

## Partial Molecular Characterization of Two Simian Immunodeficiency Viruses (SIV) from African Colobids: SIVwrc from Western Red Colobus (*Piliocolobus badius*) and SIVolc from Olive Colobus (*Procolobus verus*)

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**In order to study primate lentivirus evolution in the *Colobinae* subfamily, in which only one simian immunodeficiency virus (SIV) has been described to date, we screened additional species from the three different genera of African colobus monkeys for SIV infection. Blood was obtained from 13 West African colobids, and HIV cross-reactive antibodies were observed in 5 of 10 *Piliocolobus badius*, 1 of 2 *Procolobus verus*, and 0 of 1 *Colobus polykomos* specimens. Phylogenetic analyses of partial *pol* sequences revealed that the new SIVs were more closely related to each other than to the other SIVs and especially did not cluster with the previously described SIVcol from *Colobus guereza*. This study presents evidence that the three genera of African colobus monkeys are naturally infected with an SIV and indicates also that there was no coevolution between virus and hosts at the level of the *Colobinae* subfamily.**

Simian immunodeficiency viruses (SIVs) are found naturally in an extensive number of African primate species, and serological and/or molecular evidences for SIVs have been reported in at least 30 African nonhuman primates (13, 25). The sequence similarity of fully characterized viruses allows the classification of SIVs into six approximately equidistant phylogenetic lineages: (i) SIVcpz, from chimpanzees (*Pan troglodytes*) (together with human immunodeficiency virus type 1 [HIV-1]), (ii) SIVsm, from sooty mangabeys (*Cercocebus atys*) (together with HIV-2), (iii) SIVagm, from African green monkeys (members of the *Chlorocebus aethiops* superspecies), (iv) SIVsyk, from Sykes' monkeys (*Cercopithecus mitis*), (v) SIVlhoest/SIVsun, from l'Hoest (*Cercopithecus lhoesti*) and Sun-tailed monkeys (*Cercopithecus solatus*), and (vi) SIVcol, from a guereza colobus (*Colobus guereza*) (2, 5, 7, 8, 11, 14, 15, 16, 28).

Phylogenetic studies of primate lentiviruses provide several evidences that some SIV lineages have coevolved with their hosts, like SIVagm in the four African green monkey species and SIVlhoest/SIVsun within the *Cercopithecus lhoesti* superspecies (1, 2, 21). But there are also multiple examples of cross-species transmissions from simians to humans and between different simian species (4, 18, 30). HIV-1 and HIV-2 are of zoonotic origin, with their closest simian relatives in the common chimpanzee (*Pan troglodytes*) and the sooty mangabey (*Cercocebus atys*), respectively (11, 13, 16). Patas monkeys in West Africa and chacma baboons in South Africa are infected with an SIV from the local sympatric African green monkey species (4, 30). In addition, full-length genome sequencing of

SIVs from sabaeus monkeys (SIVsab) (17), red-cap mangabeys (SIVrcm) (3), mandrills (SIVmnd2) (26), and greater spotted monkeys (SIVgsn) (9) revealed a possible recombinant structure of their genomes. These observations suggest that both cross-species transmission and coinfection with highly divergent viral strains have existed since the beginning of the evolution of primate lentiviruses.

With the exception of SIVcpz, from chimpanzees, all SIVs identified to date originate from African primates belonging to the *Cercopithecoidea*, or Old World monkeys. *Cercopithecoidea* are subdivided into two distinct subfamilies, *Colobinae* and *Cercopithecinae* (10). SIVcol, isolated from *Colobus guereza*, is the only SIV obtained from a representative from the *Colobinae* subfamily and is very divergent from all known SIVs, possibly reflecting a divergence of the host lineages. Colobids separated from the other Old World monkeys at least 11 million years ago (24) and are subdivided into an African and an Asian group. The living African colobids are represented by three genera, namely, *Colobus* (or black and white colobus), *Piliocolobus* (or red colobus), and *Procolobus* (or olive colobus) (12). All contemporary species of the African colobids are restricted to the tropical and mountain forest belt of Africa.

In order to study primate lentivirus evolution in the *Colobinae* subfamily, we screened additional samples from the different genera of the African group for SIV infection.

We studied West African colobids from the Taï National Park, located in southwestern Ivory Coast near the border with Liberia; blood was obtained from the animals studied between 1997 and 2000. This park is the largest remaining area of primary forest in West Africa. Following the isolation of a new strain of Ebola virus in Taï Forest, in November 1994, the World Health Organization (WHO) conducted a collective study in the Taï National Park in order to identify the natural

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reservoir and vectors of the Ebola virus. During this project, samples were also collected from nonhuman primates using two methods. (i) Blood was obtained from live animals after they were darted by using teleinjection rifles (Telinject GUT 50) and a mixture of ketamine and médétomidine antagonized by atipamezole. Once the monkeys were fully anesthetized, two blood smears were obtained and a blood specimen was collected on a dry tube (Vacutainer). The monkeys were then marked on the tail with peroxidase, numbered (M001 for the first one caught, M002 for the second, and so on), and then resuscitated with atipamezole. (ii) The bodies from nonhuman primates that were found dead on the forest floor by sanitary surveillance patrols or by primatologists working in the Tai National Park were collected by the WHO staff and transported to a field laboratory in order to conduct a complete autopsy. Kidney, spleen, lung, liver, and lymph node samples and intestinal tissue were collected for histological examination and virological and serological studies. The sera and tissue samples were initially stored in liquid nitrogen and were later stored at  $-70^{\circ}\text{C}$ . The identification of the monkeys was done in the field and confirmed by analysis of the skulls.

From 1996 until 2001, 43 nonhuman primates were sampled in the Tai National Park, of which 22 were *Colobinae*. Blood samples were obtained from three different species, representing the three genera: Western red colobus (*Ptilocolobus badius*;  $n = 10$ ), Western black and white colobus (*Colobus polykomos*;  $n = 1$ ), and olive colobus (*Procolobus verus*;  $n = 2$ ). Sera were tested for the presence of HIV and SIV antibodies by using the INNO-LIA HIV confirmation test (Innogenetics, Ghent, Belgium) as previously described (25). This test configuration includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip. Five (50%) of the 10 western red colobus (*P. badius*) samples reacted strongly with HIV core antigens, four did so with p24, and one did so with p17; one olive colobus (*P. verus*) sample cross-reacted strongly with gp36, the HIV-2 transmembrane protein, but the Western black and white colobus (*C. polykomos*) had no HIV cross-reactive antibodies (Fig. 1).

PCR was performed to examine the samples with HIV cross-reactive antibodies for the presence of SIV sequences. DNA was isolated from whole blood or peripheral blood mononuclear cells by using a Qiagen DNA extraction kit (Qiagen, Courtaboeuf, France), and viral RNA was extracted from plasma by using a QIAamp viral RNA kit (Qiagen). We first amplified a fragment of 650 bp in the *pol* region by using DR1 and PolOR for the first round and Polis4/UNIPOL2 for the second round of amplification, with PCR conditions as previously reported (8). These highly cross-reactive *pol* primer pairs have been previously shown to amplify sequences from a wide variety of divergent HIV and SIV strains (25).

We were able to amplify a 650-bp fragment in four of the five cross-reactive *P. badius* samples as well as from the one *P. verus* sample. The *P. badius* sample for which no virus could be amplified was subjected to a single round of PCR using primers designed to amplify introns 4 and 5 of the nuclear glucose-6-phosphate dehydrogenase (G6PD) gene (1,450 bp) as previously described; this failed to yield a G6PD amplification product, suggesting DNA degradation and/or the presence of PCR inhibitors in this sample (25).

PCR products obtained with the HIV and SIV primers were

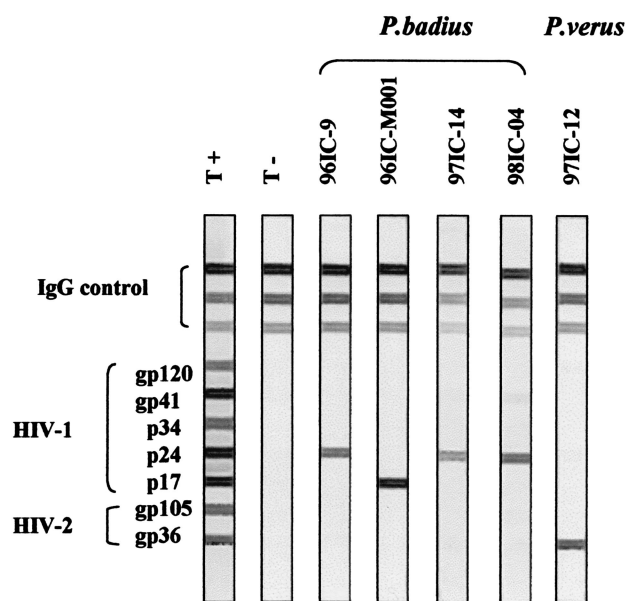


FIG. 1. Detection of HIV-1 and HIV-2 cross-reactive antibodies in sera from *Ptilocolobus badius* and *Procolobus verus* by a line immunoblot (INNO-LIA HIV Confirmation; Innogenetics, Ghent, Belgium). The five HIV-1 antigens include synthetic peptides for the exterior envelope glycoprotein (gp120) as well as recombinant proteins for the transmembrane envelope glycoprotein (gp41), integrase (p31), core (p24), and matrix (p17) proteins. The HIV-2 antigens include synthetic peptides for the exterior envelope glycoprotein (gp120) as well as recombinant gp36 protein. All assays were performed in accordance with the manufacturer's instructions, with alkaline phosphatase-labeled goat anti-human immunoglobulin G used as the secondary antibody. Plasma samples were scored as positive if they recognized at least one HIV antigen with an intensity equal to or greater than the assay's cutoff; samples which exhibited weaker but still visible reactivities with at least two HIV antigens were scored as indeterminate; samples were scored as negative if they yielded no reactivity or only a single band of less intensity than the assay's cutoff. Plasma samples from HIV-1- and HIV-2-negative and -positive individuals are shown as controls on the left. The 3+, 1+, and  $\pm$  bands (respectively, the topmost, middle, and lower bands labeled IgG control) that are evident on the top portions of all test strips control for sample addition (presence of plasma immunoglobulin) and test performance (binding of secondary antibody).

cloned into the pGEM-TEasy vector (Promega) and subsequently sequenced by using cycle sequencing and dye terminator methodologies (ABI PRISM Big Dye terminator cycle sequencing ready reaction kit with AmpliTaq FS DNA polymerase [PE Biosystems, Warrington, England]) on an automated sequencer (ABI 373, stretch model; Applied Biosystems). Newly derived SIV nucleotide sequences were aligned with reference sequences representing the different SIV lineages and other partial SIV sequences available in the *pol* region by using CLUSTAL W with minor manual adjustments, bearing in mind the protein sequences (27). Gaps and ambiguous regions in the alignment were omitted from further analyses. A phylogenetic tree was constructed by using the neighbor joining method, and the reliability of branching orders was tested by using the bootstrap approach (27). Sequence distances were calculated by using Kimura's two-parameter method to correct for superimposed hits (19). Phylogenetic analysis of the 650-bp fragment confirmed SIV infection (Fig.

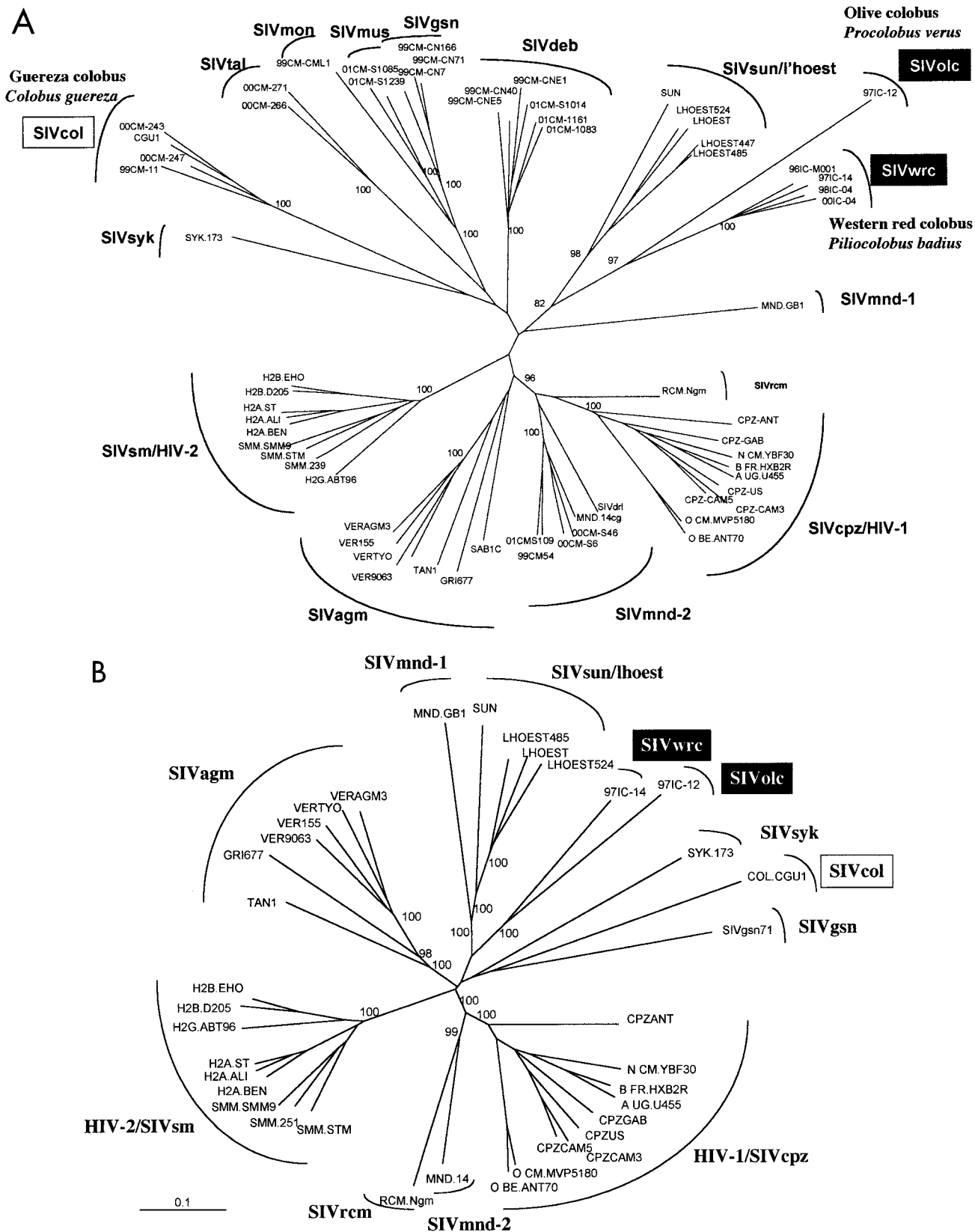


FIG. 2. Phylogenetic tree analyses of the new SIV sequences SIVwrc, from a western red colobus (*Ptilocolobus badius*), and SIVvolc, from an olive colobus (*Procolobus verus*). A 650-bp (A) fragment and a 2,000-bp (B) fragment were amplified in *pol*, sequenced, and subjected to phylogenetic tree analysis by using the neighbor joining method. The positions of the SIV sequences that were derived from the present study (boxed in black) are shown in relation to HIV and SIV reference sequences representing the different known SIV sequences available in this region of the genome. Branch lengths are drawn to scale (the bar for panel B indicates 10% divergence). The numbers at the nodes indicate the percent bootstrap values supporting the cluster to the right (only values of >80% are shown).

TABLE 1. Percent amino acid identities in the *pol* region (661 aa) between SIVwrc (SIVwrc-971C-14) and SIVolc (SIVolc-971C-12) to representatives of other SIV lineages

SIV lineage	% identity with:	
	SIVwrc	SIVolc
SIVolc	59.8	
SIVwrc		59.8
SIVcol	50.8	50.8
SIVlhoest	59.5	57.1
SIVsun	58.7	56.3
SIVmnd-1	61.9	54.1
SIVsmPBj	59.3	54.2
SIVcpzGAB	58.9	54.8
SIVcpzANT	58.9	55.2
SIVagmVER	56.3	52.5
SIVagmGRI677	55.7	51.2
SIVagmTAN	55.5	53
SIVsyk	53.2	50.5
SIVgsn-99CM71	54.7	50.2
SIVmnd-2	59	56.8
SIVrcmNig	60.2	56.7

2A) and showed that viral sequences from *P. badius* (SIVwrc) and *P. verus* (SIVolc) each formed species-specific monophyletic clusters. The new SIVs obtained from two different genera in the *Colobinae* subfamily were more closely related to each other than to the other SIVs. Interestingly, the new sequences were not at all related to the SIVcol strain obtained from a guereza colobus (*C. guereza*) from Cameroon.

A 2,000-bp fragment, corresponding to part of the reverse transcriptase and integrase of the *pol* gene and amplified with DR1 and PolOR as the outer primers and with DR4 and specific primers designed on the basis of the 600-bp fragment as the inner primers, was then sequenced for representatives of SIVwrc (SIVwrc-97CI14) and SIVolc (SIVolc-97CI12) (6, 8). The phylogenetic tree analysis shows that the two new SIVs form a separate well supported cluster, although they are only distantly related to each other (Fig. 2B). Amino acid identities between SIVwrc-97CI14 and SIVolc-97CI12, as well as to representatives of the other primate lentivirus lineages, were calculated (Table 1). SIVwrc-97CI14 and SIVolc-97CI12 showed 59.8% amino acid identity, which is close to the identities observed with the majority of the other primate lentiviruses. Surprisingly, the lowest homology was seen with the SIVcol strain, with only 50.8% amino acid identity.

This study presents evidence that in the *Colobinae* subfamily, the three African genera are naturally infected with an SIV. At least one representative from each genus is infected, namely, *Colobus guereza* in Cameroon and *Piliocolobus badius* and *Procolobus verus* in Ivory Coast. If we consider that all of these viruses have the same rate of evolution in their respective hosts, it seems that these viruses have not evolved in a host-dependent fashion at the level of the *Colobinae* subfamily, since representative viruses from the three genera do not cluster together in the region studied. However full-length genome sequencing of the new SIVolc and SIVwrc sequences is necessary to see to what extent they are pure or recombinant SIVs. We also have to take into account the geographical origin of the SIV-harboring species which we are comparing. It is important to note that the African colobid species are reflected

by their geographic distribution; e.g., the olive colobus is a relict species confined to the forest of West Africa, and the red colobus (*Piliocolobus* spp.) once ranged all over the forested areas from Africa, but their regional differentiation shows that their scattered distribution is of long standing.

SIVcol from the guereza colobus from Cameroon is divergent from all known SIVs. This could suggest an ancient infection and a different evolution of SIVcol in its host species, perhaps reflecting a more pure lineage relative to other SIV lineages. On the other hand, the clustering of SIVwrc with SIVolc could also suggest a cross-species transmission between them or infection from a common source. Colobus monkeys share habitats with *Cercopithecus* species and with mangabeys; therefore, an exchange of ancestral SIVs between these species could have been possible in the past. Monkeys living in tropical forests often form aggregations consisting of multiple species, i.e., polyspecific associations. This has been particularly well documented in the Tai National Park, where associations between olive colobus and diana monkeys (*Cercopithecus diana*) and associations between red colobus and diana monkeys are frequent (22). Moreover, SIV seropositivity has been previously described in diana monkeys (20). Importantly, such associations cannot be extrapolated to similar primate populations in another location; e.g., by contrast, a study on the same species complex on Tiwai Island in Sierra Leone reveals no associations between red colobus and diana monkeys (23). This illustrates that data on primate behavior are necessary to understand primate lentivirus evolution and to determine between which primate species cross-species transmission and superinfection have or have not been possible (29).

In order to understand the evolution of SIVs in the *Colobinae* subfamily, it will be important to identify and compare SIVs from *Colobus* and *Piliocolobus* species from West, Central, and East Africa to find out whether coevolution between viruses and hosts occurred or whether hosts became infected along with other cohabiting monkeys in the past. Screening of the other sympatric species from the *Cercopithecinae* and *Colobinae* subfamilies (e.g., *Cercopithecus diana* and *Colobus polykomos* from the Tai forest) is also required.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the sequences in this study are as follows: AY138265 (SIVwrc-961C-M001), AY138266 (SIVwrc-981C-04), AY138267 (SIVwrc-001C-04), AY138268 (SIVwrc-971C-14), and AY138269 (SIVolc-971C-12).

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