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**Virus Evolution**

*DOI:*
10.1093/ve/vew011

Published: 25/05/2016

Publisher's PDF, also known as Version of record

*Virus Evolution*, 2016, 2(1): vew011

doi: 10.1093/ve/vew011
Research article

# Genetic diversity of STLV-2 and interspecies transmission of STLV-3 in wild-living bonobos

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## Abstract

There are currently four known primate T-cell lymphotropic virus groups (PTLV1-4), each of which comprises closely related simian (STLV) and human (HTLV) viruses. For PTLV-1 and PTLV-3, simian and human viruses are interspersed, suggesting multiple cross-species transmission events; however, for PTLV-2 this is not so clear because HTLV-2 and STLV-2 strains from captive bonobos (*Pan paniscus*) form two distinct clades. To determine to what extent bonobos are naturally infected with STLV, we screened fecal samples (n = 633) from wild-living bonobos (n = 312) at six different sites in the Democratic Republic of Congo (DRC) for the presence of STLV nucleic acids. STLV infection was detected in 8 of 312 bonobos at four of six field sites, suggesting an overall prevalence of 2.6% (ranging from 0 to 8%). Six samples contained STLV-2, while the two others contained STLV-3, as determined by phylogenetic analysis of partial *tax* and Long Terminal Repeats (LTR) sequences. The new STLV-2 sequences were highly diverse, but grouped with previously identified STLV-2 strains as a sister clade to HTLV-2. In contrast, the new STLV-3 sequences did not cluster together, but were more closely related to STLVs from sympatric monkey species. These results show for the first time that fecal samples can be used to detect STLV infection in apes. These results also show that wild-living bonobos are endemically infected with STLV-2, but have acquired STLV-3 on at least two occasions most likely

by cross-species transmission from monkey species on which they prey. Future studies of bonobos and other non-human primate species in Central Africa are needed to identify the simian precursor of HTLV-2 in humans.

Key words: STLV-2, STLV-3, bonobos, DRC, feces

## 1. Introduction

Primate T-cell lymphotropic viruses (PTLVs) comprise both simian (STLVs), and human (HTLVs) T-cell lymphotropic viruses. Today, four PTLV groups, one to four, have been described, each comprising simian and human viruses. Some authors (Liégeois et al. 2008; Switzer et al. 2009) have even suggested a fifth lineage of PTLVs, consisting of the single SLTV-MarB43 isolated from a *Macaca arctoides*. The International Committee on Taxonomy of Viruses currently considers this virus as a divergent PTLV-1. HTLV-1 is highly endemic in certain geographic regions (Nicolas et al. 2015) and has infected between 10 and 20 million people worldwide (Gessain and Cassar 2012). HTLV-2 is less widespread, but has been identified in pygmy populations in Central Africa as well Amerindian tribes in North and South America, and also circulates among intravenous drug users in the USA and Europe (Gessain et al. 1995; Salemi et al. 1999; Mauclere et al. 2011; Alcantara et al. 2003). HTLV-3 has only recently been described in a handful of individuals from Cameroon, while only a single case of HTLV-4 infection has been reported in a Cameroonian hunter (Mahieux and Gessain 2011; LeBreton et al. 2014). Only about 5% of HTLV-1 infections are associated with disease, including adult T-cell leukaemia/lymphoma and tropical spastic paraparesis (Gessain et al. 1995; Kannian and Green 2010; Gessain and Mahieux 2012). HTLV-2 is not strongly associated with a particular disease. However, a long-term follow up of 387 HTLV-2 seropositive patients in the US revealed a modification of their hematological parameters, including a significant increase of their lymphocytes and platelets counts as compared with matched seronegative controls (Bartman et al. 2008). No information concerning pathogenicity is currently available for HTLV-3 and -4 (Calattini et al. 2006; Switzer et al. 2006).

STLV-1 has been documented in more than thirty Old World primate species, including monkeys and apes, in sub-Saharan Africa and Asia, while STLV-3 has only been identified in several African monkey species (Mahieux and Gessain 2011). In contrast, STLV-2 and STLV-4 seem to be much less widespread as determined by extensive screening of different primate species throughout West- and Central Africa. STLV-4 has thus far only been found in western lowland gorillas (*Gorilla gorilla gorilla*) in Cameroon (LeBreton et al. 2014), and STLV-2 seems to be restricted to captive but wild-born bonobos (*Pan paniscus*) housed in zoos and primate centers in Europe and the USA (Giri et al. 1994; Vandamme et al. 1996; Digilio et al. 1997). Some PTLV lineages can be further subdivided into subtypes that cluster by geography, rather than by host species, suggesting transmission among non-human primates (NHP). Co-circulation of different STLV types, as well as different STLV-1 and/or STLV-3 subtypes, within the same species in the same geographic area has also been documented (Liégeois et al. 2008, 2012). Importantly, HTLV-1 and HTLV-3 strains fall within the radiation of STLV-1 and STLV-3 viruses, respectively, providing evidence for multiple past and recent interspecies transmission events from NHPs to humans. Similarly, STLV-4 from gorillas in Cameroon is closely related to the single HTLV-4 strain isolated from a Cameroonian hunter, again suggesting cross-species transmission. In contrast, HTLV-2 and STLV-2 viruses form distinct clades in phylogenetic trees, with no evidence for interspecies transmission. Moreover, the two STLV-2 strains obtained from captive bonobos, one in the USA and one in Belgium, exhibit a relatively high genetic diversity, suggesting that additional strains and lineages that have not yet been identified may exist. Despite the higher genetic distance between HTLV-2 and STLV-2, STLV-2 from bonobos is considered the simian counterpart of HTLV-2.

To examine the genetic diversity of STLV-2 and to explore the simian origin of HTLV-2 in humans, we screened wild living bonobos for STLV infection, covering a large part of their geographic range in the Democratic Republic of Congo (DRC). Like all apes, bonobos are highly endangered and the presence of pathogens in wild populations can only be studied non-invasively, that is, by analysis of fecal samples. This approach, developed in 2003 (Santiago et al. 2003), has allowed us to identify the reservoirs of the different groups of HIV-1 in chimpanzees and gorillas, as well as has revealed an African ape origin of *Plasmodium falciparum* and *Plasmodium vivax* (Keele et al. 2006; Van Heuverswyn et al. 2006; Liu et al. 2010; Liu et al. 2014). Although we did not find the simian precursor of HTLV-2, we show that STLV-2 infection is widespread in wild-living bonobos and demonstrate for the first time STLV-3 infection in an ape species.

## 2. Material and Methods

### 2.1 Sample collection and study sites

The bonobo habitat spans an area of ~500,000 square kilometers in the DRC, bordered in the north and the west by the Congo River, in the south by the Kasai River and by the Lualaba River in the east. We collected 633 fecal samples between March 2006 and June 2012 at six different forest sites: Balanga (BN) (n = 85), Ikela (IK) (n = 49), Kokolopori (KR) (n = 78), Lui-kotal (LK) (n = 41), Malebo (ML) (n = 268), and Lomako-Yokokala (LA) (n = 112) (Fig. 1). Overall, fecal samples were collected primarily around night nests or feeding sites, but also opportunistically on trails. For almost all samples, the GPS position and estimated time of deposition were recorded. About 20 g of dung were collected in a 50-ml tube containing 20 ml of RNAlater (Applied Biosystems/Ambion, Austin, TX). These tubes were kept at base camps at ambient temperature for an average of 4 weeks and were subsequently transported to a central laboratory for storage at −20 or −80 °C.

### 2.2 Host species confirmation and individual identification

For all fecal samples, the host species origin was determined as described previously, by amplifying a 450-bp fragment spanning the 12S gene (van der Kuyl et al. 1995) or by amplification of a 499-bp fragment of the mitochondrial D-loop region (Li et al. 2012). Microsatellite analysis was done on all STLV-positive samples and a subset of STLV-negative fecal samples as previously described in Li et al. (2012) to estimate the number of

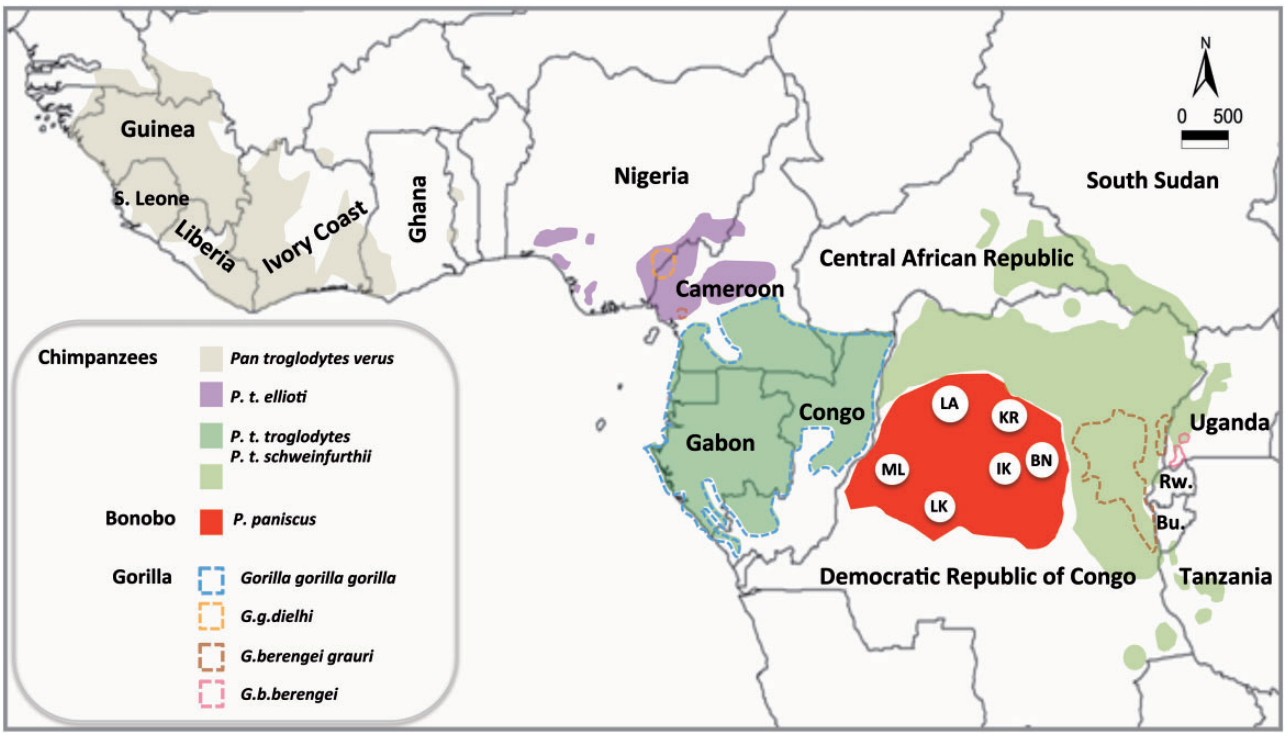


**Figure 1.** Distribution area of African great apes and the bonobo (*Pan paniscus*) range (in red) in DRC are depicted. The location of bonobo fecal sample collection sites in DRC are indicated with circles and a two-letter code: BN, Balanga; IK, Ikela; KR, Kokolopori; LK, Lui-kotal, ML, Malebo and LA, Lomako-Yokokala . Rwanda (Rw) and Burundi (Bu) are indicated with the first two letters of the country's name.

sampled individuals and to identify the number of positive bonobos. For sex determination, a region of the amelogenin gene was amplified (Keele et al. 2006). Homozygous loci were amplified at least four times to minimize allelic dropout, and independent DNA extractions were analyzed when multiplex PCR reactions yielded poor results. We also amplified 130-bp and 300-bp fragments of the 16S gene of mtDNA to check for possible contaminants of bonobo fecal samples with other mammalian DNA in the presence of chimpanzee-specific PCR blockers as described previously in Calvignac-Spencer et al. (2013). These primers sets specifically block the amplification of *P. paniscus* and *P. troglodytes* DNA.

### 2.3 Molecular detection of STLV infection in fecal samples

Fecal DNA was extracted from 2 ml of feces-RNAlater mixture using the QIAamp Stool DNA Mini kit (Qiagen, Valencia, CA), as previously described on an automated QIAcube System (QIAGEN GmbH, Hilden, Germany). DNA was subsequently subjected to nested PCR using *tax* (100–220-bp) consensus and PTLV lineage specific primers, which previously identified a wide diversity of new STLV lineages in different NHP species (Vandamme et al. 1997). The universal primers for the first round (targeting a region of 303-bp in the *tax* gene), amplify all known PTLV groups and in the second round, we used 4 different sets of primers: universal primers that allow amplification of a 220-bp fragment for all known types of PTLV or specific primers (spanning 100—151 bp in the *tax* region), which allow specific amplification of PTLV-1, 2 or 3. PCR conditions were as previously described in Liégeois et al. (2008). In addition, we used also PTLV-4 *tax* specific nested PCR (423 bp in the first round and 275 bp in the second round) (LeBreton et al. 2014) to screen 378 of 633 samples for which sufficient DNA was available for STLV-4. PCR conditions were the same as previously described in LeBreton et al. (2014). In order to increase success of PCR amplification, bovine serum albumin (BSA) was added at the final concentration of 0.2 μg/ml in the first PCR round, and we repeated two or three times the nested PCR before considering the sample as negative. BSA increases PCR success in complex samples, such as those obtained from faecal materials, by blocking DNA polymerases inhibitors present in these materials. The *tax* PCR-positive samples were further characterized in the LTR (386–689-bp) region using specific primers for the corresponding PTLV types (Liégeois et al. 2008; Courgnaud et al. 2004; Ahuka-Mundeke et al. 2012). Table 1 shows the different primers that were used in this study. After purification with the Geneclean Turbo Kit (Qbiogene, Inc., Carlsbad, CA), PCR products were directly sequenced using an automated sequencer (3130xl Genetic Analyzer, Applied Biosystems, Foster City, CA).

### 2.4 STLV prevalence estimation

The prevalence of STLV infection was estimated based on the proportion of STLV PCR-positive samples, but correcting for sample degradation and redundant sampling as identified by microsatellite analysis and described in previous surveys (Li et al. 2012). Samples studied in this study are a subset of those described in reference Li et al. (2012). 95% confidence limits were calculated based on binomial sampling (Wilson 1927).

### 2.5 Phylogenetic analyses

Newly derived nucleotide sequences were compared with previously published PTLV reference sequences. Sequences were aligned using MEGA 4 (Tamura et al. 2007), and where necessary, minor manual adjustments were performed. Sites that could not

**Table 1.** Primers used for STLV detection and characterization

| Primers | Primer sequences[a] | gene | Amplicon size (bp) | References |
|---|---|---|---|---|
| *Universal* | | | | |
| AV45 | GGACGCGTTRTCRGCTC | Tax | 303 | Vandamme et al., (1997), |
| AV46 | KGGRGAIAGYTGGTAKAGGTA | Tax | | Liégeois et al. (2008) |
| AV42 | CTCCCCTCCTTCCCCAC | Tax | 221 | |
| AV43 | CCASRKGGTGTAIAIGTTTTGG | Tax | | |
| *STLV-1* | | | | |
| AV49 | CCCTCCTTCCTCCAGGCCAT | Tax | 100 | Vandamme et al. (1997), |
| AV80 | GGTCTGGAAAAGACAGGGTTG | Tax | | Liégeois et al. (2008) |
| *STLV-2* | | | | |
| AV50 | TCAATCAATGCGGAAGCACACC | Tax | 151 | Vandamme et al. (1997), |
| AV81 | TAGGTATAGGCATACTACGGT | Tax | | Liégeois et al. (2008) |
| *STLV-2PP*[b] | | | | |
| AV52 | ACGGGTGCCTATACCCAACTC | Tax | 117 | Vandamme et al. (1997), |
| AV83 | GGTACAAGCAAACTACGGTTC | Tax | | Liégeois et al. (2008) |
| *STLV-3* | | | | |
| AV51 | ACAATTGCCTCGAGCTCACCC | Tax | 117 | Vandamme et al. (1997), |
| AV82 | GAGGCACACGACGGAGCTG | | | Liégeois et al. (2008) |
| *STLV-1* | | | | |
| 8255not | TTGAAGAATACACCAACATCCC | LTR | 467 | Mahieux et al. (1997), |
| LTRU5E | CGCAGTTCAGGAGGCACCAC | | | Courgnaud et al. (2004), |
| 8255not | TTGAAGAATACACCAACATCCC | LTR | 433 | Liégeois et al. (2008) |
| 420LTR | GAACGCGACTCAACCGGCCGTGGAT | | | |
| Enh280 | TGACGACAACCCCTCACCTCAA | LTR | 477 | Mahieux et al. (1997), |
| 5PTLR | TCCCGGACGAGCCCCCAA | | | Courgnaud et al. (2004), |
| TATAbox | CAGGAGTCTATA AAAGCGTGG | LTR | 418 | Liégeois et al. (2008) |
| 5PLTR | TCCCGGACGAGCCCCCAA | | | |
| *STLV-2* | | | | |
| BSQF2 | ACCGTCTCACACAAACAATCCC | LTR | 505 | Switzer et al. (1995) and this |
| STLVLTR1 | ACTACGKRAGTCGYAGCCBAGCTC | | | study |
| BSQF2 | ACCGTCTCACACAAACAATCCC | LTR | 386 | |
| STLVLTR2 | GCRYTGWRSTTTCGDTTTCRG | | | |
| *STLV-3* | | | | |
| P3MPLF1 | CVACCACTGCTACRACCCCCAAG | LTR | 678 | Ahuka-Mundeke et al. (2012) |
| P3MPLR1 | AAGAYACWCCCCCTTCCGAAAC | | | |
| P3MPLF2 | AAGAYACWCCCCCTTCCGAAAC | LTR | 589 | |
| P3MPLR2 | CCGTCTCGRGGYTCATCATC | | | |

[a]5′-3′.
[b]STLV-2PP: STLV-2 Pan paniscus.

be aligned unambiguously or that contained a gap in any sequence were excluded from the analyses. Maximum likelihood (ML) trees were constructed using PhyML (http://www.atgc-montpellier.fr/), with 100 bootstrap replicates (Guindon and Gascuel 2003).The TrN93 models with a gamma distribution across sites was the most appropriate model according to TOPALI (Milne et al. 2009) and was used for ML analyses for STLV sequences.

### 2.6 GenBank accession numbers

Nucleotides sequences generated in this study have been deposited in GenBank under the following accession numbers: KU529977–KU529984.

## 3. Results

### 3.1 STLV infection rates in wild bonobos

Combining universal and type specific *tax* primers to PCR amplify STLV sequences from NHP samples has identified a wide variety of new STLV lineages (Vandamme, Salemi, and Desmyter 1998; Liégeois et al. 2008; LeBreton et al. 2014). Here, we found eight (1.3%) of 633 bonobo fecal samples to be positive for *tax* specific sequences (Fig. 1 and Table 2). For six samples (PP-KR70, PP-KR77, PP-ML5489, PP-ML5538, PP-ML5560, and PP-LA7817) a 220 bp fragment was amplified with universal primers and for two samples (PP-KR78, PP-LK684) only a short *tax* fragment (110-bp) was amplified with PTLV-2 specific primers. Among the eight *tax*-positive samples, sequence analysis identified six (PP-KR70, PP-KR77, PP-KR78, PP-LK684, PP-ML5538, and PP-LA7817) to contain STLV-2 and two (PP-ML5489, PP-ML5560) to contain STLV-3 by BLAST and/or phylogenetic tree analysis (Fig. 2). The eight STLV-positive samples were derived from eight different animals as shown by microsatellite and/or mitochondrial analysis. The characteristics of these eight samples are shown in Table 3. Microsatellite analysis showed that the three positive samples from the KR site were derived from three different individuals; for one individual, three samples were collected with only a single positive for STLV, while for the two remaining samples only one fecal sample was collected, which was STLV-positive. At the MB site, the three samples were also derived from different individuals because the STLV-2 and STLV-3 infected samples were derived from different mtDNA haplotypes (Li et al. 2012).

**Table 2.** STLV infection in wild bonobos from the DRC

| Collection site[a] | Number of fecal samples collected | Estimated number of individuals[b] | Number of STLV tax PCR-positive samples | Number of STLV positive bonobos | STLV prevalence (95% CI)[c] | Number STLV-2 positive bonobos | Number STLV-3 positive bonobos |
|---|---|---|---|---|---|---|---|
| BN | 85 | 42 | 0 | 0 | 0 (0–8.4) | 0 | 0 |
| IK | 49 | 24 | 0 | 0 | 0 (0–13.8) | 0 | 0 |
| KR | 78 | 39 | 3 | 3 | 7.7 (2.6–20.3) | 3 | 0 |
| LK | 41 | 20 | 1 | 1 | 5 (0.9–23.6) | 1 | 0 |
| ML | 268 | 132 | 3 | 3 | 2.3 (0.8–6.5) | 1 | 0 |
| LA | 112 | 55 | 1 | 1 | 1.8 (0.3–9.6) | 1 | 2 |
| TOTAL | 633 | 312 | 8 | 8 | 2.6 (1.3–5) | 6 | 2 |

[a]Collection sites are indicated with a two letter code and their locations are shown in Figure 1.
[b]Number of individuals as determined by microsatellite analysis, see Methods and in Li et al. (2012).
[c]Prevalence of STLV infection based on the proportion of STLV-positive bonobos.

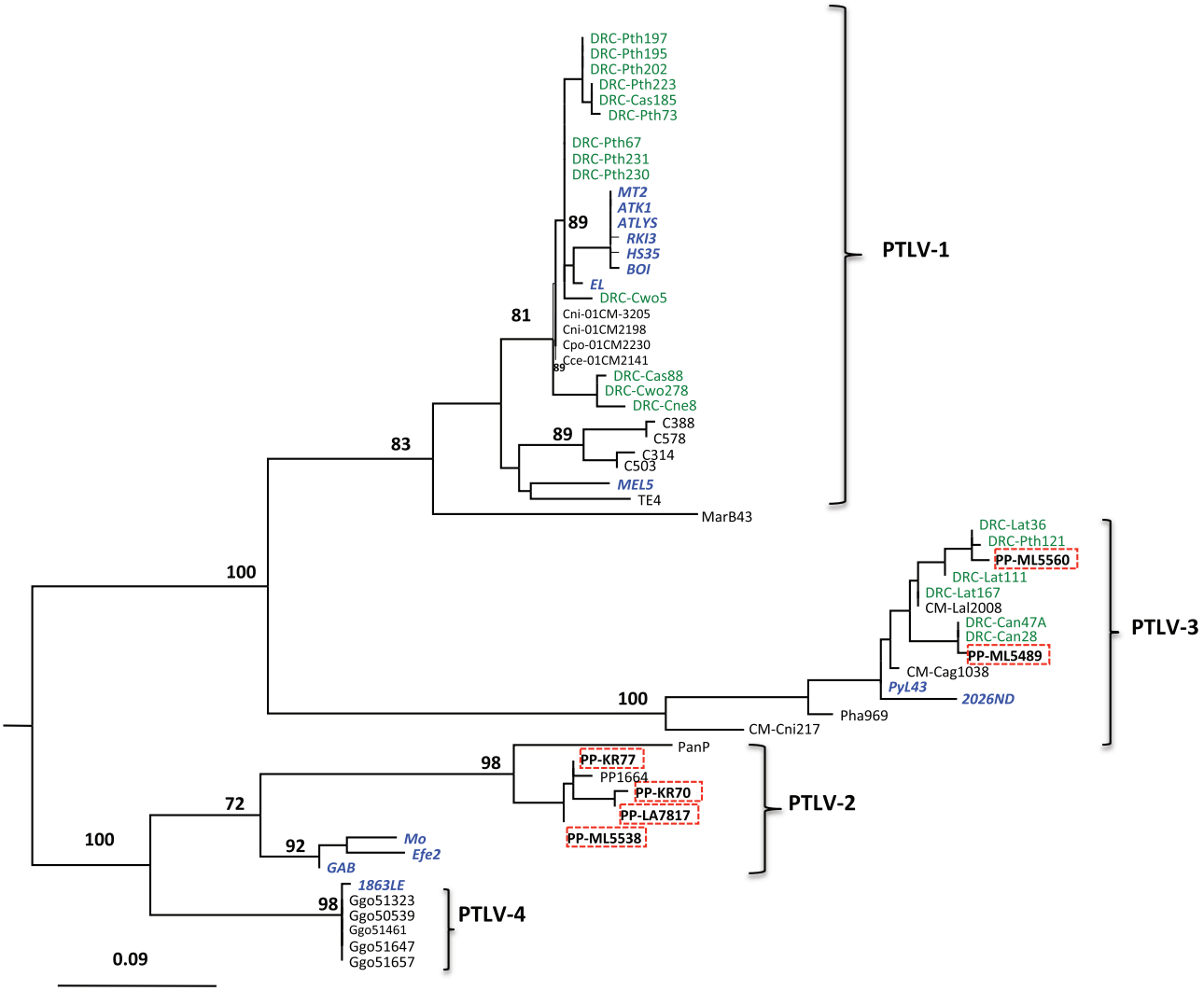


**Figure 2.** Mid-point rooted PTLVphylogeny inferred from 202-bp *tax* sequences. The tree was generated using ML methods, with the TN93 nucleotide substitution model with gamma-distributed rates among sites. Numbers correspond to internal branch support derived from 100 bootstrap replicates. Scale bar represents the number of nucleotide substitutions per site. The different PTLV types are indicated. New PTLV-1 sequences are highlighted in dotted red lines boxes, strains isolated in humans are highlighted in blue font. STLV sequences from the DRC are highlighted in green. Different NHP species are indicated with the following abbreviations; Pth, *Piliocolobus tholloni*; Cas, *Cercopithecus ascanius*; Cwo, *Cercopithecus wolfi*; Cni, *Cercopithecus nictitans*; Cpo, *Cercopithecus pogonias*; Cce, *Cercopithecus cephus*; Cne, *Cercopithecus neglectus*; Lat, *Lophocebus atterrimus*; Lal, *Lophocebus albigena*; Can, *Colobus angolensis*; Cag, *Cercocebus agilis*; Pha, *Papio hamadryas*; Ggo, *Gorilla gorilla*.

**Table 3.** Characteristics of STLV PCR-positive bonobo samples

| ID | Species | Sites | PCR detection | | | STLV types |
|---|---|---|---|---|---|---|
| | | | *tax* (<200 bp) | *tax* (>200 bp) | LTR | |
| PPKR70 | *Pan paniscus* | Kokolopori (KR) | Positive | Positive | *Negative* | STLV-2 |
| PPKR77 | *Pan paniscus* | Kokolopori (KR) | Positive | Positive | *Negative* | STLV-2 |
| PPKR78 | *Pan paniscus* | Kokolopori (KR) | Positive | *Negative* | *Negative* | STLV-2 |
| PPLK684 | *Pan paniscus* | Lui-Kotal (LK) | Positive | *Negative* | *Negative* | STLV-2 |
| PPML5489 | *Pan paniscus* | Malebo (ML) | Positive | Positive | Positive | STLV-3 |
| PPML5538 | *Pan paniscus* | Malebo (ML) | Positive | Positive | Positive | STLV-2 |
| PPML5560 | *Pan paniscus* | Malebo (ML) | Positive | Positive | *Negative* | STLV-3 |
| PPLA7817 | *Pan paniscus* | Lomako-Yokokala (LA) | Positive | Positive | *Negative* | STLV-2 |

STLV infection was thus identified in four of six forest sites (Table 2), with STLV-2 identified at four sites, (ML, LK, KR, LA), and STLV-3 identified at one site (ML) (Fig. 2). The overall prevalence of STLV infection was estimated for each field site based on the proportion of STLV-positive bonobos and by correcting for repeated sampling. Microsatellite analysis from previous studies indicated that on average each bonobo had been sampled 2.03 times (Li et al. 2012), indicating that we screened at least 312 bonobos among which at least eight (2.6%; 95% CI: 1.3–5.0) were infected with STLV. We used this correction factor here because the 312 samples of this work are a subset of those studied in the arrticle by Li et al. (2012). The gender was determined by amilogenin PCR on selected samples. These informations were not available for the eight STLV positive samples identified here. Infection rates, which likely represent an underestimate of the true prevalence, ranged from no detectable infection at the two eastern most sites (IK and BN) to almost 8% at the western most site (ML).

### 3.2 Genetic diversity of STLV in bonobos

To study the genetic diversity of the identified STLV strains, we used a 200-bp *tax* fragment for phylogenetic tree analysis (a *tax* fragment amplified with PTLV-2 specific primers was only 100–117-bp and thus too short to yield reliable results). The phylogenetic tree of the 220-bp *tax* fragment confirmed the PTLV type identification with the type specific primers. The new STLV-2 strains clustered with two strains previously identified in captive bonobos (Vandamme et al. 1996; Van Brussel et al. 1998). They formed a cluster with the PP1664 strain, though not significantly supported, suggesting presence of two different lineages and a relatively high STLV-2 genetic diversity (Fig. 2). The mean genetic distance between the new strains and the two previously characterized PP1664 and PanP strains were 0.022 and 0.131, respectively, which is 4.4–26.2 higher than the distance between, for example, HTLV-4 1863LE and the five gorillas STLV-4 strains. Only a subset of STLV-3 sequences are included in the phylogenetic tree of the *tax* region and the studied fragment is short, but the new STLV-3 strains, PP-ML5489 and PP-ML5560, were closer to recently described STLV-3 strains from DRC monkey species, including the Angolan colobus (*Colobus angolensis*), Tsuapa red colobus (*Piliocolobus tholloni*), and the black mangabey (*Lophocebus aterrimus*), than they were to each other (Ahuka-Mundeke et al. 2012). To exclude the possibility that the two samples PP-ML5489 and PP-ML5560 bearing STLV-3 strains were contaminated with feces from other primates, we performed two sets of PCR that amplified a 130- and a 300-bp mtDNA fragment in the presence of three primers sets that prevent the amplification of chimpanzee DNA (Calvignac-Spencer et al. 2013). In the presence of blockers, no amplification occurred for sample PP-MB5489. For the sample PP-ML5560, faint PCR amplicons were obtained (130- and 300-bp). In the absence of blockers, both samples yielded PCR products at right positions. Sequencing of the PCR products identified *P. paniscus* in both blocking and normal PCR conditions (data not shown). This failure to fully block bonobos' DNA might be due to the fact that the PCR primers used were primarily designed to block *P. troglodytes*. Subsequently, they were adapted to *P. paniscus* DNA.

We also attempted to amplify a fragment in the LTR region. However, among the eight *tax* PCR-positive samples, LTR fragments were only obtained for one STLV-2 (PP-ML5538) and one STLV-3 (PP-ML5489) sample. Sequence and phylogenetic analysis of the LTR region showed that in contrast to the *tax* region, the new STLV-2 strain was equidistantly related to PP1664 and PanP strains in LTR (Fig. 3), yielding genetic distances of 0.052 and 0.068, respectively. The new STLV-3 strain from PP-ML5489 clustered in the LTR fragment with strains from the STLV-3 subtype B from west and central Africa, and formed a separate lineage with STLV-3 strains from monkey species native to the DRC, that is, Angolan colobus, black mangabeys, and Tshuapa red colobus (Fig. 4).

## 4. Discussion

Although previous studies of bonobos identified two strains of STLV-2, the relevance of these findings to wild populations remained unclear because only few animals were screened and all were housed in primate facilities in Europe and the USA (Giri et al. 1994; Vandamme et al. 1996). Here we conducted a comprehensive survey of a large number of wild-living bonobos at eight field sites throughout their natural range, revealing that both STLV-2 and STLV-3 infections are endemic in these communities. We find that STLV-2 infection is common and widespread among wild bonobos, strongly suggesting that this ape species represents a natural reservoir for this virus. We also identified two cases of STLV-3 infection, which has not previously been identified in apes. Because these viruses were more closely related to viruses circulating in monkey species than to each other, it is likely that they represent the result of recent cross-species transmissions from hunted prey species.

Targeting viral DNA rather than RNA, we show for the first time that fecal samples can be used to amplify STLV sequences. We targeted DNA instead of RNA, because RNA viral loads are extremely low, even in blood and progression to disease is measured by quantifying HTLV-1 proviral load in peripheral blood mononuclear cells (Kuramitsu et al. 2015). Moreover, several studies in humans infected with HTLV-1 and Indian rhesus macaques experimentally infected with a replicative form of HTLV-2 showed presence of the proviral DNA in the gastrointestinal tract and epithelial cells of asymptomatic patients (Tokunaga

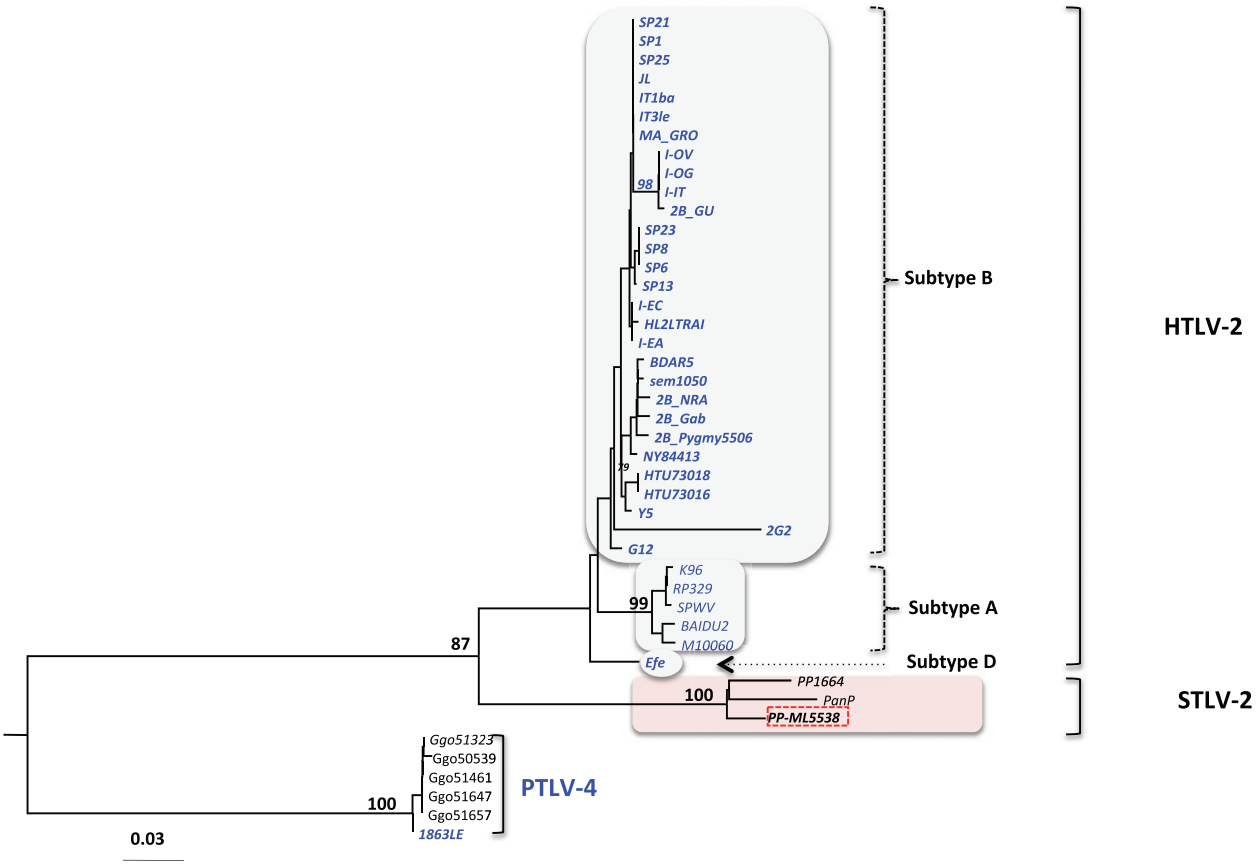


**Figure 3.** Mid-point rooted PTLV-2 phylogeny inferred from 313-bp LTR sequences. The tree was generated using ML methods with the TN93 nucleotide substitution model with gamma-distributed rates among sites. Numbers correspond to internal branch support derived from 100 bootstrap replicates. Scale bar represents the number of nucleotide substitutions per site. The new STLV-2 sequence is highlighted in dotted red line box. Human-derived strains are highlighted in blue font.

et al. 1993; Hidaka et al. 2004). A study reporting on experimental infection of Indian rhesus macaques with HTLV-2 (Gordon et al. 2010), showed evidence of comparable levels of HTLV-2 DNA in blood, lymphoid tissues, and the gastrointestinal tract. Hence, the presence of HTLV and STLV DNA in feces is thus very likely.

In previous studies of primate bushmeat (Ahuka-Mundeke et al. 2012), we observed that commercial available serological assays were not sufficiently sensitive to reliably identify divergent STLV strains in primate blood samples. Here, we used PCR to screen bonobo fecal DNA for evidence of STLV infection by targeting a small fragment of the *tax* gene with universal and type-specific primers. We specifically targeted *tax* gene (and LTR) because (1) these are the two regions in which most, if not all, PTLV lineages have sequences available, thus making comprehensive BLAST and phylogenies possible and, (2), in DNA extracts derived from faecal material, it is exceedingly difficult to PCR-amplify long fragments and *tax* and LTR PCR used in the present study allowed the amplification of fragments not exceeding 500-bp. This strategy was clearly more sensitive than fecal antibody testing since all STLV PCR- positive fecal samples were antibody-negative when subjected to the confirmatory INNOLIA HTLV assay (data not shown). Molecular screening also confirmed the infection directly and provided immediate information concerning the infecting STLV type. Overall, nearly 2.6% of all bonobos tested were STLV sequence-positive. However, this is likely a gross underestimate because viral DNA is probably less abundant in fecal samples as compared with blood. Indeed, not all longitudinal samples from known STLV-infected animals were positive by PCR, and, only two of six fecal samples, which were *tax* PCR (<200-bp) positive, were also positive for the longer LTR fragment (>300-bp) (Table 3). Nevertheless, we showed for the first time that STLV can be amplified from fecal DNA. Our previous study of monkey bushmeat from the DRC indicated an overall STLV prevalence of 7.9% (26/330) in seven different monkey species, ranging from 1 to 20% according to the species (Ahuka-Mundeke et al. 2012). Similarly, the STLV infection rate in primates from Cameroon was 7.2%, also with differences depending on the species analyzed (Liégeois et al. 2008). These overall prevalence rates are higher than the one estimated in this study and could be due to the host species, and or the type of sample analyzed. However, the current data argue strongly that STLV-2 is widespread and common in bonobos. Moreover, it seems likely that this virus is actively transmitted within and between wild communities, since studies of two independent colonies of captive bonobos identified STLV-2 in more than one animal (4/8 in the Antwerp zoo and 2/3 in a US primate center) (Vandamme et al. 1996). Finally, we found a relative high genetic diversity among the newly identified STLV-2 strains, suggesting a long-standing virus–host relationship.

Although we confirmed bonobos as the natural host of STLV-2, our results did not resolve the origin of HTLV-2. This is because in contrast to PTLV-1 and 3, and possibly also PTLV-4, where STLV and HTLV strains are intermixed, STLV-2 and HTLV-2 strains formed two genetically distinct clusters. It is

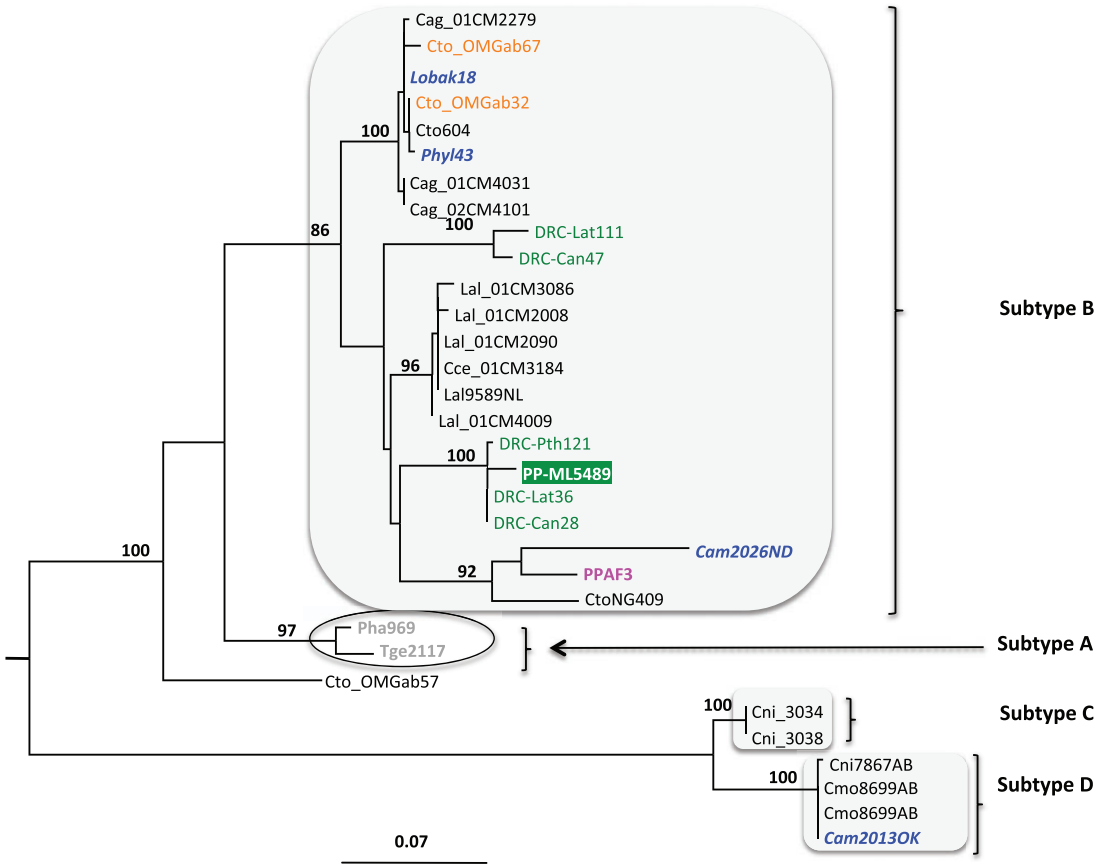


**Figure 4.** Mid-point rooted PTLV-3 phylogeny inferred from 380-bp LTR sequences. The tree was inferred using ML methods with the TN93 nucleotide substitution model with gamma-distributed rates among sites. Numbers correspond to internal branch support derived from 100 bootstrap replicates. Scale bar represents the number of nucleotide substitutions per site. The different PTLV-3 subtypes are indicated. The new STLV-3 sequence is highlighted in green box. STLV-3 and HTLV-3 sequences are highlighted in colors according to their geographic origin, green for DRC, black from Cameroon, orange for Gabon, magenta for Senegal and grey for Ehtiopia/Eritrea. Different NHP species are indicated with the following abbreviations; Cag, *Cercocebus agilis*; Cto, *Cercocebus torquatus*; Lat, *Lophocebus atterrimus*; Can, *Colobus angolensis*; Lal, *Lophocebus albigena*; Pth, *Piliocolobus tholloni*; Cni, *Cercopithecus nictitans*; Cmo, *Cercopithecus mona*; Ph, *Papio hamadryas*; Ppa, *Papio anubis*; Tge, *Theropithecus gelada*. HTLV-3 strains are highlighted in blue font.

possible that the last common ancestor of humans and chimpanzees harbored an STLV-2-like virus, which subsequently coevolved with its respective hosts. However, the apparent lack of STLV-2 in common chimpanzees renders this possibility highly unlikely. It is also possible that other more divergent STLV-2 lineages circulate in bonobos which have not yet been sampled, or that STLV-2 originated in an unknown monkey species, which transmitted to both humans and bonobos. The latter possibility seems less likely given the large number of samples (> >2,500) from different NHP species in Central Africa (Cameroon, DRC and Gabon) that have already been tested and were negative for STLV-2, while new STLV-1 and 3 variants were discovered (Nerrienet et al. 2001; Courgnaud et al. 2004; Liégeois et al. 2008, 2012; Sintasath et al. 2009; Ahuka-Mundeke et al. 2012). HTLV-2, albeit rare, has been reported to infect humans in these countries (Letourneur et al. 1998; Vandamme et al. 1998; Mauclere et al. 2011].

Although STLV-3 is known to infect a wide variety of African monkey species (Mahieux and Gessain 2011), it has never been identified in African great apes, due the scarcity of this virus in apes or the their insufficient screening. Here, we show for the first time that STLV-3 can infect wild bonobos and that the new STLV-3 sequences (PP-ML5489 and PP-ML5560) cluster with STLV sequences derived from monkey species which are sharing the same habitat as bonobos in DRC, that is, angolan colobus, black mangabeys and Tshuapa red colobus (Ahuka-Mundeke et al. 2012). Our previous studies showed that STLV-3 strains from the DRC form different geographic clusters and do not intermix with STLV-3 strains from other regions like Cameroon (Ahuka-Mundeke et al. 2012). Although known for being primarily vegetarian and frugivorous, recent studies have described that in addition to duikers and forest squirrels, bonobos also hunt small monkeys, including Wolf 's monkey, red tailed monkey, black mangabey, and Tshuapa red colobus, (Hohmann and Fruth 2002; Surbeck and Hohmann 2008; Sakamaki et al. 2016) or other monkey species, including *C. wolfi* and *C. ascanius*, present at Malebo site. All of these monkey species are naturally infected with STLV-1 and 3 (Ahuka-Mundeke et al. 2012). Although we do not know yet the extent of hunting by bonobos at ML site where we identified the STLV-3 strains, they most likely acquired STLV-3 by predation, as it was also described for STLV-1 in chimpanzees and red colobus from the Taï forest in Ivory Coast (Leendertz et al. 2011). Although we did not detect STLV-1 and STLV-4 in the subset of bonobo fecal samples that we screened, we cannot exclude the possibility that bonobos could also carry viruses from these lineages, especially STLV-1 variants from their prey species.

In conclusion, our study shows that fecal samples can be used to screen wild-living endangered apes for STLV infection, although the resulting rates likely represent a gross underestimate

of the true viral prevalences. We also show that bonobos are endemically infected with diverse strains of STLV-2, suggesting a long-standing virus host relationship. The lack of data on gender and filiation on the six STLV-2 positive bonobos could not be used to further support the endemicity of these viruses. However, given that wild bonobos from four different sites are found STLV infected supports this notion of endemicity. Finally we show that bonobos are susceptible to STLV-3 infection and may have acquired members of this lineage by cross-species transmission. What remains unknown is whether there is bonobo-to-bonobo transmission of STLV-3 and whether this ape species also harbors members of STLV-1 and STLV-4 lineages. Similarly, the primate origin of HTLV-2 in humans remains to be deciphered.

## Acknowledgements

We thank the staff of the World Wildlife Fund for Nature (WWF/DRC), the Institut National de Recherches Biomédicales (INRB, Kinshasa, DRC), the Bonobo Conservation Initiative, and Vie Sauvage, Didier Mazongo, Octavie Lunguya, Muriel Aloni, and Valentin Mbenzo-Abokome for field work in DRC.

## Funding

This study was supported by grants from the NIH (R37 AI50529), the Agence Nationale de Recherches sur le SIDA (ANRS), France (grants No. ANRS 12182, ANRS 12555, and ANRS 12325) and the Institut de Recherche pour le Développement (IRD), France; The Arthure L Greene Fund and Harvard University.

**Conflict of interest:** None declared.

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