

1 **High prevalence, co-infection rate and genetic diversity of retroviruses in wild red colobus**
2 **monkeys (*Ptilocolobus badius badius*) in Taï National Park, Côte d'Ivoire**

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29 **ABSTRACT**

30 Simian retroviruses are precursors of all human retroviral pathogens. However, little is
31 known about the prevalence and co-infection rates as well as the genetic diversity of major
32 retroviruses - simian immunodeficiency viruses (SIV), simian T-cell Lymphotropic viruses
33 type 1 (STLV-1) and simian foamy viruses (SFV) - in wild populations of non-human primates.
34 Such information would contribute to the understanding of the natural history of
35 retroviruses in various host species. Here we estimate these parameters for wild West
36 African red colobus monkeys (*Piliocolobus badius badius*) in the Taï National Park, Côte
37 d'Ivoire. We collected samples from a total of 54 red colobus monkeys; blood and/or
38 internal organs from 22 monkeys, and additionally, muscle and other tissue samples from
39 further 32 monkeys. PCR analyses revealed a high prevalence of SIV, STLV-1 and SFV in this
40 population: 82%, 50% and 86%, respectively. Forty-five percent of the monkeys were co-
41 infected with all three viruses while another 32% were co-infected with SIV in combination
42 with either STLV or SFV. As expected, phylogenetic analyses showed a host-specific pattern
43 for SIV and SFV strains. In contrast, STLV-1 strains appeared to be distributed in genetically
44 distinct and distant clades, which are unique to the Taï forest and include strains previously
45 described from wild chimpanzees in the same area. The high prevalence of all three
46 retroviral infections in the *P. b. badius*, represents a source of infection to chimpanzees, and
47 possibly humans, who hunt them.

48 **INTRODUCTION**

49 Lentiviruses and Deltaretroviruses infecting African non-human primates have received
50 considerable attention as they are the precursors of all pathogenic human retroviruses;
51 Human Immunodeficiency Viruses (HIV-1/HIV-2) and Human T-cell Lymphotropic Viruses-1
52 (HTLV-1s). These human infections are the results of past zoonotic transfers of Simian
53 Immunodeficiency Viruses (SIV) and Simian T-cell Lymphotropic Viruses-1 (STLV-1) from
54 wild monkeys and apes into local human populations, presumably through primate hunting
55 and handling of primate bushmeat (13, 19, 43, 46, 56, 57, 59). Via the same route, zoonotic
56 transmission of Simian Foamy Viruses (SFV), Spumaretroviruses whose exact pathogenicity
57 in human hosts is still unknown, has also been shown (64). The increasing contact between
58 humans and wild primates implies that further zoonotic transmission of retroviruses is likely
59 to happen (42, 63). Studying the occurrence and circulation of simian retroviruses such as
60 SIV, STLV-1 and SFV in wild primate populations enables us to better understand retrovirus
61 evolution in primates, but also provides tools for monitoring possible future retroviral
62 zoonotic events.

63 Systematic studies on SIV, STLV-1 and SFV in wild primates are relatively rare. Many use
64 bushmeat samples, which can vary in their quality and are prone to cross-contamination
65 from butchering and storage with other carcasses. Confiscated primates are also not
66 representative for the wild situation since the animals are caught at a young age when the
67 occurrence of different retroviruses may be extremely low (24). The technical possibilities
68 for the detection of various pathogens in non-invasive samples such as urine and faeces
69 have greatly improved and are frequently used; however, in general the sensitivity of
70 detection methods is higher when using blood and tissue samples (25, 32, 47). Such samples
71 can be collected if fresh carcasses are found, or through anaesthetizing live primates for

72 sampling purpose, animal translocation or medical intervention such as snare removal. The
73 practical and ethical issues of each of the sampling methods have been discussed elsewhere
74 (12, 14).

75 Red colobus monkeys [*Procolobus (Piliocolobus)*] are interesting subjects for retroviral
76 infection studies for a number of reasons. First, they are widely distributed (yet in a
77 fragmented manner) from East to West Africa, which suggests that red colobus species and
78 sub-species, or more likely ancestor(s) of these, could have been key hosts in transmitting
79 retroviruses across tropical Africa (4, 54). Secondly, as they are herbivore primates, hunting
80 of other primates as a route of infection can be excluded. Finally, these monkeys are
81 frequently hunted by humans and chimpanzees and represent a possibly large reservoir for
82 retroviruses and other pathogens that ought to be investigated further (2, 45).

83 Very little information is available about the prevalence and co-infection of SIV, STLV-1 and
84 SFV in wild red colobus monkeys across Africa. In other colobine monkeys only SIV has been
85 documented; in olive colobus (*Procolobus verus*) in Côte d'Ivoire and in black and white
86 colobus (*Colobus guereza*) in Cameroon (7, 8). Based on faecal samples from habituated
87 adult individuals, the prevalence of SIV in West African red colobus monkeys (local sub-
88 species: *P. badius badius*) (SIVwrc) has been estimated to a minimum of 26% in the Taï
89 National Park, Côte d'Ivoire, but the authors recognised the low sensitivity of viral RNA
90 detection in faecal samples (34). Another study conducted on the same population revealed
91 that five out of ten blood samples were SIV-positive (7). These results highlight that the most
92 reliable prevalence data are based on analyses of blood/tissue samples, although such
93 sampling is not always feasible for reasons discussed above. Published prevalence
94 information concerning STLV-1 and SFV in wild red colobus monkeys (STLV-1wrc and SFVwrc)
95 in the same area is restricted to results obtained from analyses of a limited number of blood

96 and necropsy samples collected as a part of studies whose focus was on cross-species
97 transmission of these two viruses to chimpanzees (27, 28). However, these samples
98 indicated a high prevalence of STLV-1wrc and SFVwrc in the red colobus monkey population
99 (56% and 90%, respectively). A recent study from Uganda, East Africa, estimated the
100 prevalence of SIV, STLV-1 and SFV in another red colobus species (*P. rufomitratus*
101 *tephrosceles*) to be 22.6%, 6.4%, and 97%, respectively (15). The study was performed using
102 blood samples collected from anaesthetised wild red colobus monkeys living in their natural
103 habitat, which allowed reliable assessment of the prevalence and genetic diversity of these
104 three retroviruses.

105 The preliminary data from Taï indicate that there might be a great variation in the
106 prevalence of retroviruses across the African continent, even in closely related species of
107 wild primates. Here, we aimed at generating reliable prevalence and co-infection data for
108 SIVwrc, STLV-1wrc and SFVwrc based on the analysis of blood and tissue samples from wild
109 western red colobus monkeys. We expected that this would allow for proper comparison of
110 retroviral prevalence in the allied species *P. b. badius* and *P. r. tephrosceles*.

111

112 MATERIALS AND METHODS

113 **Study site and animals.** Field work was conducted in the evergreen rainforest of the Taï
114 National Park in Côte d'Ivoire, West Africa (5°15'-6°07'N, 7°25'-7°54'W). The West African
115 red colobus monkey (*P. b. badius*) is the most abundant primate species in this forest and
116 shares its habitat with eight other diurnal primates: chimpanzee (*Pan troglodytes verus*),
117 sooty mangabey (*Cercocebus atys*), black-and-white colobus (*Colobus polykomos*), olive
118 colobus (*Procolobus verus*), Diana monkey (*Cercopithecus diana*), lesser spot-nosed monkey
119 (*C. petaurista*), Campbell's monkey (*C. campbelli*) and greater spot-nosed monkey (*C.*

120 *nictitans*) (36). Samples were collected from wild non-habituated West African red colobus
121 monkeys within an area of approximately 100 km².

122 **Sample collection.** Blood samples were collected from 10 adult red colobus monkeys under
123 general anaesthesia (28). At the same time other biological samples and anatomical
124 measurements were collected. The samples were centrifuged shortly after collection, and
125 the cell rich layer (buffy coat) was frozen immediately in liquid nitrogen. As part of the
126 chimpanzee health monitoring project, veterinarians perform necropsies on all carcasses of
127 any species found in the forest. Since the year 2001 necropsy samples were collected from
128 12 adult red colobus monkey carcasses and transported on ice directly to the camp.
129 Chimpanzees in the area regularly hunt red colobus (2) and sometimes leave behind pieces
130 of muscle or other tissues from their prey. The veterinary project has collected such tissue
131 samples from observed hunts of 32 individual red colobus monkeys as soon as the
132 chimpanzees had left the site. These samples were collected using single use gloves,
133 transported at ambient temperature and preserved at camp a maximum of 12 hours after
134 the death of the monkey. For the necropsies and chimpanzee meal remains, multiple
135 samples were collected if possible and up to three samples per individual were analysed. All
136 samples were stored in liquid nitrogen at the field site and later transported on dry ice to
137 Robert Koch-Institute, Berlin, where samples were stored at -80°C until analyses. All parts of
138 the study were performed under permission of the Ministry of Research and the National
139 Park authorities of Côte d'Ivoire.

140 **DNA extraction, PCR and sequencing.** DNA was extracted using either DNA tissue kit or DNA
141 blood kit (Qiagen, Hilden, Germany), respectively.

142 Samples were tested for SIV with a semi-nested PCR with primers specifically designed for
143 the detection of *pol* regions of SIV from the western red colobus/olive colobus group (SIVwrc

144 S1 [CAT GGC AAA TGG ATT GTA CTC A], SIVwrc R2 [GTG CCA TTG CTA ATG CTG TTT C], SIVwrc
145 S3 [CCA AAT TCT TGT TCT ATC CCT AAC C], and SIVwrc R3 [AGC AAA AAT CAT ATC AGC AGA
146 AGA T]). These primers were based on SIVwrc and SIVolc sequences published by Courgnaud
147 and colleagues (7). We used SIVwrc S1 and SIVwrc R2 in the first round PCR, and SIVwrc S1
148 and SIVwrc R3 (expected amplicon size approximately 250 bp), and SIVwrc S3 and SIVwrc R2
149 (expected amplicon size approximately 300 bp) in two parallel semi-nested PCRs. The cyclor
150 conditions were 94°C for 5 minutes, 30x[94°C for 15 seconds, 55°C for 30 seconds, 72°C for
151 30 seconds], 72°C for 10 minutes, then cooling to 4°C. SIVwrc negative samples and one
152 sample from each SIVwrc positive individual were also tested with a generic SIV PCR known
153 to detect most primate Lentiviruses, to determine if the monkeys also carried other types of
154 SIV (6). We used the primers DR1 (TRC AYA CAG GRG CWG AYG A) and DR2 (AIA DRT CAT
155 CCA TRT AYT G) in the first round PCR and primers DR4 (GGI ATW CCI CAY CCD GCA GG) and
156 DR5 (GGI GAY CCY TTC CAY CCY TGH GG) in a nested PCR. The cyclor conditions were 94°C
157 for 2 minutes, 30x[94°C for 15 seconds, 50°C decreasing by 0.5°C each cycle to 35°C for 30
158 seconds, 72°C for 1 minute], 15x[94°C for 15 seconds, 50°C for 30 seconds, 72°C for 1
159 minute], 72°C for 5 minutes, then cooling to 4°C. The expected amplicon size was 194 bp.
160 Samples were tested for proviral DNA of STLV-1 by a *tax* specific real time PCR (23). We used
161 the primers SK43 (CGG ATA CCC AGT CTA CGT GT) and SK44 (GAG CCG ATA ACG CGT CCA
162 TCG) and the probe HTLV TAX TM (6FAM-CGC CCT ATG GCC ACC TGT CCA GA XT P), and the
163 cyclor conditions were 95°C for 10 minutes, 45x[95°C for 15 seconds, 60°C for 35 seconds].
164 The expected amplicon size was approximately 190 bp. A fragment of the LTR region was
165 then sequenced from positive samples as this region of Primate T-cell Lymphotropic Virus 1
166 (PTLV-1) genome evolves more rapidly and is frequently used for phylogenetic analyses. We
167 used the primers S10, H and X (26, 27) derived from nucleotides 7929-7948, 8756-8735, and

168 8296-8316, respectively, from the prototype HTLV-1 sequence ATK (Accession number
169 J02029) (48). We used Primer S10 (GGC CCT AAT AAT TCT ACC CG) and Primer H (AGT TCA
170 GGA GGC ACC ACA GGC G) for the first round, and the primers Primer X (GAG CTC GAG CAG
171 ATG ACA ATG ACC ATG AG) and Primer H in a semi-nested PCR. The cycler conditions were
172 94°C for 5 minutes, 35x[94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 min (30 seconds
173 for semi-nested PCR)], 72°C for 10 minutes, then cooling to 4°C. The expected amplicon size
174 for the semi-nested PCR was 450 bp.

175 Samples were tested for SFV with a PCR specifically designed to amplify a fragment of
176 SFVwrc *pol* (28). We used the primers SFVwrc 1s (CAT ACA ATT ACC ACT CCA AGC CT),
177 SFVwrc 2as (CAG ACA AAT CCA GTC ATA CCA TC), SFVwrc 3s (CTC AGT ACT GGT GGC CAA
178 ATC TTA GA) and SFVwrc 4as (CCA GTC ATA CCA TCG ACT ACT ACA AGG). In the first round
179 PCR we used primers SFVwrc S1 and SFVwrc 2as, then for two parallel semi-nested PCRs we
180 used primers SFVwrc S1 and SFVwrc 4as (expected amplicon size approximately 430 bp), and
181 SFVwrc S3 and SFVwrc 2as (expected amplicon size approximately 270 bp), and the cycler
182 conditions were 96°C for 5 minutes, 40x[96°C for 1 minute, 56°C for 30 seconds, 72°C for 1
183 min], 72°C for 10 minutes, then cooling to 4°C. One sample from each individual was also
184 tested with a generic SFV PCR (15) to check for the presence of other non-red colobus strains
185 of SFV. We used the primers SIF2 (TAG CWG AYA ARC TTG CCA CCC AAG G) and SIR1 (GTC
186 GTT TWA TIT CAC TAT TTT TCC TTT CCA C) in the first round, and primers SIF3 (CCA ARC CTG
187 GAT GCA GAG YTG GAT CA) and SIR3 (ACT TTG GGG RTG RTA AGG AGT ACT G) in a nested
188 PCR. The cycler conditions were 95°C for 5 minutes, 40x[95°C for 30 seconds, 45°C for 45
189 seconds, 72°C for 1 min], 72°C for 10 minutes, then cooling to 4°C. The expected amplicon
190 size for was approximately 480 bp.

191 PCR products were visualised with gel electrophoresis before being purified. Gel extraction
192 was performed when necessary. Sequencing was performed in both directions using the
193 Sanger method, with all PCR products being sequenced on both strands. Comparison to the
194 public database using NCBI BLAST (1) always confirmed that the expected proviral sequences
195 had been amplified.

196 **Prevalence and co-infection of SIV, STLV-1 and SFV, and correlation between infections.**

197 The prevalence of the three retroviruses was calculated in Stata (Stata/SE 10.0 for Windows,
198 Stata Corp, College Station TX), as well as the corresponding 95% confidence interval (CI) for
199 proportions (normal approximation). We calculated the percentage of monkeys with single
200 infections of the individual viruses, and in order to investigate if infections were linked to
201 each other, we also calculated the percentage of individual monkeys with dual (for all
202 possible viral combinations) or triple infections. Kendall tau-b test, including Fisher exact
203 test, was used to determine the degree of correlation between the infections. Prevalence
204 and co-infection of SIV, STLV-1 and SFV, and correlation between infections were calculated
205 on basis of the results obtained from buffy coat samples and necropsy samples only. The
206 samples collected after chimpanzee meals were considered not to be of sufficient quality to
207 be included in these analyses. These samples were however used to obtain additional
208 nucleotide sequences for the phylogenetic analyses.

209 **Sequence analyses**

210 Newly generated sequences were added to datasets considered to encompass the overall
211 genetic diversity of SIV, STLV-1 and SFV. Alignments were edited manually using SeaView v4
212 (16). To allow detection of saturation, the number of transitions and transversions versus
213 divergence was first plotted in DAMBE (65). If distances calculated under the global time
214 reversible (GTR) model are chosen as a measure of divergence, then both transitions (ts) and

215 transversions (tv) should increase in a linear manner with GTR distance. In general, ts should
216 also accumulate faster than tv. However, with GTR distance increasing, multiple
217 substitutions are expected to occur at the same sites, ultimately coming to losing the initial
218 correlation. Saturation should thus translate into plateauing plots and tv outnumbering ts
219 (30). While the PTLV-1 dataset was apparently exempt of saturation, the SIV and SFV
220 datasets exhibited clear patterns of ts saturation at the 3rd position of codons. Accordingly,
221 we performed all following analyses on SIV and SFV datasets stripped for this position (a
222 conservative approach). All three datasets were haplotyped (reduced to unique sequences)
223 using FaBox (60). The overall process resulted in our starting datasets being composed as
224 follows: i) SIV *pol*: 75 taxa; 152 bp, ii) PTLV-1 LTR: 47 taxa, 422 bp; and iii) SFV *pol*: 51 taxa,
225 252 bp.

226 Nucleotide substitution models to which data were a better fit were then selected using
227 jModeltest v0.1.1 (17, 44). According to the Akaike information criterion (AIC), comparisons
228 of model likelihoods were most favourable to GTR+I+G (SIV), Hasegawa Kishino and Yano
229 (HKY)+I+G (PTLV-1) and GTR+G (SFV). Phylogenetic analyses were performed in both
230 maximum likelihood (ML) and Bayesian frameworks, under the appropriate model of
231 nucleotide substitution. ML analyses were performed on the PhyML webserver
232 (<http://www.atgc-montpellier.fr/phyml/>; (17, 18)). Equilibrium frequencies, topology and
233 branch lengths were optimised, the starting tree was determined using BioNJ and both
234 nearest neighbour interchange (NNI) and subtree pruning and regrafting (SPR) algorithms of
235 tree search were used (keeping the best outcome). Branch robustness was assessed by
236 performing non-parametric bootstrapping (500 replicates). Bayesian analyses were
237 performed using BEAST v1.5.3 (11). Besides allowing modelling nucleotide substitution
238 processes, BEAST also allows for modelling rate variation among tree branches and tree

239 shape. All analyses were run under the assumption of a relaxed, uncorrelated lognormal
240 clock. For SIV and SFV datasets, analyses were performed assuming 2 different tree shape
241 speciation models (Yule and birth-death processes). Given the expected relatively shallow
242 depth of the PTLV-1 phylogenetic tree, a speciation model (birth-death process) and a
243 coalescent model (constant population size) were employed. Two runs of 10,000,000
244 generations were run per dataset per tree shape model (i.e. four runs total for each dataset).
245 Trees and numerical values taken were sampled every 1,000 generations. Tracer v1.5 was
246 used to check that individual runs had reached convergence, that independent runs
247 converged and that chain mixing was satisfactory (effective sample size values >200) (11).
248 Trees sampled in duplicate runs were then gathered into a single file (after removal of a
249 visually conservative 10% burn-in period) using LogCombiner v1.5.3 (distributed with BEAST)
250 and the information of 18,000 trees per dataset per tree shape model summarized onto the
251 maximum clade credibility tree using TreeAnnotator v1.5.3 (distributed with BEAST).
252 Posterior probabilities were taken as a measure of branch robustness.
253 For Bayesian analyses, no major discrepancy in topology or branch support was detectable
254 using different tree shape models. ML and Bayesian methods globally supported congruent
255 topologies with consistent branch supports (even though bootstrap and posterior probability
256 are not directly comparable (10)). All xml files (including sequence alignments) used for
257 Bayesian analyses are available at
258 <http://sebastienalvignac.fr/emergingzoonoses/index.html>. Figures summarising
259 phylogenetic analyses were drawn using FigTree v1.3.1
260 (<http://tree.bio.ed.ac.uk/software/figtree/>).
261 **Nucleotide sequence accession numbers.** All sequences generated in this study were
262 deposited in GenBank under the accession numbers XXXXX-XXXXX (in progress).

263

264 **RESULTS**

265 **Prevalence of SIV, STLV and SFV.** All three viruses were detected by specific PCR and
266 confirmed with sequencing and BLAST. Further, all newly sequenced SIV, STLV-1 and SFV
267 strains could be linked to strains previously found in red colobus. Results are summarised in
268 table 1. SIVwrc was detected in 8 out of 10 anaesthetised monkeys and in 10 out of 12
269 carcasses. The overall prevalence was 82% (95% CI 66-98%). STLVwrc was detected in 6
270 anaesthetised monkeys and in 5 carcasses. The overall prevalence was 50% (95% CI 29-71%).
271 SFVwrc was detected in all anaesthetised monkeys and in 9 carcasses. The overall
272 prevalence was 86% (95% CI 72-100%). In the samples from chimpanzee meal remains
273 (attributed to 32 red colobus monkeys), SIV, STLV-1 and SFV were detected in 5, 9 and 6
274 individuals, respectively.

275 **SIV, SFV and STLV-1 co-infections and correlation between infections.** Forty-five percent
276 (n=10) of the monkeys were co-infected with all three viruses, 27% (n=6) were infected with
277 SIV in combination with SFV, and 5% (n=1) were infected with SIV in combination with STLV-
278 1. Fourteen percent (n=3) were infected with SFV only, and 5% (n=1) were infected with SIV
279 only. Five percent (n=1) were negative for all viruses. Of note, no monkey was infected with
280 STLV-1 only or with the virus combination STLV-1 and SFV. There was no statistically
281 significant correlation between any of the infections (Kendall tau-b coefficient and, Fisher
282 exact p-value 0.47 and 0.09 for SIV/STLV-1, 0.16 and 0.47 for SIV/SFV, and 0.13 and 1.00 for
283 STLV-1/SFV).

284 **Phylogenetic analyses.** To infer the phylogenetic relationships of the newly described SIV,
285 STLV-1 and SFV strains with previously characterised strains, we used ML and Bayesian
286 methods. The main pattern of SIV host-specificity was retrieved by these analyses (figure 1).

287 SIVwrc sequences from *P. b. badius* were found to form a clade with SIVwrc strains
288 previously identified from *P. b. badius* from the same area and *P. b. temminckii* from
289 Gambia, the branch defining the bipartition receiving reasonable statistical support (Bp 57,
290 pp 0.99; figure 1). The sister taxon to this group appeared to be the strain identified from
291 Kibale *P. r. tephrosceles*, which also grouped with other SIVwrc with reasonable statistical
292 support (Bp 66, pp 0.97; figure 1). SIVolc, a strain identified from an olive colobus monkey
293 (*Procolobus (Procolobus) verus*) appeared as completing a big colobine clade in the ML
294 analysis (Bp 58) but was found to group with the SIVlho/sun clade in Bayesian analyses,
295 though with very low branch support (pp 0.57).

296 The general design of PTLV-1 strains clustering into geographical sub-types was retrieved by
297 the analyses whose results are summarised in figure 2. Into that scheme, our STLVwrc
298 sequences from *P. b. badius* did not form one monophyletic group. Sequences were
299 distributed in three distinct clades, corresponding to subtype I or nested into subtype J
300 (figure 2) as defined by Junglen et al. 2010. In all cases, these clades were well supported
301 and comprised chimpanzee strains (Bp 98-99, pp 1; figure 2). The *P. r. tephrosceles* strain did
302 not exhibit a close relationship to any of the STLVwrc strains found in Taï, nor did it show
303 particular affinity to any of the previously described PTLV-1 sub-types (figure 2).

304 SFV phylogeny exhibited the expected pattern of marked host-specificity together with
305 plausible long-term co-speciation (figure 3). SFVwrc from *P. b. badius* respected that rule,
306 forming a monophyletic group supported by reasonable Bp and pp values (Bp 54, pp 1,
307 figure 3). *P. r. tephrosceles* strains clustered together with high support (Bp 79, pp 1, figure
308 3). The existence of a colobine clade was also reasonably supported (Bp 54, pp 1), though its
309 inner branching order could not be determined (figure 3).

310

311 **DISCUSSION**

312 **Prevalence of SIV, STLV-1 and SFV.** Most data on retroviruses in wild primates are derived
313 from studies based on bushmeat samples or confiscated and captive populations, and might
314 therefore not be representative for the wild situation. Therefore the main data we use here
315 for comparison are based on a study by Goldberg et al. (2009) using samples obtained from
316 wild primates under anaesthesia.

317 We estimate that the prevalence of SIVwrc in *P. b. badius* in Taï National Park in Cote
318 d'Ivoire is 82%. This is somewhat higher than previously estimated for this population (26%
319 based on non-invasive samples from 53 individuals and 50% based on 10 blood samples),
320 which could be due to differences in test material and sample sizes (7, 35). However, our
321 results confirm that this population has one of the highest prevalence of this virus in wild
322 non-human primates to date. Further, the prevalence in *P. b. badius* is more than three
323 times higher than in the closely related species of red colobus monkey, *P. r. tephrosceles*,
324 living in Kibale, Uganda. The fact that there is no overlap in the 95% CI of the estimated SIV
325 prevalence in the Kibale study and the present study (8-37% and 66-98% for Kibale and Taï,
326 respectively) shows that there is a significant difference between these two
327 populations/species (15). Previous studies have shown that SIV prevalence varies greatly
328 between species, and that some populations, such as mandrills in Cameroon and sooty
329 mangabeys in Côte d'Ivoire, have a high frequency of SIV infection (estimated prevalence
330 79% [95% CI=54-99%] and 59% [95% CI=35-88], respectively) (46, 51, 58). The effect that the
331 high SIV occurrence might have on the *P. b. badius* population is unknown. In general,
332 natural SIV infections have been considered non-pathogenic and even asymptomatic
333 throughout the course of infection (49). However, it has recently been discovered that SIV in
334 wild chimpanzees (SIVcpz) has a negative effect on health and fertility and causes AIDS-like

335 immunopathology in wild chimpanzees from Gombe National Park, Tanzania (22). It is
336 possible that a similar effect will be found in other primate species when additional
337 prospective, long term follow-up studies of SIV-infected wild primates become available. It
338 should be underlined that this type of study requires continuous investigation, and the
339 discovery of SIV-pathogenicity in the Gombe chimpanzees was only possible because good
340 demographic data had been obtained from a long-term field study.

341 Also for STLV-1 we found that the prevalence is much higher in *P. b. badius* than in *P. r.*
342 *tephrosceles*, with no overlap found in the 95% CI: 50% in *P. b. badius* (95% CI=29-71%) and
343 6.4% in *P. r. tephrosceles* (95% CI=0-15%) (15). It appears that there is a great deal of
344 variation in the estimated prevalence of STLV-1 in wild primates, ranging from 0-89%,
345 depending on species and region (9, 25, 37, 50). Interestingly, with the comparisons possible
346 from our study it is striking to observe such extreme differences also between closely related
347 primate species. This probably means that both genetic diversity and retroviral prevalence
348 are determined by geographical location for STLV-1s. However, further studies on additional
349 populations are needed to adequately assess if the prevalence in *P. b. badius* is unusually
350 high or if that of *P. r. tephrosceles* is extraordinarily low.

351 In contrast to SIV and STLV-1, there was not much difference in the prevalence of SFV in our
352 study (86%, 95% CI=72-100%) compared with that of *P. r. tephrosceles* (97%, 95% CI=90-
353 100%) (15). High SFV prevalence has also been found in other wild primate populations,
354 where infections can reach 100% (3, 33). The fact that this generally high prevalence is
355 unique to SFV (when compared to SIV and STLV-1) might be explained by a lesser sensitivity
356 to the behavioural differences that exist between species and even populations.

357 The results from our study, as well as those from the red colobus study in Uganda, are based
358 on relatively small sample sizes (15). However, samples from wild red colobus monkeys and

359 primates in general are difficult to obtain, and one should not refrain from discussing
360 possible reasons for differences in viral prevalence between populations, based on the data
361 we have to date. Virus biology can be one possible reason for the observed difference in SIV
362 and STLV-1 prevalence since these viruses in the Tai and Kibale populations are all distinct.
363 Behavioural differences should also be considered, although no further demographic data
364 are available for our study animals and the routes of retroviral transmission in wild primates
365 are not fully understood (20, 38). In general, the intense social behaviour of red colobus
366 monkeys could give the opportunity for frequent retrovirus transmission. The monkeys live
367 in promiscuous multi-male groups of about 50 individuals, the males fight each other to
368 mate receptive females throughout their adult lives and frequent aggressive harassment of
369 mating couples occurs (36, 52). There are, however, behavioural differences between the
370 red colobus monkey populations/species found in Tai and Kibale that might explain, at least
371 partly, the difference in the prevalence of SIV and STLV-1 at these sites. First, the Tai *P. b.*
372 *badius* population has a defined breeding season of 5-6 months every year, whereas the
373 Kibale *P. r. tephrosceles* population breed all year around (A. Korstjens, pers. comm.). During
374 the intense breeding season in Tai, the receptive females mate with virtually all males
375 available. In Kibale, there are receptive females available all year around and the average
376 number of males in the group is lower than in Tai (3.5 versus 10), which makes monopoly of
377 dominating males easier (36, 40, 52). This means that the *P. b. badius* females in Tai overall
378 mate with a larger number of partners, which is a risk factor in the spread of sexually
379 transmitted diseases (41). Second, although a precise comparison is difficult, it appears that
380 there is more aggression associated with breeding in Tai than in Kibale because of the
381 intense male competition over access to females during a restricted breeding period (A.
382 Korstjens, pers. comm.). Frequent fighting facilitates close contact between individuals and

383 hence represents a risk of viral transmission. Finally, the *P. b. badius* males in Tai were also
384 more frequently seen with red, ulcerated penises than the males in Kibale (A. Korstjens,
385 pers. comm.). This could be a sign of other sexually transmitted diseases which could
386 ultimately make retroviral transmission easier. Further studies are required to diagnose and
387 determine the extent of sexually transmitted diseases in this red colobus population as well
388 as investigate their possible effect on retrovirus transmission.

389 **Co-infection and correlation of viruses.** There was a high degree of co-infection of the
390 retroviruses in the *P. b. badius* monkeys, as nearly half of the individuals were triple-infected
391 and nearly one third were dually infected with SIV and one of the other retroviruses. This is
392 not surprising considering the relatively high prevalence of all the individual viruses. In
393 comparison, the level of co-infections in the *P. r. tephrosceles* population was substantially
394 lower (3% of the monkeys had triple infections, and 23% had dual infections which all
395 included SFV), as would be expected with the relatively lower prevalence of both SIV and
396 STLV-1 (15). Interestingly, in the Tai *P. b. badius* population no individual included in the co-
397 infection analysis was positive for STLV-1 alone or in combination with SFV; all the STLV-1-
398 positive individuals were at the same time infected with SIV. It is possible that STLV-1 is
399 frequently transmitted together with SIV, however there was no significant correlation
400 among any of the viruses in the Tai *P. b. badius* population. Also in the Kibale *P. r.*
401 *tephrosceles* population, no correlation was found among these viruses (15).

402 **Phylogeny.** With data accumulating, the mechanisms of retrovirus evolution have taken
403 more precise and distinctive shapes. SFV and SIV mostly show species-specific distribution,
404 either as a result of host-parasite co-speciation or preferential host-switching (5, 53, 61). In
405 contrast to SFV and SIV, the distribution of the genetic diversity of STLV-1 seems to be more

406 complex, which most likely reflects more frequent cross-species transmission of this virus
407 between different primate species than for Lenti and Spumaretroviruses (21).

408 Also in our study, SIV and SFV phylogenies both exhibited the pattern of host-specific
409 association of these retroviruses. This was especially true for SIVwrc and SFVwrc sequences
410 determined from *P. b. badius*. In the SIV tree, SIVwrc sequences from *P. b. badius* are
411 clustered together with those determined from habituated *P. b. badius* in the same area and
412 in *P. b. temminckii* in Gambia (34, 35). Of note, these red colobus populations are both called
413 Western red colobus, but are believed to belong to different sub-species (54). In the
414 phylogeny of mitochondrial DNA, *P. b. temminckii* appears as being nested into the genetic
415 diversity of *P. b. badius*. SIVwrc therefore conforms to the same pattern. In the SFV tree,
416 SFVwrc strains clusters into one clade but are this time associated with one strain previously
417 identified as coming from a chimpanzee, most probably as the result of cross-species
418 transmission event linked to chimpanzee hunting behaviour (28). For both SIV and SFV,
419 strains identified from *P. r. tephrosceles* in Kibale grouped with strains of *P. b. badius* in Tai
420 but were never interspersed with them, suggesting reciprocal monophyly. As this conforms
421 to the host phylogeny (54), this might be explained by host-parasite co-speciation for
422 SFVwrc; a common process for retroviruses of this genus (53). For SIVwrc, preferential host-
423 switching (5) would seem a much more likely explanation given the presumed overall short
424 time scale of primate Lentiviruses evolution (62).

425 In contrast to SIV and SFV, the phylogeny of STLV-1 is thought to be linked to geography
426 rather than to host species (55). Given this geographical component and the slow
427 evolutionary rate of STLV-1 (29) one could expect relatively low genetic variation on small
428 geographical scales. However, we found that the novel strains of STLV-1wrc in *P. b. badius*
429 living in a relatively small area of the Tai rainforest showed high genetic variability, being

430 distributed into three distinct lineages (of which one shows some affinity to STLV-1sm
431 described from sooty mangabeys from Sierra Leone, whereas STLV-1krc from Kibale *P. r.*
432 *tephrosceles* show no affinity to any of these lineages). Thus Tai strains were found in the
433 two main lineages constituting the recently described subtype J as well as in the group
434 formed by subtype I sequences (21). This is a considerable extension of their known genetic
435 diversity as only two Tai STLV-1wrc strains have been described so far (27). Importantly, all
436 new STLV-1wrc strains were interspersed with those found from *P. t. verus* living in the area.
437 This confirms previous findings of cross-species transmission of STLV-1 from the red colobus
438 monkeys to the chimpanzees, and underlines that the major part of the diversity of STLV-1
439 strains found in those chimpanzees possibly stems from hunting and eating *P. b. badius* (27).
440 To the author's knowledge a comparable pattern of genetic diversity has so far only been
441 found in two other herbivore primate species (*C. nictitans* and *C. cephus* (31)). Under the
442 hypothesis that geographical proximity rather than host specificity is the main determinant
443 of the presence of a given strain in a given species, it is tempting to make the assumption
444 that Tai *P. b. badius* are infected with a high variety of STLV-1 strains because Tai rainforest
445 is a place of high endemicity for STLV-1. This might in turn indicate that STLV-1 has been
446 circulating in that forest zone for a longer period than in other regions, or has not been
447 submitted to similar degrees of bottleneck effect. It would therefore be interesting to
448 investigate in more detail the overall STLV-1 diversity in Tai, and also other areas of tropical
449 Africa since at the moment only a two point comparison is possible. Increasing the sampling
450 of *P. b. badius* as well as getting samples from other Tai primate species, of which some live
451 in close contact with red colobus monkeys (36, 39), could be a first step into the process.

452

453 **CONCLUSION**

454 This study shows that retroviral infections with SIV, STLV-1 and SFV are common in red
 455 colobus monkeys (*P. b. badius*) in the Taï National Park, Côte d'Ivoire. Comparing our results
 456 with those obtained from a study of a sister species (*P. r. tephrosceles*) in Uganda shows that
 457 the prevalence of these retroviruses in wild primates can vary dramatically, even between
 458 closely related species. We further demonstrate a high genetic variability of STLV-1 in this
 459 herbivore monkey species, which might be taken as an indication that Taï is a hotspot of
 460 diversity for this retrovirus.

461

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694 **Table 1. Overview of red colobus PCR results for SIV, STLV-1 and SFV.** For SIV and SFV two
 695 different primer sets were used; where results agree only one result is put in the table; wrc =
 696 tested with specific primers for according red colobus virus; gen = generic primers for virus.
 697 * No material available to test four and five individuals with generic primers for SIV and SFV,
 698 respectively. CMR = Chimpanzee meal remain.

699

700 **Figure 1. Maximum likelihood tree based on the analysis of SIV partial *pol* sequences (152**

701 **bp).** The topologies of Bayesian maximum clade credibility trees obtained under two
 702 different tree priors were similar when considering shallow evolutionary depths (deep
 703 branching patterns were not similar). Branches leading to strains isolated from red colobus
 704 monkeys in Côte d'Ivoire are blue, those leading to strains isolated from Ugandan red
 705 colobus monkeys are red, and the one branch leading to a Gambian strain is green. Major
 706 SIV groups are cartooned to improve readability: in every case the number of cartooned
 707 strains is indicated between parentheses after group names. Numbers above branches
 708 represent bootstrap values (Bp); italicized numbers below branches represent posterior
 709 probability values (pp) obtained using the birth-death model. Bp and pp are only indicated
 710 where Bp \geq 50 and pp \geq 0.95. * Strains identified in the present study. Note that this tree is
 711 mid-point rooted, due to the lack of information regarding to the position of the root in SIV
 712 phylogeny. tal: talapoin monkey (*Miopithecus talapoin*); den: Dent's monkey (*Cercopithecus*
 713 *denti*); deb: De Brazza's guenon (*Cercopithecus neglectus*); syk: Sykes' monkey (*Cercopithecus*
 714 *mitis*); mon: mona monkey (*Cercopithecus mona*); gsn: greater spot-nosed guenon
 715 (*Cercopithecus nictitans*); mus: mustached guenon (*Cercopithecus cephus*); asc: red-tailed
 716 guenon (*Cercopithecus ascanius*); col: mantled guereza (*Colobus guereza*); agm: African
 717 green monkey (*Chlorocebus aethiops*); cpz: chimpanzee (*Pan troglodytes*); rcm: red-capped

718 mangabey (*Cercocebus torquatus*); smm: sooty mangabey monkey (*Cercocebus atys*); lho:
 719 L’Hoest’s monkey (*Cercopithecus lhoesti*); sun: sun-tailed guenon (*Cercopithecus solatus*);
 720 mnd: mandrill (*Mandrillus sphinx*); drl: drill (*Mandrillus leucophaeus*); olc: olive colobus
 721 monkey (*Procolobus verus*); wrc: Western red colobus (*Piliocolobus badius badius*); krc:
 722 Eastern red colobus (*Piliocolobus rufomitratu s tephrosceles*); wrcPBT: Western Red colobus
 723 (*Piliocolobus badius temminickii*).

724

725 **Figure 2. Maximum likelihood tree based on the analysis of PTLV-1 partial LTR sequences**

726 **(422 bp)**. The topologies of Bayesian maximum clade credibility trees obtained under two
 727 different tree priors were similar when considering shallow evolutionary depths (deep
 728 branching patterns were not similar). Branches leading to strains isolated from red colobus
 729 monkeys in Côte d’Ivoire are blue, those leading to strains isolated from Ugandan red
 730 colobus monkeys are red. Major PTLV-1 groups are cartooned to improve readability: in
 731 every case the number of cartooned strains is indicated between parentheses after group
 732 names. Numbers above branches represent bootstrap values (Bp), italicized numbers below
 733 branches represent posterior probability values (pp) obtained using the birth-death model.
 734 Bp and pp are only indicated where Bp≥50 and pp≥0.95. * Strains identified in the present
 735 study. Three of these strains were actually identified in more than one individual: **wrc15**
 736 (published as STLVwrc (27))= wrc129*= wrc212* (plus ptr-Dorry); **wrc66***=wrc72*;
 737 **wrc126***=wrc211*. sm: sooty mangabey monkey (*Cercocebus atys*); ptr: chimpanzee (*Pan*
 738 *troglodytes*); mnd: mandrill (*Mandrillus sphinx*); cae: African green monkey (*Chlorocebus*
 739 *aethiops*); msy: Barbary macaque (*Macaca sylvanus*); wrc: Western red colobus (*Piliocolobus*
 740 *badius badius*); krc: Eastern red colobus (*Piliocolobus rufomitratu s tephrosceles*).

741

742 **Figure 3. Maximum likelihood tree based on the analysis of SFV partial *pol* sequences (252**
 743 **bp).** The topologies of Bayesian maximum clade credibility trees obtained under two
 744 different tree priors were similar. Branches leading to strains isolated from red colobus
 745 monkeys in Côte d'Ivoire are blue, those leading to strains isolated from Ugandan red
 746 colobus monkeys are red. Non-colobine tips are cartooned to improve readability: in every
 747 case the number of cartooned strains is indicated between parentheses after family, sub-
 748 family or genus names. Numbers above branches represent bootstrap values (Bp), italicized
 749 numbers below branches represent posterior probability values (pp) obtained using the
 750 birth-death model. Bp and pp are only indicated where $Bp \geq 50$ and $pp \geq 0.95$. * Strains
 751 identified in the present study. Two of these strains were actually identified in more than
 752 one individual: **wrc125=wrc3*=wrc12=wrc45*=wrc68*=wrc71*=wrc126=wrc127**
 753 **=wrc128=wrc129=wrc130=wrc131=wrc213*=wrc236*=wrc276*** (15 wrc sequences plus ptr-
 754 Leo); **wrc133= wrc132***. ptr: chimpanzee (*Pan troglodytes*); krc: Eastern red colobus
 755 (*Piliocolobus rufomitratu tephrosceles*); wrc: Western red colobus (*Piliocolobus badius*
 756 *badius*).
 757

Red colobus ID	Origin of samples	Samples tested	SIV (wrc/gen)	STLV	SFV (wrc/gen)
124	Darted	Buffy coat	+	+	+
125	Darted	Buffy coat	+	+	+
126	Darted	Buffy coat	+	+	+
127	Darted	Buffy coat	+	-	+
128	Darted	Buffy coat	+	+	+
129	Darted	Buffy coat	+	+	+
130	Darted	Buffy coat	+	+	+
131	Darted	Buffy coat	-	-	+
132	Darted	Buffy coat	+	-	-/+
133	Darted	Buffy coat	-	-	+
3	Necropsy	Spleen, lymph node	+	-	-/+
12	Necropsy	Spleen, lymph node	+	+	+
23	Necropsy	Lung, heart	-	-	-
28	Necropsy	Liver	+	-	+/-
43	Necropsy	Spleen, lung, muscle	+	+	-
66	Necropsy	Lung, unidentified tissue	+	+	+
71	Necropsy	Lung	-	-	+
72	Necropsy	Liver, spleen	+	+	+
213	Necropsy	Spleen, kidney, muscle	+	-	+
236	Necropsy	Lymph node	+	-	+
268	Necropsy	Lymph node, intestine	-/+	-	-
276	Necropsy	Liver, lung	+	+	+
15	CMR	Muscle, bone marrow	+/-	+	-
29	CMR	Blood	-	+	-
30	CMR	Blood in RNA later	-	+	-
45	CMR	Blood	-	-	+/-
68	CMR	Trachea, lymph node	-	+	+
204	CMR	Blood	+ /no material	-	- /no material
210	CMR	Muscle, blood	-	+	+
211	CMR	Muscle	-	+	-
212	CMR	Muscle	+/-	+	-
269	CMR	Muscle, brain	-/+	-	-
278	CMR	Muscle, bone marrow	-	+	+ /no material
279	CMR	Muscle, bone marrow	-	-	+
280	CMR	Muscle	+/-	+	+ /no material
Remaining 19 red colobus monkeys	CMR	Muscle x7, blood x7, other tissue x7	- *	-	- *





