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### Repository Citation

Calvignac, Sebastien; Terme, Jean-Michel; Hensley, Shannon M.; Jalinot, Pierre; Greenwood, Alex D.; and Hanni, Catherine, "Ancient DNA Identification of Early 20th Century Simian T-Cell Leukemia Virus Type 1" (2008). *Biological Sciences Faculty Publications*. 284. [https://digitalcommons.odu.edu/biology\\_fac\\_pubs/284](https://digitalcommons.odu.edu/biology_fac_pubs/284)

### Original Publication Citation

Calvignac, S., Terme, J. M., Hensley, S. M., Jalinot, P., Greenwood, A. D., & Hanni, C. (2008). Ancient DNA identification of early 20th century simian T-cell leukemia virus type 1. *Molecular Biology and Evolution*, 25(6), 1093-1098. doi:10.1093/molbev/msn054

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# Ancient DNA Identification of Early 20th Century Simian T-Cell Leukemia Virus Type 1

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The molecular identification of proviruses from ancient tissues (and particularly from bones) remains a contentious issue. It can be expected that the copy number of proviruses will be low, which magnifies the risk of contamination with retroviruses from exogenous sources. To assess the feasibility of paleoretrovirological studies, we attempted to identify proviruses from early 20th century bones of museum specimens while following a strict ancient DNA methodology. Simian T-cell leukemia virus type 1 sequences were successfully obtained and authenticated from a *Chlorocebus pygerythrus* specimen. This represents the first clear evidence that it will be possible to use museum specimens to better characterize simian and human T-tropic retrovirus genetic diversity and analyze their origin and evolution, in greater detail.

## Introduction

Three retroviruses, the human T-cell leukemia virus type 1 (HTLV-1) and the human immunodeficiency viruses type 1 and 2 (HIV-1 and HIV-2), are known to be human pathogens (Barre-Sinoussi et al. 1983; Gessain et al. 1985; Clavel 1987). These viruses originated from cross-species transmissions of simian T-cell leukemia viruses (STLV) and simian immunodeficiency viruses (SIV) from a yet unidentified Asian Primate (Van Dooren et al. 2007), the chimpanzee (*Pan troglodytes*; Keele et al. 2006) and the gorilla (*Gorilla gorilla*; Van Heuverswyn et al. 2006), and the sooty mangabey (*Cercocebus atys*; Hirsch et al. 1989). SIV and STLV infect a wide range of nonhuman African primates at a naturally high rate (VandeWoude and Apetrei 2006). Their cross-species transmission to humans may be frequent in Central Africa (Kalish et al. 2005; Wolfe et al. 2005), constituting a potentially serious threat to human health. Investigating STLV and SIV genetic diversity, which will allow for a better understanding of their evolution, is thus of prime interest. Such investigations have previously relied on the use of blood or scat samples, which are difficult to obtain from wild individuals. A poorly explored alternative is the use of the bones from the numerous museum specimens gathered over the last 2 centuries.

In contrast to mitochondrial or nuclear DNA, which can be found in any nucleated cell of the bone, proviral DNA of T-cell tropic retroviruses such as SIV or STLV is only expected to be found in a tiny fraction of all cells present in bones (i.e., CD4<sup>+</sup> T-cells and macrophages). This makes proviral DNA amplification from bones an unusually challenging task. Furthermore, the only ancient proviral sequences published at this time (HTLV-1 sequences from a 1,500-year-old mummy bone; Li et al. 1999; Sonoda et al. 2000) are highly controversial (Gessain et al. 2000; Vandamme et al. 2000). Thus, the question of the feasibility of paleoretrovirological studies remains unanswered.

The authenticity of ancient sequences can be ascertained by using standards widely accepted by the ancient

DNA community (Hofreiter et al. 2001; Pääbo et al. 2004). Following a strict, yet classical, ancient DNA methodology based on these standards, this study aimed to assess whether it is feasible to retrieve and authenticate ancient proviral DNA sequences from the bones of museum specimens. To address this question, we focused on museum specimens of a convenient model, the species complex *Chlorocebus* sp. (their vernacular name African green monkeys [AGM] will be used hereafter). Numerous populations belonging to this complex are indeed infected at high rates of prevalence with 2 retroviruses (SIV<sub>AGM</sub> and STLV-1; Meertens et al. 2001; VandeWoude and Apetrei 2006). Attempts to extract and identify proviral DNA from AGM samples were successful in unambiguously authenticating STLV-1 pX and long terminal repeat (LTR) sequences from an early 20th century specimen. These results open up museum specimen-based studies of retroviral genetic diversity and evolution.

## Materials and Methods

The following section reports experimental conditions in Lyon, France. The latter are essentially similar to those that were followed in Norfolk, VA. Noteworthy, the replication in Norfolk was fully independent and performed from samples that never transited by any of Lyon molecular labs. For a complete description of both experimental conditions and results obtained in Norfolk, refer to supplementary materials and methods S1 [Supplementary Material online] and to MacPhee et al. (2005).

## Sample Description

Bone fragments were sampled from 6 distinguishable individuals identified as *Cercopithecus aethiops* (the genus name has been changed to *Chlorocebus* and several cryptic species have been defined recently; Groves C, personal communication and Groves [2001]) in the catalog of the Royal Museum of Tervuren (Belgium). All sampled skeletons were complete or nearly complete and had been stored at room temperature after an initial cleaning by desiccation. All individuals came from Central Africa, which is the putative geographical origin of both retrovirus epizootics. Specimen characteristics are detailed in table 1.

Key words: ancient DNA, STLV-1, SIV, *Chlorocebus*.

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*Mol. Biol. Evol.* 25(6):1093–1098. 2008

doi:10.1093/molbev/msn054

Advance Access publication February 23, 2008

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**Table 1**  
**Sample Description and Results of the Various PCR Attempts**

Sample Name (Lyon laboratory)	Sample Name (Tervuren museum)	Sample Provenance	Date of Sampling	Mitochondrial DNA (12S)	Nuclear DNA (CD4)	Nuclear DNA Copies ( $10^3$ ) per Gram of Tissue	Proviral DNA (SIV)	Proviral DNA (STLV-1)
CH351	RG4023	Kerio (0°40'N–35°35'E)	May–June 1914	+	+	23.2 ± 8.4 <sup>a</sup>	–	–
CH352	RG2149	Lume (3°31'S–37°43'E)	June 1913	+	+	42 ± 3.7	–	+
CH353	RG23452	Busumba (11°19'S–27°18'E)	July 1955	+	+	603 ± 60	–	–
CH354	RG22087	Kakkanda (10°40'S–26°36'E)	December 1953	+	+	244 ± 35	–	–
CH356	RG37479	Ngamba (0°24'N–29°34'E)	May 1953	+	+	382 ± 129	–	–
CH357	RG36970	Utukuru (4°21'N–29°53'E)	February 1952	+	+	170 ± 49	–	–

NOTE.—All specimens belong to the genus *Chlorocebus*. Numbers of nuclear CD4 copies are expressed as mean ± standard deviation. The symbol “+” indicates that PCR attempts yielded at least 2 independent PCR products that delivered clones whose sequences corresponded to the correct species or viral group.

<sup>a</sup> This number takes into account a negative assay (>36 cycles).

### Retrovirus-Targeting Primer Assay

Targeting proviral DNA fragments means a priori targeting very low-copy DNA fragments. DR4/DR5 (Clewley et al. 1998) and SK43/SK44 (Kwok et al. 1988) (2 primer pairs that respectively amplify short fragments of SIV and STLV-1 genomes in all known groups of SIV and STLV-1) were first assessed on a known, decreasing concentration of DNA templates by quantitative polymerase chain reaction (Q-PCR). Q-PCR amplifications were performed in a building physically isolated from the one in which the ancient DNA laboratory was located. Assays were performed on 10 to 1,000 templates using a kit (Light Cycler FastStart DNA Master<sup>PLUS</sup>, Roche Applied Science, Penzberg, Germany) following manufacturer's instructions. Cycling conditions were as follows: 95 °C for 15 min and 60 cycles at 94 °C for 15 s, 45–58 °C for 25 s, and 72 °C for 25 s. As DR4/DR5 did not fulfil our requirements (very low-copy DNA amplification, i.e., 1 to 10 copies per reaction), DR5 was used with a newly designed primer, SIVintf2 (see supplementary table S1, Supplementary Material online), with which good performance were shown under the same conditions than above.

### Ancient DNA Handling

#### *Ancient DNA Extraction*

Extractions and manipulations were performed in specific ancient DNA facilities. *Chlorocebus* sp. bones were first reduced to a powder in liquid nitrogen. The powder obtained (0.5–1 g) was then digested for 18 h with proteinase K (1 mg/mL) in buffer (0.5 M ethylenediaminetetraacetic acid, 0.5% N-lauroyl-sarcosine) under constant agitation at 55 °C (Loreille et al. 2001). Following this treatment, samples were centrifuged (1200 × g) and supernatants washed 3 times with phenol–chloroform–isoamylalcohol (25:24:1) (Orlando et al. 2002). Finally, DNA was concentrated on a Centricon 30 column and eluted in an ultrapure water volume of 80 and 100 µL. A further DNA purification was done using Qiaquick kit (Qiagen, Hilden, Germany) when too much inhibition of the PCR reaction was detected (Hänni et al. 1995).

#### *Specific Ancient DNA Controls*

Two kinds of controls specific to ancient DNA analyses were included in our experiments. When several AGM individuals were extracted at the same time, cross-

contamination was monitored. This entailed monitoring additional samples from other species (lemurs and sheep) that were extracted with the AGM samples. To monitor airborne contaminations, an aerosol control was also included in all PCR assays. It consisted of a tube which was kept open throughout the manipulation (Loreille et al. 2001). Both controls were used in amplification attempts with all AGM and retrovirus primer pairs.

#### *Ancient DNA Amplification and Sequencing*

Nine different short (<300 bp) fragments (AGM 12S, AGM CD4, SIV<sub>AGM</sub> *pol* and STLV-1 pX, and STLV-1 LTR fragments) were targeted using the 9 primer pairs described in supplementary table S1 (Supplementary Material online). All amplifications from ancient specimens were performed from 1 µL of 10-fold diluted DNA extract in a total volume of 25 µL using AmpliTaq Gold (PerkinElmer, Wellesley, MA). Cycling conditions were as follows: 94 °C for 5 min; 50–60 cycles at 94 °C for 30 s, 45–58 °C for 30 s, and 72 °C for 45 s; and at 72 °C for 7 min. PCR products were then subcloned using Topo TA cloning kit for sequencing (Invitrogen, Hamburg, Germany). To test insert size, clones were picked and amplified by PCR with Mastermix (Eppendorf, Hamburg, Germany) using the following cycling conditions: 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and at 72 °C for 5 min. Products of the expected size were sequenced on both strands by Genome Express, Grenoble. The sequence of a given PCR product was determined as the consensus of the sequences of its clones. The sequence of a given fragment for a given individual was determined as the consensus of the PCR product sequences.

#### *Quantification of the Nuclear DNA Content of Ancient Specimens*

Q-PCR was performed on 10-fold diluted samples using primer pair SCCD4-f5/SCCD4-r5 (see supplementary table S1, Supplementary Material online) and the same kit as described above. Bovine serum albumin was added to the reaction so that the concentration would be 1 mg/mL. Cycling conditions were as follows: 95 °C for 15 min and 60 cycles at 94 °C for 15 s, 58 °C for 25 s, and 72 °C for 25 s. Ten-fold serial dilutions of known concentration CD4 PCR product (from 100,000 to 100 copies) served to define

the linear regression from which ancient extracts content in nuclear DNA was deduced.

### Sequence Alignment and Analysis

Sequences were aligned by eye using the software SEAVIEW (Galtier et al. 1996). In order to identify the different consensus sequences, a Blast analysis was then performed.

Phylogenetic analyses were performed to infer relationships of all AGM 12S sequences obtained in this study with previously published *Cercopithecus erythrotis*, *Cercopithecus mona*, and *Cercopithecus preussi* and AGM sequences (van der Kuyl et al. 1995) as well as to infer relationships of the CH352 LTR sequence with 2 Asian HTLV-1/STLV-1 sequences and 43 African HTLV-1/STLV-1 sequences (Van Dooren et al. 2001). The appropriate model of evolution was determined using MrModeltest 2.0 (Nylander 2004), a Modeltest-modified version (Posada and Crandall 1998) adapted to options available with Bayesian analysis softwares. According to MrModeltest, the best-fitting models of substitution were, respectively, HKY + I and HKY + I + G. For each data set, 3 maximum likelihood (ML) analyses were performed with PHYML (Guindon and Gascuel 2003) using the online interface <http://atgc.lirmm.fr/phyml/> (Guindon et al. 2005). The transition/transversion ratio and the proportion of invariable sites were estimated and the starting tree was determined by a BioNJ analysis of the data sets (default settings). Using optimization options, 500 bootstrap (Bp) replicates were performed. For both data sets, all 3 runs gave similar tree topologies and Bp values. Bayesian analyses were also performed using MrBayes v3.1 (Ronquist and Huelsenbeck 2003). Four independent runs of, respectively, 1,000,000 generations and 2,000,000 generations each were performed under the models described above. Burn-in periods of respectively 100,000 generations and 50,000–350,000 generations were determined graphically using Tracer 1.2 (Rambaut and Drummond 2003), a software that allows easy plotting of all parameters against the number of generations. For both data sets, all 4 runs gave similar tree topologies and posterior probability (pp) values.

### Results

Ancient DNA analyses requires a precise strategy to control for contamination from modern sources (Hofreiter et al. 2001; Pääbo et al. 2004). The one adopted is described step by step, together with the obtained results, in the following paragraphs.

DNA extraction and pre-PCR protocols (preparation of PCR mixes) were performed in isolated, positive pressure, regularly bleached, and UV-irradiated rooms. None of the rooms used had ever contained AGM or provirus samples or DNA extracts prior to the experimentation. To further exclude the possibility of contamination with exogenous modern DNA, each extraction included a blank and, when necessary, a cross-contamination control (see Materials and Methods and Loreille et al. [2001]). The various PCR assays reported here always included the necessary controls including a classical PCR control and an

aerosol one (see Specific Ancient DNA Controls and Loreille et al. [2001]). Importantly, all controls were consistently negative.

Replication of the results is the basis of any ancient DNA experiment. It allows for the detection of inconsistent sequence changes that may result from the process of decay DNA unavoidably undergoes postmortem (Hofreiter et al. 2001; Pääbo et al. 2004). Thus, samples were only considered positive for a given PCR if a minimum of 2 PCR products could be obtained (see Ancient DNA Amplification and Sequencing). To highlight and identify artifactual mutations induced by ancient DNA modifications, all PCR products were also subcloned (see Ancient DNA Amplification and Sequencing).

As explained in the introduction, proviral DNA is less than a single copy per cell (as not every cell is infected in a given population). In contrast, mitochondrial DNA is present in thousands of copies per cell while all nucleated cells contain 2 copies of the nuclear genome. To strengthen the probability that the proviral sequences we hoped to obtain would be authentic, the AGM bone extracts were screened for their mitochondrial DNA content (12S rRNA-encoding gene; see supplementary table S2, Supplementary Material online) and nuclear (CD4 gene; see supplementary table S2, Supplementary Material online) DNA fragments. As success rates on ancient templates are known to increase when targeting fragments of decreasing lengths (Pääbo et al. 2004), the nuclear DNA was purposely chosen for being shorter than the mitochondrial one (248 bp from the mitochondrial 12S rRNA gene, 201 bp from the nuclear CD4 gene). The latter was also used to quantify the amount of nuclear DNA in all of the 6 samples. All 6 samples yielded mitochondrial and nuclear sequences that matched AGM sequences available in GenBank (see supplementary alignment S3, Supplementary Material online). Phylogenetic analyses of the mitochondrial sequences also confirmed the expected phylogeography of AGM species (Colin Groves, personal communication and Groves [2001]; Supplementary fig. S4, Supplementary Material online). The quantification of nuclear DNA from each extract by Q-PCR (using the same primer pair as above) revealed a clear time dependency: the 2 oldest specimens (captured in 1913 and 1914) contained less nuclear DNA than the 4 youngest (captured in 1952, 1953, and 1955). However, all 6 samples were considered good candidates for proviral DNA research as nuclear DNA could be detected.

In order to be confident that the amplification would be successful from any infected individual, highly conserved parts of the SIV<sub>AGM</sub> and STLV-I progenomes (respectively pol and pX genes) were targeted. One primer pair per virus was used to try amplifying fragments shorter than those that had already been amplified from the AGM genomes (143 bp from the pol gene and 159 bp from the pX gene). These 2 primer pairs had previously been shown to allow amplification from very low concentration targets (see Retrovirus-Targeting Primer Assay and supplementary table S1, Supplementary Material online). Thirty-one PCR attempts per extract were performed with each primer pair. No PCR-positive individual could be identified with the SIV<sub>AGM</sub> targeting pair. One individual (CH352, see table 1) was identified as PCR positive with the STLV-1 targeting pair. Four



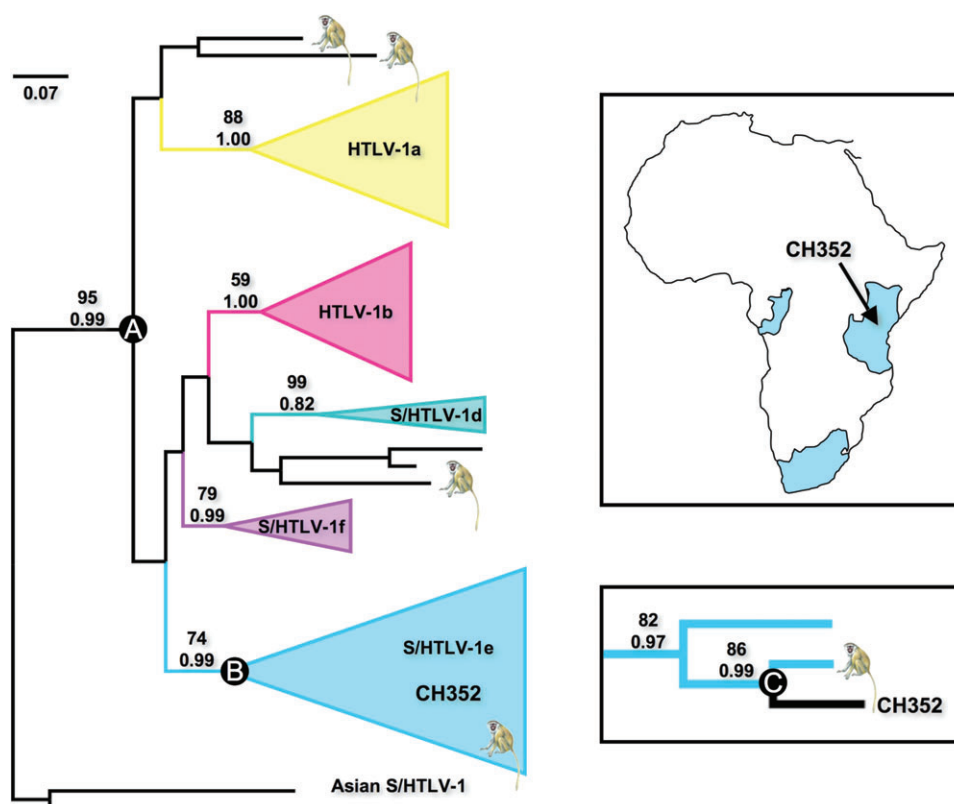


FIG. 1.—Phylogenetical analyses of 467-bp-long LTR. Bp and pp values are shown above nodes corresponding to S/HTLV-1 groups. STLV-1 sequences obtained from *Chlorocebus* sp. individuals are labeled with an AGM picture. The part of the clade S/HTLV-1e containing the CH352 LTR sequence is magnified on the right of the phylogenetic tree. Just above, countries in which at least 1 HTLV-1e virus has been described are shaded blue on a map of Africa. The arrow indicates the geographical origin of CH352 (near the border between Kenya and Tanzania).

subcloned PCR products allowed for the determination of a consensus pX sequence (see Ancient DNA Amplification and Sequencing) that exhibited 100% identity with modern STLV-1/HTLV-1 sequences (as determined by BlastN analysis).

Targeting conserved regions increases the probability of successful amplification of very divergent retroviruses. However, it only allows the recovery of poorly informative sequences (see the criticisms of Li et al. [1999] in Gessain et al. [2000]; Vandamme et al. [2000]). Those might be difficult to distinguish from potential contaminants. To further exclude the possibility of a contamination with a modern STLV-1/HTLV-1 strain, it was thus necessary to obtain phylogenetically informative sequences from CH352. Five fragments of the LTR, covering a total of 467 bp, were targeted (see supplementary table S2 and alignment S5, Supplementary Material online). A total of 184 clones obtained from 21 PCR products allowed for the determination of a consensus sequence that differed by at least one substitution to all available HTLV-1/STLV-1 sequences (according to a BlastN analysis). This is in agreement with both the low rate of substitution of HTLV-1/STLV-1 ( $10^3$ – $10^4$  lower than the rate of substitution of HIV-1; Lemey et al. 2005) and the number of substitutions observed in a pedigree-based study (5 substitutions for a 419- to 1,109-year period; Van Dooren et al. 2004). Phylogenetic analyses performed on a 46-sequence alignment of the LTR region (see Sequence Alignment and Analysis; Van Dooren et al. 2001)

confirmed 1) the African origin of the CH352 provirus because the latter nests into the African STLV-1/HTLV-1 (fig. 1, node A; ML Bp: 95, Bayesian pp: 1), 2) its accurate geographical origin (Kenya, see table 1) because the CH352 LTR sequence belonged to the Congo, Kenya, Tanzania, and South Africa endemic clade HTLV-1e (fig. 1, node B; Bp: 74, pp: 0.99), and 3) the species designation of its carrier because the CH352 LTR sequence was most closely related to a sequence obtained from a modern *C. aethiops* individual (accession number AF012730; fig. 1, node C; Bp: 86, pp: 0.99). Thus, the phylogenetic analyses of the CH352 provirus LTR sequence confirms the information available about the sample CH352.

Finally, the sequences of 2 of the LTR fragments were replicated in an independent laboratory from an independent sample of CH352 (see Materials and Methods). A BlastN analysis of the 176-bp-long sequence revealed that only 2 sequences were 100% similar to that of the CH352 provirus (AY026848 and AF117282), both having been identified from individuals belonging to the complex *Chlorocebus* sp.

## Discussion

Both technical and phylogenetical reasons argue in favor of the authenticity of the proviral sequences described here. Experiments were performed in ancient DNA

dedicated facilities following an adapted methodology which included 1) the use of specific ancient DNA controls, 2) the replication of the results in the laboratory, 3) the screening of samples by targeting templates of expectedly decreasing concentrations, 4) the identification of provirus-positive individuals by using primer pairs targeting conserved regions, 5) the determination of phylogenetically informative sequences, and finally 6) the replication of a part of the results in an independent laboratory. Li et al. (1999) controversial sequences did not fulfil requirements (1), (5), and (6). We therefore provide the first clear evidence that amplifying proviral DNA from early 20th century bones is possible if a systematic strategy to ascertain the authenticity of the isolated sequences is followed. Other related substrates such as bone marrow or dental pulp (which were already used for the search of other pathogens, reviewed in Drancourt and Raoult [2005]) could contain even higher concentrations of proviral DNA and should also be considered for future studies.

Only one sample was PCR positive for STLV-1 proviral DNA and none for SIV<sub>AGM</sub>. This is far lower than the expected high rates of prevalence of both infections (Meertens et al. 2001; VandeWoude and Apetrei 2006). One could conclude that very few of the museum specimens were well enough preserved to detect proviruses. However, there are at least 2 reasons to be optimistic about the future rate of success of comparable experiments. First, most of the sampling could well have been composed of uninfected individuals (or alternatively of infected individuals with low proviral loads). Only 6 individuals were sampled and this is too small a collection to be considered representative. In addition, the Q-PCR results show that the only STLV-1-positive individual is 1 of the 2 samples with the least concentrated nuclear DNA. The failure to amplify STLV-1 DNA fragments from the 4 other individuals therefore does not reflect the fact that they did not contain enough DNA to still yield amplifiable proviral DNA but rather that these individuals were probably uninfected. Second, the primer pairs that we used to detect infected individuals may not have been efficient enough for amplifying the proviral DNA present in the extracts. Their detection threshold was shown to be very low in the “ideal” conditions of a Q-PCR on modern templates (see Retrovirus-Targeting Primer Assay) but it might well be that inhibitory molecules present in the extract of ancient bones hamper this ability. Furthermore, although we targeted conserved viral regions, the possibility of unexpected mismatches with the templates cannot be ruled out.

Museum specimens provide a unique opportunity to gather information about T-cell tropic retrovirus genetic diversity and evolution. Our results should thus encourage research aimed at obtaining proviral sequences from additional museum specimens.

## Supplementary Material

Supplementary materials and methods S1, tables S1 and S2, alignment S3, figure S4, and alignment S5 are available at *Molecular Biology and Evolution* online (<http://mbe.oxfordjournals.org/>).

## Acknowledgments

We are greatly indebted to Sandrine Hughes, Ambre Spencer, François-Loïc Cosset, and Vincent Laudet for critical reading of the manuscript. We also thank Marilyne Duffraisie for her technical help in the laboratory. We are deeply indebted to the Royal Museum of Tervuren (Belgium) and in particular to Emmanuel Gilissen and Wim Wendelen for having provided us with the samples used in this study. This study was supported by the Agence Nationale pour la Recherche contre le SIDA, the Centre National de la Recherche Scientifique, the Ecole Normale Supérieure de Lyon, and the Université Claude Bernard Lyon I. S.C. has a grant from the French Ministry for Education and Research. J.-M.T. has a grant from the French Ligue contre le Cancer. S.M.H. and A.D.G. were supported by a grant from the National Science Foundation OPP0117400.

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Robin Bush, Associate Editor

Accepted February 19, 2008