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Coinfection of Ugandan Red Colobus (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles*) with Novel, Divergent Delta-, Lenti-, and Spumaretroviruses[▽]

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Nonhuman primates host a plethora of potentially zoonotic microbes, with simian retroviruses receiving heightened attention due to their roles in the origins of human immunodeficiency viruses type 1 (HIV-1) and HIV-2. However, incomplete taxonomic and geographic sampling of potential hosts, especially the African colobines, has left the full range of primate retrovirus diversity unexplored. Blood samples collected from 31 wild-living red colobus monkeys (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles*) from Kibale National Park, Uganda, were tested for antibodies to simian immunodeficiency virus (SIV), simian T-cell lymphotrophic virus (STLV), and simian foamy virus (SFV) and for nucleic acids of these same viruses using genus-specific PCRs. Of 31 red colobus tested, 22.6% were seroreactive to SIV, 6.4% were seroreactive to STLV, and 97% were seroreactive to SFV. Phylogenetic analyses of SIV polymerase (*pol*), STLV *tax* and long terminal repeat (LTR), and SFV *pol* and LTR sequences revealed unique SIV and SFV strains and a novel STLV lineage, each divergent from corresponding retroviral lineages previously described in Western red colobus (*Procolobus badius badius*) or black-and-white colobus (*Colobus guereza*). Phylogenetic analyses of host mitochondrial DNA sequences revealed that red colobus populations in East and West Africa diverged from one another approximately 4.25 million years ago. These results indicate that geographic subdivisions within the red colobus taxonomic complex exert a strong influence on retroviral phylogeny and that studying retroviral diversity in closely related primate taxa should be particularly informative for understanding host-virus coevolution.

The discovery of lentiviruses closely related to the pandemic strain of human immunodeficiency virus type 1 (HIV-1; group M) in central African chimpanzees (*Pan troglodytes troglodytes* [16]) has created considerable interest in the natural history of primate retroviruses, as has the discovery of lentiviruses related to HIV-2 in West African sooty mangabeys (*Cercocebus atys* [25]). The diversity of naturally circulating simian retroviruses is now known to be high, with most African primates harboring simian immunodeficiency viruses (SIVs) (1), simian T-cell lymphotrophic viruses (STLVs) (36), and simian foamy viruses (SFVs) (60). Nevertheless, our knowledge of the full range of primate retrovirus diversity remains incomplete due to limited taxonomic and geographic sampling of potential hosts.

Although phylogenetic relationships of SFV lineages generally coincide with patterns of evolutionary relatedness among hosts (60), notable examples of natural cross-species transmission have been documented for each of the three retrovirus groups (3, 8, 16, 29, 33, 64, 66–68). For SIV, some lineages seem to have cospeciated with simian hosts, but this appears to be the exception rather than the rule, with frequent host switching most parsimoniously explaining congruent host and SIV evolutionary histories (7). In contrast, STLV phylogeny typically parallels geography rather than host phylogeny, demonstrating the ease with which cross-species transmission events can occur with deltaretroviruses (11, 67). Several examples of recombination among phylogenetically distinct *Lentivirus* lineages have also been described (1) and, more rarely, for SFV (37), suggesting that cross-species transmission and multiple infection have been common throughout simian retroviral evolutionary history.

Old World monkeys are divided into two major taxonomic groups: colobines and cercopithecines, corresponding to the subfamilies *Colobinae* and *Cercopithecinae*, respectively (13).

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To date, retroviruses have been characterized more thoroughly for the cercopithecine primates, which are more taxonomically diverse than the African colobines (22) and are known to harbor zoonotic retroviruses (8, 67, 68). The African colobines consist of three groups whose classification remains in debate: the black-and-white colobus, the olive colobus, and the red colobus. Groves (21) recognizes all three groups as separate genera (*Colobus*, *Procolobus*, and *Piliocolobus*, respectively). However, Grubb et al. (22) recognize only two genera (*Colobus* and *Procolobus*) and consider both the olive colobus and the red colobus as subgenera of *Procolobus* (*Procolobus* [*Procolobus*] and *Procolobus* [*Piliocolobus*], respectively). Here, we follow the Grubb et al. (22) classification because it is emerging as the consensus view.

Courgnaud et al. (10) first described a highly divergent SIV from black-and-white colobus (SIVcol; *Colobus guereza*) in Cameroon, perhaps reflecting the deep phylogenetic split between colobines and cercopithecines approximately 15 million years ago (Ma) (54). Full-length genome analyses of SIV from Temminck's red colobus (SIVpbt; *Procolobus badius temminckii*) in The Gambia (38) and olive colobus (SIVolc; *Procolobus verus*) and western red colobus (SIVwrc; *Procolobus badius badius*) from Côte d'Ivoire (35), show deep divergences even within the African colobine SIVs. However, the clustering of SIVpbt, SIVolc, and SIVwrc with SIV from L'hoest's monkeys (SIVlho; *Cercopithecus lhoesti*) in Central Africa and not SIVcol raises questions about the evolutionary history of colobine SIVs that may be resolved by analysis of SIVs from additional colobine populations (35, 38).

Our current understanding of the diversity and evolutionary history of African colobine retroviruses is biased by incomplete geographic sampling. All colobine retroviruses characterized to date come from western Africa, whereas colobine primates are distributed throughout equatorial Africa, with a number of morphologically and genetically distinct taxa existing in geographically disparate locales (62). Most notably, the poorly resolved taxonomic complex of red colobus monkeys contains a number of endemic East African forms, their distributions separated by current biogeographic barriers or reflecting ancient patterns of speciation and dispersal (22, 62). Such sets of related but phylogeographically distinct taxa may offer a unique opportunity to understand diversity in simian retroviruses from a comparative perspective.

This study describes the occurrence and phylogenetic position of retroviruses naturally circulating in a population of East African red colobus (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles*) from Uganda. We hypothesized that simian retroviruses in this eastern red colobus population would be phylogenetically divergent from previously characterized West African colobine retroviruses, reflecting deep phylogenetic splits between some eastern and western populations in the red colobus taxonomic complex (62). Our study also examines the prevalence and diversity of SIV, STLV, and SFV in this population, which offers novel insights into the natural history of multiple simian retroviruses cocirculating in a single geographically defined population of wild colobine primates.

MATERIALS AND METHODS

Study site and primate social groups. The study took place in Kibale National Park, Uganda (795 km²; 0°13′ to 0°41′N, 30°19′ to 30°32′E; Fig. 1; 5, 55). Kibale
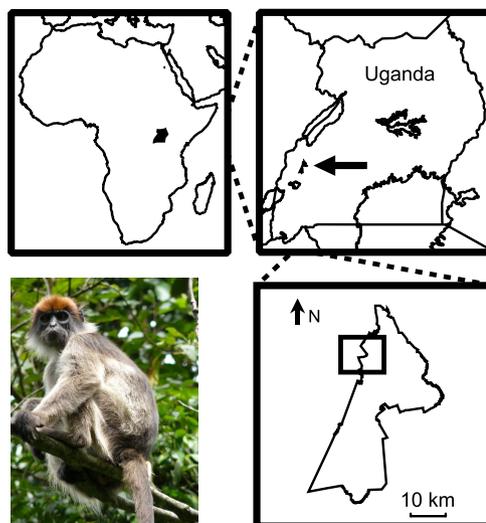

FIG. 1. Map showing (clockwise from upper left): Uganda within Africa, Kibale National Park within Uganda (arrow), the study site within Kibale National Park (box), and male Kibale red colobus (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles*).

is notable for its high species diversity and density of primates (6). At least 11 different primate species live in Kibale, including baboons (*Papio anubis*), gray-cheeked mangabeys (*Lophocebus albigena*), L'hoest's monkeys (*Cercopithecus lhoesti*), blue monkeys (*Cercopithecus mitis*), red-tailed monkeys (*Cercopithecus ascanius*), black and white colobus (*Colobus guereza*), red colobus, vervet monkeys (*Chlorocebus aethiops*), eastern chimpanzees (*Pan troglodytes schweinfurthii*), and a number of nocturnal prosimian primates. Red colobus from Kibale have been studied almost continuously since 1970 (4, 56). We focused on two red colobus social groups, LM and SC. At the time of sampling, the social groups of LM and SC consisted of 127 and 45 animals, respectively. The home ranges of the two groups overlapped extensively.

Specimen collection and preparation. Between 13 and 24 June 2006, 31 red colobus (13 males and 18 females, all adult or subadult) were sampled from the LM (*n* = 18) and SC (*n* = 13) groups (Table 1). Animals were chemically immobilized in the field by intramuscular injection of 4.56 to 9.62 mg of tiletamine-zolezepam (Telazol; Fort Dodge Animal Health, Fort Dodge, IA)/kg of body weight using plastic darts (Pneudart, Williamsport, PA) and a variable-pressure compressed air rifle (model 178 projector; Pneudart). A general physical exam was performed on each animal, and the rectal temperature and respiratory and heart rates were monitored throughout the anesthetic period. Blood samples were collected from the femoral vein into Vacutainers containing sodium-EDTA, plasma was separated in the field by centrifugation, and plasma and whole-blood samples were stored in liquid nitrogen for transport to North America. Animals were placed in a cloth bag to recover from anesthesia and, when they were assessed to be completely ambulatory, were released near easily climbable trees and vines within visual range of the social group. Animal protocols were approved by the McGill University Animal Care Committee prior to specimen and data collection.

Serological assays. Initial screening for STLV antibodies in plasma was performed by using a Vironostika HTLV-1/2 Micro-ELISA system (Organon-Teknika, Durham, NC). Seroreactive samples were then tested in a WB test (HTLV Blot 2.4; Genelabs Diagnostics, Singapore) that contains disrupted human T-cell leukemia virus type 1 (HTLV-1) virions, a gp21 recombinant protein (GD21) common to both HTLV-1 and HTLV-2, and two HTLV-type specific recombinant envelope (*env*) proteins, MTA-1 and K55, which allow differentiation of HTLV-1 and HTLV-2, respectively. Plasma samples were screened for HIV/SIV antibodies by using the Bio-Rad HIV-1/2 envelope (Env) and polymerase (Pol) peptide-based enzyme immunoassay (EIA) capable of detecting SIVcpz; HIV-1 groups M, N, and O; and other divergent SIVs (26, 34, 42, 58). EIA-reactive samples were further tested by using an HIV-2-based Genelabs Western blot (WB) test. Screening for SFV was performed with an in-house SFV EIA, followed by confirmatory WB testing of reactive samples as previously described (27). All serologic assays have previously shown good sensitivity in identifying divergent simian retroviruses (26, 27, 36, 42, 58, 63).

TABLE 1. Prevalence of simian retroviruses in red colobus monkeys from two social groups in Kibale National Park, Uganda

Animal	Sex[a]	Group	Positive (+) or negative (−) WB or PCR result[b]					
			HIV-2 WB[c]	SIV PCR (pol)[d]	HTLV-1/2 WB[e]	PTLV PCR (LTR, tax)[f]	SFV WB	SFV PCR (LTR, pol)[g]
3	M	LM	+	−	−	ND	+	−
6	M	LM	+	−	−	ND	+	−
12	M	LM	−	ND	−	ND	+	ND
13	F	LM	−	ND	−	ND	+	ND
14	M	LM	−	ND	−	ND	+	ND
21	M	LM	+	+ (78)	−	ND	+	−
29	M	LM	−	ND	−	ND	+	ND
41	F	LM	−	ND	−	ND	+	ND
44	F	LM	−	ND	−	ND	+	ND
46	M	LM	−	ND	−	ND	+	ND
49	M	LM	+	−	+	+ (95, 95)	+	−
52	F	LM	−	ND	−	ND	+	ND
65	F	LM	−	ND	−	ND	+	ND
67	F	LM	+	+ (76)	−	ND	+	+ (77, 84)
70	F	LM	−	ND	−	ND	+	+ (77, −)
72	F	LM	−	ND	−	ND	+	+ (77, 86)
996	M (J)	LM	−	ND	−	ND	+	ND
997	M (SAd)	LM	−	ND	−	ND	+	ND
9	F	SC	−	ND	−	ND	+	ND
11	F	SC	−	ND	−	ND	+	ND
27	M	SC	+	−	−	ND	+	−
30	F	SC	−	ND	−	ND	+	ND
31	F	SC	−	ND	−	ND	+	ND
33	F	SC	−	ND	−	ND	+	ND
34	F	SC	−	ND	−	ND	−	ND
40	M	SC	+	+ (74)	−	ND	+	+ (−, 85)
54	F	SC	−	ND	−	ND	+	ND
56	F	SC	−	ND	−	ND	+	ND
71	F	SC	−	ND	−	ND	+	ND
73	F	SC	−	ND	−	ND	+	−
999	M (SAd)	SC	−	ND	+	+ (95, 95)	+	+ (77, −)

[a] Animals 996 to 999 were juvenile (J) or subadult (SAd); all other animals were adult males (M) or females (F).
[b] ND, not done.
[c] Positive WB results showed weak Gag (p26) and strong Env (gp36 and gp80) seroreactivity (Fig. 2C).
[d] Numbers in parentheses indicate the percent nucleotide similarity determined by BLAST analysis to the homologous region of the polymerase (pol) gene of SIVwrc Pbt-05GM-X02 (GenBank accession no. AM937062) for animals 21 and 67 and the pol gene of SIVwrc-97IC-14 (GenBank accession no. AY138268) for animal 40.
[e] Positive results showed typical HTLV-1-like WB profiles.
[f] Numbers in parentheses indicate the percent nucleotide similarity determined by BLAST analysis to the homologous region of the LTR region of STLV-wrc (GenBank accession no. AY267837) and the tax gene of STLVmnd9-5 (GenBank accession no. Z71213), respectively.
[g] Numbers in parentheses indicate the percent nucleotide similarity determined by BLAST analysis to the homologous regions of the LTR and pol gene of SFV-3 (GenBank accession no. M74895).

PCR detection of simian retrovirus sequences. Nucleic acids were extracted from frozen whole blood by using NucliSens nucleic acid isolation kits (Bio-mérieux, Durham, NC). Briefly, 500 μl of whole blood were incubated in lysis buffer for 2 h at room temperature. Nucleic acids were eluted from silica suspension with wash buffer containing guanidine thiocyanate, and ethanol-precipitated nucleic acids were resuspended in water and stored at 4°C until tested. DNA quality and yield were determined by using a Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA) and a semiquantitative PCR amplification of the β-actin gene as previously described (68). To prevent contamination, DNA preparation and PCR testing were performed in separate laboratories specifically outfitted for processing and testing only nonhuman primate samples.

Colobus DNAs were screened for SFV, SIV, and STLV sequences by using retrovirus genus-specific nested PCR assays. SFV polymerase (pol) sequences (632 bp) were detected by using existing assays (50) or using the outer and internal primers SIF2 [5′-TAGC(A/T)GA(T/C)AA(A/G)CTTGCCACCCAAGG-3′] and SIR1 [5′-GTCGTTT(A/T)ATITCACTATTTTTCCTTTCCAC3′] and SIF3 [5′-CCAA(G/A)CCTGGATGCAGAG(T/C)TGGATCA-3′] and SIR3 [5′-ACTTTGGGG(A/G)TG(A/G)TAAGGAGTACTG-3′], respectively, and standard PCR conditions with an annealing temperature of 45°C for 40 cycles. A small fragment of the SFV long terminal repeat (LTR; 357 bp) was amplified by using the outer and internal primer pairs FVRU5F1 [5′-CACT(A/G)CTCGCTGCG(T/C)CGAGAGT-3′]/FVRU5R1 (5′-CCCGACTTATATTCGAGCCCCAC-3′) and FVRU5F2 [5′-GAG(A/T)CTCTCCAGG(C/T)TTGGTAAGA-3′]/FVRU5R2 (5′-CACGTTGGGCGCCAATTGTC-3′), respectively. SIV 5′ pol

sequences were amplified by using the first-round primers Polis4 and PolOR, followed by second-round primers UNIPOL1 and UNIPOL2, as previously described (10). An overlapping fragment (382 bp) of the 5′ pol region was obtained from animal 67 by using the SIVkrc-specific forward primer 67COLF3 (5′-TGGRCCTAATTTCAGAGCACAACAAAT) with the reverse primer PolOR. For animal 40, a 157-bp fragment of the pol region was amplified with the SIVkrc-specific pol outer primers 67COLF1 (5′-CCAGCAGAAGATGCCTACAACACA) and 67COLR1 (5′-TTTTGAGCTTTTCTGCTTGATCCC) and the internal primers 67COLF2 (5′-CAG GAT TGT TCC TGT TAA AAT TAG CCT) and 67COLR2 (5′-CCT TGA CCT TGT GGA TTA TAT GGG) using standard PCR conditions. Colobus DNAs were screened for PTLV tax sequences (222 bp) using previously described primers and conditions (63, 67). PCR amplification of overlapping regions of the 5′ and 3′ STLV-1 LTR (611 bp) was performed using primers and conditions reported previously (41, 67).

PCR products were purified with QiaQuick PCR or gel purification kits (Qiagen, Valencia, CA) and were directly sequenced on both strands by using ABI Prism BigDye terminator kits and an ABI 3130xl sequencer (Foster City, CA) or after cloning into a TOPO vector (Invitrogen).

Viral sequence analysis. Percent nucleotide divergence and similarity values were determined by using the Bestfit and GAP programs in the Genetics Computer Group (GCG) Wisconsin package and/or by BLAST analysis (69). Sequences were aligned by using the CLUSTAL W program (61), followed by manual editing and removal of indels. Nucleotide substitution saturation was assessed by using pairwise transition and transversion versus divergence plots

using the DAMBE program (70). Unequal nucleotide composition was measured by using the TREE-PUZZLE program (49). Genetic recombination was evaluated using the Recombination Detection Program v3.0 (RDP3) (40). Nucleotide substitution models and parameters were estimated from the edited CLUSTAL W sequence alignments by using Modeltest v3.7 (44). For the SFV and SIV alignments, a variant of the general time reversible (GTR) model, allowing six different substitution rate categories (SFV: $r_{A \leftrightarrow C}$ = 4.82, $r_{A \leftrightarrow G}$ = 11.86, $r_{A \leftrightarrow T}$ = 3.42, $r_{C \leftrightarrow G}$ = 2.58, $r_{C \leftrightarrow T}$ = 11.86, and $r_{G \leftrightarrow T}$ = 1; SIV: $r_{A \leftrightarrow C}$ = 4.7, $r_{A \leftrightarrow G}$ = 11.85, $r_{A \leftrightarrow T}$ = 3.64, $r_{C \leftrightarrow G}$ = 2.54, $r_{C \leftrightarrow T}$ = 11.85, and $r_{G \leftrightarrow T}$ = 1.0), with gamma-distributed rate heterogeneity (SFV, α = 0.914; SIV, α = 0.896) and an estimated proportion of invariable sites (SFV = 0.294, SIV = 0.284), was determined to fit the data best. The best model for the PTLV LTR data was GTR, with six different rate substitutions ($r_{A \leftrightarrow C}$ = 2.32, $r_{A \leftrightarrow G}$ = 24.0, $r_{A \leftrightarrow T}$ = 1.56, $r_{C \leftrightarrow G}$ = 0.95, $r_{C \leftrightarrow T}$ = 13.2, and $r_{G \leftrightarrow T}$ = 1) and gamma-distributed rate heterogeneity (α = 0.437).

Phylogenetic trees were inferred by using Bayesian analysis implemented in the BEAST software package (14). Two independent BEAST runs consisting of 10 million Markov Chain Monte Carlo (MCMC) generations with sampling every 1,000 generation, an uncorrelated log normal relaxed molecular clock, and either the constant coalescent or the Yule process of speciation as tree priors, were used to infer the viral tree topologies. Convergence of the MCMC was assessed by calculating the effective sampling size of the runs using the Tracer program (A. Rambaut and A. J. Drummond, Tracer v1.4 [http://beast.bio.ed.ac.uk/tracer]). The tree with the maximum product of the posterior clade probabilities (maximum clade credibility tree) was chosen from the posterior distribution of 9,001 sampled trees (after burning in the first 1,000 sampled trees) with the program TreeAnnotator version 1.4.6 included in the BEAST software package (14). Trees were viewed and edited by using FigTree v1.1.2 (A. Rambaut, FigTree [http://beast.bio.ed.ac.uk/FigTree]).

Phylogeny of host taxa. Ting (62) recently performed a comprehensive study of African colobine molecular phylogeny and divergence dates using a "penalized likelihood" method, which combines a likelihood model with a penalty function (47), to infer the latest possible date by when African colobine mitochondrial lineages diverged, given unequal evolutionary rates. We reanalyzed a taxonomic subset of the Ting data set (62) in order to infer best estimates for the timing of divergence events between red colobus populations where SIV has been detected, including viruses in the present study, to use more recently developed Bayesian methods to test the accuracy of the previously inferred dates, and to provide a range of dates under which cospeciation of colobine and viral lineages might have occurred.

Our initial alignment contained 18 3,831-bp concatenations of primate mitochondrial DNA (mtDNA) that include the NADH3, NADH4L, NADH4, and NADH5 genes, from samples available in locations where African colobines are known to harbor SIV. However, transitions and transversions plateaued at the third codon position, indicating sequence saturation typical of mtDNA protein coding genes (data not shown). In contrast, transitions and transversions increased linearly for the first and second codon positions without reaching a plateau, indicating they still retained phylogenetic signal (data not shown). The BEAST program was therefore used to infer phylogenetic relationships and divergence dates of host NADH sequences using only first and second codon positions, resulting in a final alignment of 2,548 bp.

Modeltest selected a variant of the TrN model with gamma-distributed (Γ) rate heterogeneity (α = 0.5) and an estimated proportion of invariable sites (I = 0.339) to best fit the first plus second codon alignment. Since the TrN model is not specifically implemented in the BEAST program but is nested within the GTR model family, we used the GTR + I + Γ model instead. Two independent BEAST runs of 10 million MCMC generations and sampling every 1,000th generation were performed as for the viral sequences. The relaxed molecular clock was calibrated with the three independent primate calibration dates: a human/chimpanzee split (4.2 to 8.0 Ma), an Old World monkey/hominoid split (20.6 to 30.0 Ma), and a *Theropithecus/Papio* split (4.0 to 6.0 Ma). The more recent limits of these calibration point ranges are based on the fossil record (17, 30, 31), whereas the more ancient limits were inferred from present knowledge of primate evolution (12, 46, 52, 53).

Nucleotide sequence accession numbers. The GenBank accession numbers for the new simian retrovirus sequences obtained in the study are FJ006934 to FJ006948.

RESULTS

Serologic detection of simian retroviruses. SFV antibodies were observed in all but one of the 31 (97%) red colobus plasma specimens (Table 1). As depicted in Fig. 2A, diagnostic seroreactivity to the Gag doublet proteins (p71/p68) was observed in all SFV-infected monkeys. In contrast, only two red colobus males (animals 49 and 999; 6.4%) were seroreactive to HTLV antigens (Table 1). Both samples gave WB profiles similar to that observed in control HTLV-1-infected sera, including seroreactivity to the HTLV-1-type specific Env peptide MTA-1 (Fig. 2B) and were thus classified as being STLV-1-infected. Seven monkeys (animals 3, 6, 21, 27, 40, 49, and 70; 22.6%) were reactive in both the HIV-1/2 EIA and the HIV-2 WB tests, and all seven showed strong reactivity to Env proteins (gp36 and gp80) but weak reactivity to the Gag protein (p26; Fig. 2C). These results are consistent with the detection of SIV antibodies that are cross-reactive to the genetically similar HIV-2 antigens used in the WB assays (26, 34, 42). All seven samples showed either no or only weak seroreactivity to p24 Gag in an HIV-1 WB assay (data not shown), suggesting an absence of infection with HIV-1/SIVcpz-like viruses. The majority of HIV-2-seropositive samples (*n* = 5) were from the LM group, and six of seven were males, which is consistent with transmission of SIV via aggressive behaviors such as biting (20, 43). All SIV- and STLV-1-infected monkeys were also SFV seropositive (Table 1). The plasma from one animal (i.e., animal 49) was positive for SIV, STLV-1, and SFV (Table 1).

Identification of novel, divergent simian retroviruses in Kibale red colobus. SFV *pol* sequences were detected in 3 of 11 (27%) DNA samples available from SFV WB positive colobus (Table 1). The *pol* sequences from these three red colobus monkeys (animals 40, 67, and 72) were distinct from each other, sharing 98.3 to 99.0% identity, and were equally divergent from all Old World monkey SFV sequences in GenBank, including those from *Colobus guereza* and *Procolobus badius* animals (84.0 to 86.0% identity). Phylogenetic analysis of the new SFV *pol* sequences from animals 40, 67, and 72 showed that they formed a distinct lineage with those from *Colobus guereza* and *Procolobus badius* but were closer to the *Procolobus badius* SFV with significant posterior probabilities (P = 1.0), as would be expected with cospeciation of virus and host (Fig. 3A). SFV LTR sequences were found in five of the same 11 (45%) colobus DNA samples, giving a total of six animals that were positive for SFV sequences (54.5%, Table 1). Concordant positive *pol* and LTR PCR results were observed in 2 of the 11 (18%) colobus DNA samples (Table 1). As with the *pol* sequences, phylogenetic analysis of LTR sequences from animals 6, 67, 70, 72, and 999 revealed a colobus-specific lineage, with SFVcgu from *Colobus guereza* as a sister taxon (results not shown). SFV LTR sequences from *Procolobus badius* have not previously been reported.

PTLV *tax* and LTR sequences were detected in DNA samples from both HTLV-1 WB-positive red colobus monkeys. Nearly identical *tax* and LTR sequences (>99%) were found in each STLV-1-infected red colobus, despite being from separate social groups. By BLAST analysis, the *tax* sequences showed similarity to both HTLV-1 and STLV-1 from Africa (ca. 95 to 97%), including STLV-1wrc from *Procolobus* (*Piliocolobus*) *badius* (GenBank accession no. AY267837). Similarly, LTR sequences from these animals were similar to both HTLV-1 and STLV-1 from Africa (~95%), including STLV-1wrc found in *Procolobus* (*Piliocolobus*) *badius* (AY267837) from Cote d'Ivoire (32). Phylogenetic analysis revealed a divergent STLV-1 lineage containing both new colobus LTR

A.

B.

C.
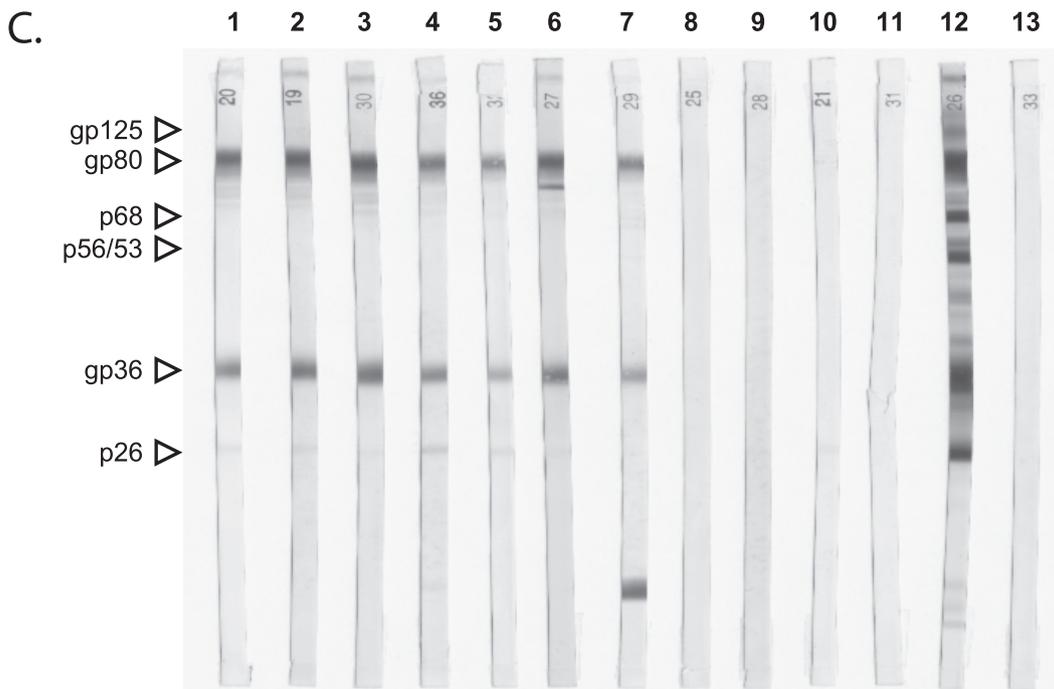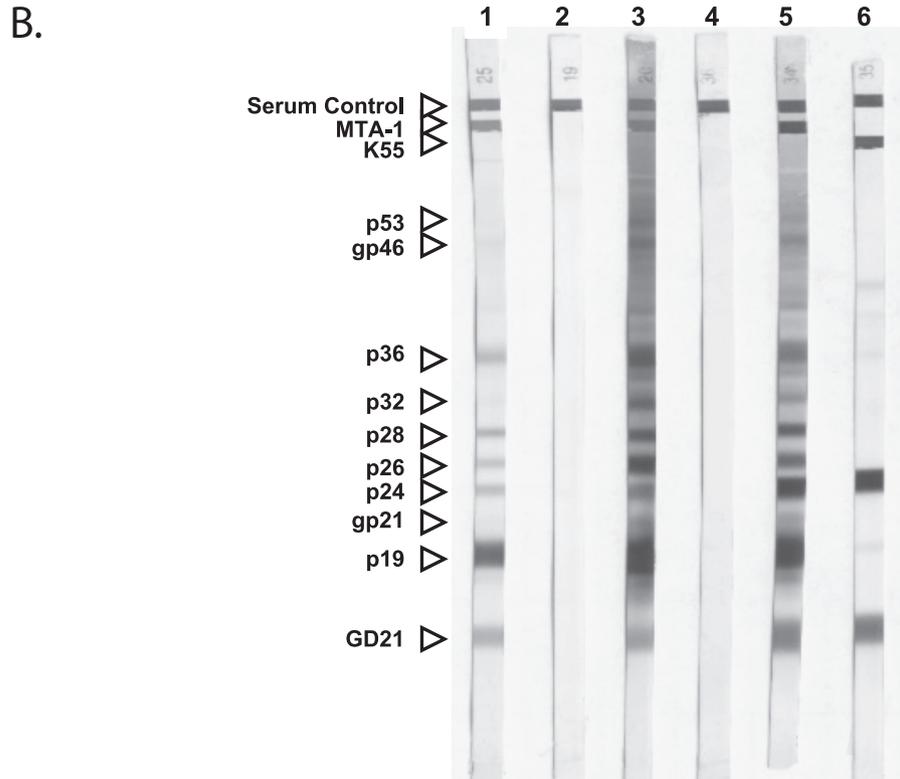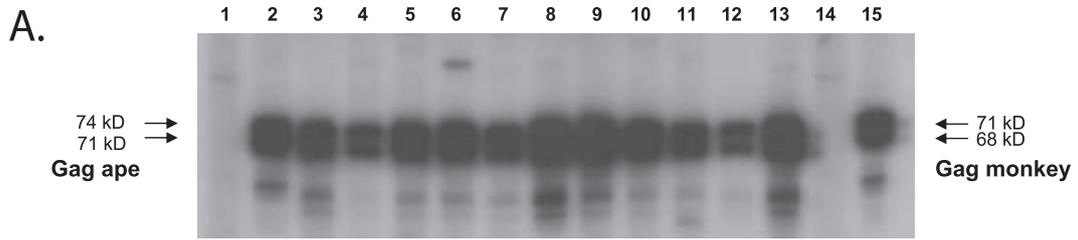
sequences distinct from STLV-1wrc but without significant posterior probabilities ($P < 0.7$; Fig. 3B). Nonetheless, similar levels of phylogenetic support were also obtained with LTR sequences from all other major PTLV-1 clades (Fig. 3B), a pattern which is typically seen in studies of PTLV-1 genotypes (36, 41, 67).

SIV sequences were identified in one (animal 67) of seven HIV-2 WB-positive red colobus monkeys (14.3%) using generic PCR primers. BLAST analysis demonstrated this 337-bp SIV *pol* sequence to be unique, showing the closest similarity (77%) to SIV from a western red colobus (SIVwrc, *Procolobus badius*, GenBank accession no. AY138266). For consistency with established SIV nomenclature and to distinguish our new virus from that found in western red colobus (SIVwrc), we tentatively designated this strain SIVkrc for SIV from a Kibale red colobus. Overlapping SIV sequences were then obtained from DNA from colobus 67 by using a combination of generic and SIVkrc-specific PCR primers to generate a 465-bp *pol* fragment. Using SIVkrc-specific primers, we then obtained a 348-bp *pol* sequence from colobus 21 that shared 97% nucleotide similarity to SIVkrc (animal 67) but was equidistant from SIV from *Colobus guereza* (SIVcol) and SIVwrc, sharing only ca. 64% identity. We were also able to amplify a shorter *pol* sequence (106 bp) from animal 40 by using additional SIVkrc-specific primers, and this sequence shared 91% nucleotide similarity to SIVkrc (animal 67) but only 74% similarity to SIVwrc (GenBank accession no. AY138268).

Phylogenetic analysis of *pol* sequences yielded a starlike tree topology (Fig. 3C), similar to previously published SIV phylogenies with the formation of the seven "classical" SIV lineages in this region composed of SIVcpz/SIVrcm/SIVdrl/mnd2 from chimpanzees (cpz), red-capped mangabeys (rcm), drill (drl), and mandrill (mnd2; type 2 genotype); SIVsmm from sooty mangabey monkeys (smm), SIVagm/tal from African green monkeys (agm) and talapoins (tal); SIVmon/gsn/mus/asc from mona (mon), greated spot-nosed (gsn), mustached (mus), and red-tailed (asc; *Colobus ascanius*) monkeys; SIVsyk/deb/den from Syke's (syk), De Brazza's (deb), and Dent's mona (den) monkeys; SIVcol from *Colobus guereza* (col); and SIVlho/sun/olc/wrc/mnd1 from L'hoest's (lho) and sun-tailed (sun) monkeys, olive colobus (olc), Western red colobus (wrc), and mandrill (mnd1; type 1 genotype) (1, 9, 10, 16, 24, 37, 66). The SIVkrc sequences clustered within the SIVlho/sun/olc/wrc/mnd1 lineage with SIVs from olive and Western red colobines (Fig. 3C). Within the colobus sublineage in this cluster, SIVs from each *Procolobus* (*Piliocolobus*) species formed distinct lineages (Fig. 3C). SIVwrcPbt from *Procolobus badius temminckii* found in The Gambia (GenBank accession no. AM937062) clustered with SIV sequences from western red

colobus monkeys (*Procolobus badius badius*) (Fig. 3C) mirroring the genetic relationships observed in the host phylogeny (Fig. 4). Like the SFV found in Kibale and western red colobus, SIVkrc appears to have codiverged from the distinct but phylogenetically related SIVwrc and SIVolc. However, except for SIVwrc and SIVkrc, the branching order of SIVolc in this clade was only weakly supported ($P = 0.75$), making it difficult to ascertain with confidence the evolutionary relationships of these red colobus SIVs within this clade using these short *pol* sequences (Fig. 3C). In contrast, SIVcol from *Colobus guereza*, formed a separate lineage distinct from all other SIVs as expected (Fig. 3C). Inclusion of *pol* sequences from other SIV-infected western red colobus from Cote d'Ivoire (SIVwrc; GenBank accession numbers AM743109 to AM743117) in the analysis shortened considerably the final alignment length, since these sequences are located at the 3′ end of the new SIVkrc *pol* sequences with only a small overlap of ~120 nucleotides (38, 39). Nonetheless, all of these recently reported SIVwrc sequences clustered with other SIVwrc sequences and not with SIVkrc or SIVolc in distance-based trees (data not shown). Although we did not detect evidence of recombination within the short *pol* sequences used for the alignment, SIVsab from *Chlorocebus sabeus* is a known recombinant in the 5′ end of *pol* and clustered with SIVsmm instead of other SIVagm sequences, as previously reported (28).

All viral sequences were found to be devoid of substitution saturation determined by pairwise transition and transversion versus genetic divergence plots using the DAMBE program (data not shown). In addition, genetic recombination was not inferred in each alignment using four different detection methods (RDP, GENECONV, Bootscan, and 3seq) in the RDP3 software package. Each sequence in each viral alignment also demonstrated equal base composition. In combination, these data suggested the presence of good phylogenetic signal, indicating that the aligned sequences were suitable for phylogenetic inference using Bayesian methods. Identical topologies were inferred using both constant coalescent and Yule process tree priors (only trees using the Yule process of speciation are shown).

Phylogeny and estimation of divergence dates of host taxa. The African colobine sequences formed three major phylogenetic lineages consisting of black-and-white, olive, and red colobus sequences with the olive and red colobus being sister taxa but with low posterior probability ($P = 0.75$; Fig. 4). Inferred mtDNA divergence dates suggest that the black-and-white colobus split from the other colobus 10.21 Ma (7.10 to 13.31 Ma), and the olive colobus and red colobus diverged from one another 8.79 Ma (6.35 to 12.08 Ma). Also, the Kibale red colobus (*Procolobus rufomitratus tephrosceles*) and the

FIG. 2. Serologic detection of antibodies to simian retroviruses in Kibale red colobus (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles*). (A) SFV WB analysis. Lanes 2 and 15 are positive control sera from an SFV-infected chimpanzee and African green monkey, respectively; lanes 1 and 14 are negative control sera from an uninfected human; lanes 3 to 13 show the representative reactivities in plasma samples from red colobus monkeys (animals 49, 9, 6, 3, 999, 996, 997, 73, 72, 30, and 29, respectively); The predicted Gag protein sizes for the monkey and ape SFVs are indicated. (B) HTLV WB analysis. Lanes 1 through 3 are from seroreactive and nonreactive plasma samples from red colobus monkeys (animals 49, 72, and 999, respectively); lane 4 is the nonreactive control; lanes 5 and 6 are the HTLV-1- and HTLV-2-reactive positive control antisera, respectively. Reactivity to HTLV-specific proteins is indicated on the left. (C) HIV-2 WB analysis. Lanes 1 to 7 and lanes 8 to 11 show plasma from seroreactive (animals 3, 6, 21, 27, 4, 49, and 67) and nonreactive (animals 11, 31, 54, and 73) red colobus monkeys, respectively; lanes 12 and 13 are assay-positive and -negative control antisera, respectively. Reactivity to HIV-2-specific proteins is indicated on the left.

FIG. 3. Phylogenetic relationships of simian retrovirus sequences from Kibale red colobus (KRC) monkeys using Bayesian inference. Sequences generated in the current study are boxed. 10,000 trees were sampled by an MCMC method under a relaxed clock model, and the maximum clade credibility tree, i.e., the tree with the maximum product of the posterior clade probabilities, was chosen. Scale bars indicate substitutions per site; branch tips are aligned because of the relaxed clock model. Posterior probabilities of >0.70 are provided on each major node. (A) SFV
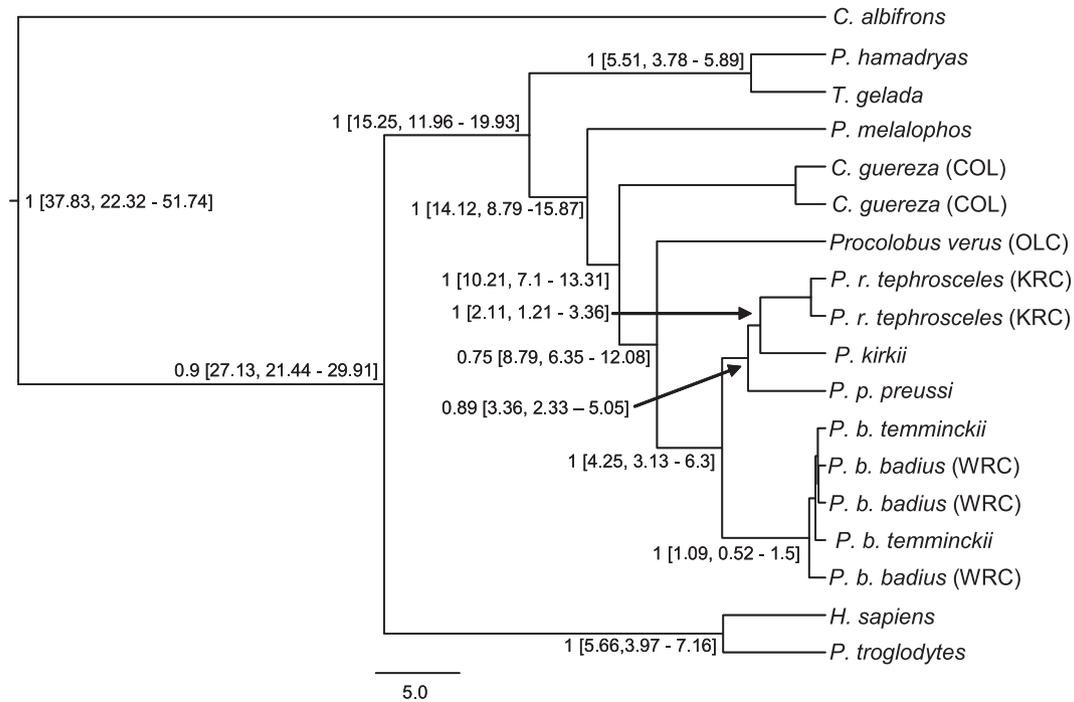
FIG. 4. Phylogenetic relationships of African colobines using first and second codon positions of NADH3, NADH4L, NADH4, and NADH5 mitochondrial genes (2,548 bp) and Bayesian inference. 10,000 trees were sampled with a MCMC method under a relaxed clock model, and the maximum clade credibility tree was chosen. Branch lengths are proportional to median divergence times in years estimated from the post-burn in trees with the scale at the bottom indicating 5 Ma. Posterior probabilities and median divergence times in Ma and 95% high posterior density intervals (inside brackets) are provided on each major node. Codes used for colobines with SIV infection are shown in parentheses. GenBank accession numbers for sequences included in the analysis are NC002763 (*Cebus albifrons*), NC001807 (*Homo sapiens*), NC001807 (*Pan troglodytes*), NC001992 (*Papio hamadryas*), EU580083 (*Theropithecus gelada*), DQ355299 (*Presbytis melalophos*), EU580051 (*Colobus guereza matschiei* from Kakamega Forest, Kenya), EU580052 (*Colobus guereza occidentalis* from Cameroon), EU580082 (*Procolobus* [*Procolobus*] *verus* from Tai National Park, Côte d'Ivoire), DQ355301 (*Procolobus* [*Piliocolobus*] *badius badius* from Sierra Leone), EU580057 and EU580058 (*Procolobus* [*Piliocolobus*] *badius badius* from Tai National Park, Côte d'Ivoire), EU580062 (*Procolobus* [*Piliocolobus*] *kirkii* from Zanzibar Island, Tanzania), EU580073 (*Procolobus* [*Piliocolobus*] *pennantii preussi* from South Korup National Park, Cameroon), EU580078 (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles* from Kibale National Park, Uganda), EU580079 (*Procolobus* [*Piliocolobus*] *rufomitratus tephrosceles* from Gombe National Park, Tanzania), EU580076 (*Procolobus* [*Piliocolobus*] *temminckii* from Abuko Nature Reserve, The Gambia), and EU580077 (*Procolobus* [*Piliocolobus*] *temminckii* from Njassang Forest Park, The Gambia).

western red colobus (*Procolobus badius badius*) populations diverged from one another at the base of the red colobus radiation 4.25 Ma (3.13 to 6.30 Ma). Nearly identical divergence dates were obtained when we excluded the New World monkey *Cebus albifrons* mtDNA sequence from the first and second cdp alignment. These dates are slightly older than those found in another study (62), with the more recent limits of our 95% high posterior density intervals strongly coinciding with

the previously inferred dates. This result was not surprising because the previous study aimed to determine the most recent dates of divergence. This difference may also have been due to the use in the present study of the relaxed molecular clock model in BEAST but the penalized likelihood model in r8s in the previous study, as well as by the use of only first and second codon positions in the BEAST analyses in the present study and the preference for overparameterization when using non-

phylogeny of 407-bp polymerase (*pol*) sequences. Primate species are indicated to the right of the tree. WRC, western red colobus (*Procolobus* [*Piliocolobus*] *badius badius*); col, mantled guereza (*Colobus guereza*). (B) Inferred PTLV-1 LTR phylogeny (511-bp). Corresponding PTLV subtypes or primate hosts are shown to the right of the tree. WRC, western red colobus (*Procolobus* [*Piliocolobus*] *badius badius*). (C) SIV *pol* phylogeny (271 bp). OLC, olive colobus (*Procolobus* [*Procolobus*] *verus*); WRC, western red colobus (*Procolobus* [*Piliocolobus*] *badius badius*); col, mantled guereza (*Colobus guereza*); mnd1 and mnd2, mandrill (*Mandrillus sphinx*); sun, sun-tailed guenon (*Cercopithecus solatus*); lho, L'Hoest's monkey; smm, sooty mangabey monkey (*Cercocebus atys*); tan, tantalus guenon (*Chlorocebus tantalus*); agm, African green monkey (*Chlorocebus aethiops*); sab, sabaeus guenon (*Chlorocebus sabaeus*); drl, drill (*Mandrillus leucophaeus*); rcm, red-capped mangabey (*Cercocebus torquatus*); cpz, chimpanzee (*Pan troglodytes*); gsn, greater spot-nosed guenon (*Cercopithecus nictitans*); asc, red-tailed guenon (*Cercopithecus ascanius*); mus, mustached guenon (*Cercopithecus cephus*); mon, mona monkey (*Cercopithecus mona*); syk, Syke's (or blue) monkey (*Cercopithecus mitis*); deb, De Brazza's guenon (*Cercopithecus neglectus*); den, Dent's monkey (*Cercopithecus denti*); tal, talapoin monkey (*Miopithecus talapoin*). An asterisk indicates the position of SIV from Temminck's red colobus (*Procolobus* [*Piliocolobus*] *badius temminckii*). Sequences of SIVkrc from animals 21 and 40) were excluded from this analysis because they considerably shortened the alignment but clustered with SIVkrc from animal 67 with highly significant bootstrap support (99%) in distance-based trees inferred from the shorter *pol* alignment (data not shown).

parametric rate smoothing in the r8s method (47). In addition, the mean substitution rate was slightly lower for the first and second codon positions (5.8×10^{-9}) versus that of the all three codon positions combined (1.47×10^{-8}), which would also increase inferred divergence times (15). The dates inferred from the two studies are therefore congruent, and they both show that the red colobus radiation, and the living colobus monkey radiation as a whole, contains several deeply divergent lineages. This includes a separation of the Kibale red colobus and western red colobus populations by ~4.25 Ma. Divergence dates and phylogenetic relationships for the non-African colobines, African noncolobines, and platyrrhines were congruent with those from past mitochondrial genomic studies (46, 54).

DISCUSSION

The presence in Kibale red colobus of simian retroviruses representing distinct and divergent strains in the *Spumavirus* (SFV) and *Lentivirus* genera (SIV), and one novel *Deltavirus* lineage (STLV) expands our understanding of the diversity and evolutionary history of naturally occurring primate retroviruses. All of the novel retroviral lineages identified in our study were distinct from the corresponding retroviral lineage from western red colobus and, for SFV and SIV, from simian retroviruses in *C. guereza* also. These observations suggest that primate retroviral diversity is extensive even among related colobine taxa.

The degree to which the various primate retroviruses have coevolved with their hosts has remained a subject of considerable debate. Although cross-species transmission of primate retroviruses has been extensively documented, geographic and ecological overlap is prerequisite for this to occur. Preferential host switching among closely related primate taxa may, in fact, explain concordances between host and viral phylogenies in the case of SIV (7). The degree to which host-virus coevolution versus preferential host switching has led to the current pattern of primate retroviral phylogeographic diversity remains unclear and probably differs for different retroviral lineages. Thus far, compelling evidence for cospeciation of simian retroviruses and their primate hosts has been shown only for SFV (37, 60).

Our results from Kibale red colobus shed light on the propensity for some primate retroviruses to cross phylogeographic boundaries. Red colobus are a diverse set of taxa, with multiple forms distributed in overlapping and disjunct ranges throughout equatorial Africa (62). Evidence to date points to deep divergences between some eastern and western populations, estimated by our own analyses to be ~4.25 million years old. The deep phylogenetic splits between STLV from Kibale red colobus and western red colobus suggest that, in the red colobus taxonomic complex, host phylogeographic subdivisions may exert a primary influence on the evolution of these red colobus retroviruses. Notably, STLV-1 from Kibale red colobus did not cluster with STLV-1 identified in western red colobus (STLV-1wrc) and thus, as is common in STLV evolution, appear to have diverged phylogeographically.

In contrast, the phylogeny of SIVs from Kibale and Western red colobus and olive colobus resembles that of their hosts, which is suggestive of cospeciation. However, the presence of a more divergent SIV in *Colobus guereza* demonstrates that host switching also played a role in shaping the evolutionary history of colobine SIVs. Combined, these results suggest that SIV was introduced into the *Procolobus/Piliocolobus* lineage less than 10 million years ago after divergence from *Colobus guereza*. Phylogeography may have also played an important role in the evolution of SIV in L'hoest monkeys and colobines. L'hoest's monkeys and *Procolobus* (*Piliocolobus*) *tephrosceles* are sympatric in western Uganda, and their SIVs cluster together with those from olive and other red colobus and suntailed monkeys (*Cercopithecus solatus*), a member of the L'hoest group endemic to Gabon. Similar phylogenetic relationships have been observed using complete SIV genomes available from these same monkeys (35). One hypothesis to explain these results is the infection of either L'hoest's or Kibale red colobus monkeys with a common ancestral SIV which subsequently switched hosts and infected other colobines and/or L'Hoest's monkey subspecies as these primates diverged and dispersed across Africa.

We note that chimpanzees sympatric with western red colobus can become infected with SFV and STLV from these monkeys, probably as a result of exposure to infectious tissues during predation (33), and that similar cross-species transmission events have been suggested for SIV (18, 32). Although Santiago et al. (48) did not detect SIV in a subset of Kibale chimpanzees, the tools used were specific for SIVcpz/HIV-1 and may have had low sensitivity for detecting divergent colobine SIVs. Thus, it remains to be determined whether chimpanzees from Kibale, or other locations in Africa, are infected with colobine SIVs, as might be predicted given their predilection for preying upon red colobus (65).

We infer from the general propensity for primate retroviruses to cross species barriers that the transmission of retroviruses from Kibale red colobus to other primates, including other red colobus taxa, would probably have occurred if such transmission were ecologically possible. Divergent STLV and SIV lineages within Kibale red colobus therefore most likely reflect long-term geographic and ecological separation of this population from western red colobus. We posit that geographic and ecological separation may be more important than biological barriers to host switching in structuring SIV and STLV phylogeny. In contrast, the inferred congruent evolutionary relationships of SFV *pol* and colobine mtDNA genes strongly support cospeciation of virus and host, extending further our understanding of the interdependent evolutionary history of SFV and simian host as demonstrated previously (60). Nonetheless, analysis of SFV from additional colobine species would be needed to evaluate this cospeciation hypothesis more fully. Similar studies have been successful in demonstrating coevolution of SFV with chimpanzee subspecies (37, 57, 60).

Our results also shed light on the dynamics of coinfection of wild primate social groups with multiple retroviruses. The LM and SC groups sampled during our study overlap extensively in their home ranges and had very similar prevalences of infection with SFV, STLV, and SIV. Within the groups, however, the apparent prevalence of each virus differed substantially, with SFV detected in nearly all individuals, but STLV and SIV occurring in less than 10 and 25% of individuals, respectively. These findings may reflect differences in modes of transmission and viral expression levels in different body fluids characteristic of the three retroviruses. Horizontal transmission in all three

retroviral groups occurs mainly by aggressive behaviors between adults, including biting and possibly grooming at sites of open wounds. Unlike SIV and STLV, which can transmit from mother to offspring, there is no clear evidence of vertical transmission for SFV (37). The higher prevalence of SFV than SIV or STLV seen in the wild colobines is similar to that seen in captive primates, demonstrating the ease with which SFV is transmitted in both settings. Additional studies are needed to examine whether these differences may be due to higher viral loads in the oral cavity of SFV-infected simians compared to SIV- and STLV-infected animals.

Although our small sample sizes precluded meaningful statistical analysis, our preliminary data suggested neither a positive nor a negative effect of infection with one retrovirus on infection with another, either at the level of the group or the individual. This trend, should it be substantiated, would suggest that the transmission dynamics of each virus can likely be understood relatively independently of the others, which may in turn reflect differences in the primary mode of transmission and levels of virus expression for each virus. Our discovery of seven individuals dually positive for SFV and SIV, two individuals positive for both SFV and STLV, and one individual positive for all three viruses further strengthens this hypothesis.

We also caution that our results are based on partial genome sequencing, which can yield inaccurate results both because of the limited information contained in short viral genomic regions (45) and because of the propensity for primate lentiviruses to recombine (37, 51). Nonetheless, genetic recombination has not been observed in primate T-lymphotropic viruses (59) and only rarely in SFV (37), and recombination was not detected in the alignments used for our analyses. Thus, recombination and the partial genomic sequences used in the present study should have had minimal, if any, effect on the inferred phylogeny of the simian retroviruses in our analyses. Likewise, the inferred SIV topologies are in agreement with results obtained by others by analysis of complete genomes (35). We also note that we recovered viral nucleic acid sequences from only a proportion of SFV- and SIV-seropositive animals and that we were therefore unable to examine the relative diversity of each virus within and among social groups. It is clear, however, that Kibale red colobus harbor far less within-population viral genetic diversity than exists between Kibale red colobus and western red colobus (39).

Red colobus are a diverse set of taxa about which molecular phylogenetic information is only now becoming available (62). Certain populations of red colobus inhabit mainland forests and are members of diverse and interconnected primate communities, while others represent remnant populations that are relatively isolated, such as *Procolobus* [*Piliocolobus*] *kirkii*, which is endemic to Zanzibar Island (56). Considering their taxonomic and geographic diversity and their importance as chimpanzee prey in certain locations, red colobus may be a favorable model system for understanding how primate retroviral phylogeny has been shaped by recent interactions among sympatric primate taxa versus ancient divergences among allopatric taxa. We also note that colobus phylogeny itself has been difficult to resolve (46, 54), even with extensive molecular sequencing as demonstrated herein. Given the ancient cospeciation of SFV and their primate hosts as described elsewhere

(37, 60), and as demonstrated here for SFV in three African colobines, we propose that SFV phylogeny may be used as a complementary source of information to understand the evolutionary history of colobine hosts, similar to what has been suggested for feline immunodeficiency virus in cougars (2).

In conclusion, our discovery of three novel retroviruses in Kibale red colobus indicates that geographic isolation and SFV cospeciation may be a strong force shaping retroviral diversity among closely related primate taxa. The different prevalence of these viruses in the same host populations suggests that cocirculating retroviruses may have different transmission dynamics in natural populations. Studying primate taxa such as red colobus with multiple host lineages and varied ecologies should facilitate natural comparisons, shedding light on host-virus coevolution and the dynamics of primate retroviral transmission in nature. The well-documented zoonotic potential of primate retroviruses (23, 67, 68), coupled with changing patterns and levels of human-primate contact in regions like our study area (19, 20), also suggests that such interactions could have important public health implications.

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