
19	19,328,842-19,328,983	Solitary	+	141 bp	AW966153	Distal-P4
20	19,788,470-19,789,056	Solitary	+	586 bp		
21	22,017,509-22,023,645	Both	C	6136 bp	AK026367	
22	22,151,328-22,157,319	Both	+	5991 bp	TTY13	IR2
23	22,401,007-22,407,033	Both	C	6026 bp		IR2
24	22,640,603-22,641,188	Solitary	+	585 bp		Proxiaml-P3/b1
25	23,056,286-23,056,871	Solitary	C	585 bp		Distal-P3/b2
26	24,250,291-24,250,876	Solitary	+	585 bp		Proxaimal-P1/b3
27	24,443,767-24,444,351	Solitary	+	584 bp		Proximal-P1/P1.2
28	26,336,826-26,337,410	Solitary	C	584 bp		Distal-P1/P1.1
29	26,530,270-26,530,855	Solitary	C	585 bp		Distal-P1/b4

Numbers are depicted in Figure 1 and highlighted numbers are for Figure 5, 6 and 7. Bolded numbers are used in Figure 3, 4. Abbreviations: Solitary, Solitary LTRs; Both, Presence of 5' and 3' LTR; N/D, Not Determined; Integration into genome with complementary (C) and sense (+) direction relative to Y chromosome reference sequences.

Table 1. The total copies of HERV-K14C in human genome

chromosome	genomic location (start/end)		orientation	HERV-K14C struct
chr1	20,126,513	20,131,239	C	HERVK14C HERVK14C
	160,655,774	160,656,356	+	LTR14C
	166,693,028	166,693,594	+	LTR14C
	193,957,243	193,957,723	C	LTR14C
	193,959,207	193,959,303	C	LTR14C
	204,098,502	204,099,978	+	HERVK14C
	224,047,008	224,049,730	C	HERVK14C HERVK14C
	231,813,555	231,814,166	C	LTR14C
	245,335,953	245,340,227	+	HERVK14C HERVK14C HERVK14C
	245,340,549	245,341,105	+	LTR14C
	245,495,846	245,496,431	+	LTR14C
copy number	11			
chr2	77,888,237	77,888,819	+	LTR14C
	81,385,066	81,385,703	C	LTR14C
	82,103,296	82,111,914	+	LTR14C HERVK14C HERVK14C
	97,947,816	97,948,402	+	LTR14C
	105,975,087	105,977,063	+	HERVK14C
	125,498,256	125,498,839	C	LTR14C
	135,995,924	135,996,510	+	LTR14C
	139,334,958	139,335,558	C	LTR14C
	141,858,545	141,859,124	+	LTR14C
	171,929,789	171,930,373	+	LTR14C
	180,809,824	180,810,409	+	LTR14C
	183,337,029	183,337,564	C	HERVK14C
	183,338,672	183,339,331	C	HERVK14C HERVK14C HERVK14C
	193,060,358	193,060,917	+	LTR14C
	194,829,129	194,829,715	C	LTR14C
copy number	15			
chr3	44,550,985	44,551,555	+	LTR14C
	59,430,258	59,430,807	C	LTR14C
	81,461,744	81,462,329	C	LTR14C
	83,750,251	83,750,836	C	LTR14C
	156,144,814	156,145,394	C	LTR14C
	164,483,672	164,484,259	C	LTR14C
copy number	6			
	19,166,820	19,167,405	+	LTR14C
	84,705,846	84,706,383	C	LTR14C
	104,556,373	104,556,918	+	LTR14C

chr4	118,120,827	118,121,411	+	LTR14C		
	130,221,478	130,222,059	+	LTR14C		
	167,414,664	167,415,227	+	LTR14C		
	171,284,078	171,284,591	C	HERVK14C		
	171,285,884	171,291,609	C	HERVK14C	LTR14C	
	176,142,249	176,147,829	C	HERVK14C	HERVK14C	HERVK14C
copy number	9					
chr5	28,583,235	28,583,463	+	HERVK14C		
	75,191,104	75,191,690	+	LTR14C		
	105,362,563	105,363,148	C	LTR14C		
copy number	3					
chr6	48,949,272	48,953,409	+	LTR14C	HERVK14C	HERVK14C
	48,953,729	48,955,321	+	HERVK14C	LTR14C	
	83,064,348	83,064,934	C	LTR14C		
	169,226,247	169,226,828	+	LTR14C		
copy number	4					
chr7	14,515,391	14,515,977	+	LTR14C		
	48,153,376	48,153,959	C	LTR14C		
	48,211,073	48,211,610	C	LTR14C		
	87,333,760	87,334,326	+	LTR14C		
	142,879,389	142,879,959	+	LTR14C		
	149,889,094	149,893,650	C	LTR14C	HERVK14C	HERVK14C
	149,941,146	149,941,638	C	HERVK14C	HERVK14C	
copy number	7					
chr8	3,336,749	3,337,320	C	LTR14C		
	17,809,894	17,816,758	C	HERVK14C	HERVK14C	
	17,817,197	17,817,628	C	HERVK14C		
	43,066,233	43,066,393	C	LTR14C		
	43,668,319	43,676,881	C	LTR14C	HERVK14C	LTR14C
	43,694,764	43,695,320	C	LTR14C		
	43,849,594	43,849,815	C	LTR14C		
	43,856,790	43,861,091	C	LTR14C	HERVK14C	
	52,253,706	52,254,292	C	LTR14C		
	79,720,601	79,720,694	+	HERVK14C		
	79,721,109	79,721,321	+	HERVK14C		
	79,721,839	79,722,663	+	HERVK14C		
	79,723,727	79,724,163	+	HERVK14C		
	79,726,865	79,729,466	+	HERVK14C	HERVK14C	LTR14C
	146,216,157	146,217,433	+	HERVK14C		
	146,225,026	146,228,909	+	HERVK14C	LTR14C	

copy number		16		
chr10	19,185,581	19,186,168	C	LTR14C
	37,645,058	37,645,639	C	LTR14C
	135,271,937	135,272,840	+	HERVK14C
copy number		3		
chr11	61,709,286	61,709,872	C	LTR14C
	73,328,224	73,331,962	C	HERVK14C
	123,645,812	123,646,381	+	LTR14C
copy number		3		
chr12	4,699,435	4,700,020	C	LTR14C
	11,772,399	11,772,483	+	HERVK14C
	36,824,542	36,825,121	+	LTR14C
copy number		3		
chr13	37,791,859	37,792,436	C	LTR14C
	84,341,679	84,349,456	+	LTR14C HERVK14C HERVK14C
copy number		2		
chr14	37,549,997	37,550,576	+	LTR14C
	47,550,158	47,550,733	+	LTR14C
	53,049,757	53,049,899	+	LTR14C
copy number		3		
chr15	37,090,527	37,091,113	+	LTR14C
	43,313,968	43,322,563	C	LTR14C HERVK14C LTR14C
	51,871,100	51,871,682	C	LTR14C
	52,674,289	52,674,863	C	LTR14C
	60,291,628	60,292,055	C	HERVK14C HERVK14C
copy number		5		
chr16	19,312,405	19,320,975	C	LTR14C HERVK14C LTR14C
	29,598,489	29,599,337	C	LTR14C LTR14C
	34,221,866	34,221,910	+	LTR14C
	62,351,692	62,352,243	+	LTR14C
copy number		4		
chr17	48,394,015	48,394,428	+	LTR14C
copy number		1		
chr18	4,517,619	4,518,204	C	LTR14C
	59,466,320	59,466,769	+	LTR14C
	75,872,504	75,873,135	+	LTR14C LTR14C
copy number		3		
	7,762,723	7,763,518	C	LTR14C LTR14C
	7,764,529	7,764,596	C	LTR14C
	7,771,942	7,772,482	C	LTR14C

chr19	20,684,962	20,685,325	C	HERVK14C	HERVK14C	
	23,925,077	23,925,423	C	HERVK14C		
	23,927,239	23,927,487	C	HERVK14C		
	23,929,343	23,929,866	C	HERVK14C		
	23,930,457	23,931,164	C	HERVK14C	HERVK14C	HERVK14C
	46,151,918	46,156,935	+	LTR14C	HERVK14C	HERVK14C
	56,593,044	56,594,558	C	HERVK14C		
	63,053,772	63,054,342	C	LTR14C		
copy number		11				
chr20	2,737,699	2,738,033	C	LTR14C		
	2,738,340	2,738,598	C	LTR14C		
copy number		2				
chr21	14,374,659	14,375,237	C	LTR14C		
	19,475,163	19,475,481	C	LTR14C		
copy number		2				
chrX	10,961,788	10,962,371	+	LTR14C		
	92,414,839	92,415,423	C	LTR14C		
	141,683,325	141,683,905	C	LTR14C		
	143,680,477	143,681,064	C	LTR14C		
copy number		4				
chrY	7,934,660	7,935,245	C	LTR14C		
	8,049,863	8,054,809	C	LTR14C	HERVK14C	HERVK14C
	8,924,104	8,928,826	+	HERVK14C	HERVK14C	HERVK14C
	9,171,317	9,177,286	C	LTR14C	HERVK14C	HERVK14C
	10,502,263	10,502,845	+	LTR14C		
	14,234,539	14,239,889	C	HERVK14C	HERVK14C	HERVK14C
	18,194,255	18,194,813	C	LTR14C		
	18,291,099	18,293,063	C	HERVK14C	LTR14C	
	18,366,094	18,366,678	+	LTR14C		
	18,374,133	18,374,713	C	LTR14C		
	18,773,844	18,774,424	+	LTR14C		
	18,781,879	18,782,463	C	LTR14C		
	18,855,499	18,857,463	+	LTR14C	HERVK14C	
	18,953,746	18,954,304	+	LTR14C		
	19,233,968	19,234,109	C	LTR14C		
	19,243,800	19,248,242	C	LTR14C	HERVK14C	HERVK14C
	19,287,743	19,293,648	+	LTR14C	HERVK14C	HERVK14C
	19,314,706	19,319,148	+	LTR14C	HERVK14C	HERVK14C
	19,328,841	19,328,982	+	LTR14C		
	19,788,469	19,789,055	+	LTR14C		

	22,017,508	22,023,644	C	LTR14C	HERVK14C	HERVK14C
	22,151,327	22,157,318	+	LTR14C	HERVK14C	HERVK14C
	22,401,007	22,407,033	C	LTR14C	HERVK14C	HERVK14C
	22,640,603	22,641,188	+	LTR14C		
	23,056,286	23,056,871	C	LTR14C		
	24,250,291	24,250,876	+	LTR14C		
	24,443,767	24,444,351	+	LTR14C		
	26,336,826	26,337,410	C	LTR14C		
	26,530,270	26,530,855	C	LTR14C		
copy number		29				
total	total copy number : 146					

Abbreviations: Integration into genome with complementary (C) and sense (+) direction relative to Y chromosome conserved intact domain is colored as orange. Truncated and mutated HERV-K14 related sequences is highlighted.

ture	length	mRNA/EST	LTR ^a
	4,727		
	582		1-1
	566		1-2
	480		
	96		
	1,477		
	2,723		
	611		1-3
HERVK14C HERVK14C	4,275	AK096414(NT2 cells neuron teratocarcinoma)	
	556	NM_207401	1-4
	585		1-5
	582		2-1
	637		2-2
LTR14C	8,619		
	586		2-3
	1,977		
	583		2-4
	586		2-5
	600		2-6
	579		2-7
	584		2-8
	585		2-9
	535		
	660		
	559		2-10
	586		2-11
	570		3-1
	549		3-2
	585		3-3
	585		3-4
	580		3-5
	587		3-6
	585		4-1
	537		4-2
	545		4-3

	584		4-4
	581		4-5
	563		4-6
	514		
	5,726		
	5,581		
	229		
	586		5-1
	585		5-2
HERVK14C	4,138		
	1,593		
	586		6-1
	581		6-2
	586		7-1
	583		7-2
	537		7-3
	566		7-4
	570		7-5
HERVK14C	4,557		
	493		
	571		8-1
	6,865	NM_201553(fibrinogen-like 1)	
	432		
	160		
	8,563		
	556		8-2
	221		
	4,302		
	586		8-3
	94		
	213		
	825		
	437		
	2,062		
	1,277		
	3,884		

	587		10-1
	581		10-2
	904		
	587		11-1
	3,739		
	569		11-2
	585		12-1
	85		
	579		12-2
LTR14C	577	DB083245	13-1
	7,778		
	579		14-1
	575		14-2
	142		
	586		15-1
	8,596		
	582		15-2
	574		15-3
	428		
	8,571		
	848		
	44		
	551		16-1
	413		
	585		18-1
	449		
	631		18-2
	795		
	67		
	540		19-1

	364		
	347		
	249		
	524		
	708		
HERVK14C LTR14C	5,018		
	1,515		
	570	AK022663	19-2
	334		
	258		
	578		21-1
	318		
	583		X-1
	584		X-2
	580		X-3
	587		X-4
	585 bp		Y-1
HERVK14C LTR14C	4946 bp		
LTR14C	4722 bp		
HERVK14C LTR14C	5969 bp		
	582 bp		Y-2
	5350 bp		
	558 bp		Y-3
	1,964 bp		
	584 bp		Y-4
	580 bp		Y-5
	580 bp		Y-6
	584 bp		Y-7
	1,964 bp		
	558 bp		Y-8
	141 bp	AW966153	
HERVK14C LTR14C	4,442 bp		
HERVK14C LTR14C	5,905 bp		
HERVK14C LTR14C	4,442 bp		
	141 bp	AW966153	
	586 bp		Y-9

HERVK14C	LTR14C	6,136 bp	AK026367	
HERVK14C	LTR14C	5,991 bp	<i>TTY13</i>	
HERVK14C	LTR14C	6,026 bp		
		585 bp		Y-10
		585 bp		Y-11
		585 bp		Y-12
		584 bp		Y-13
		584 bp		Y-14
		585 bp		Y-15

some reference sequences. LTR^a was used for analysis of phylogenetic analysis of Figure 3. Relatively hted as yellow.