

# Full molecular characterization of a simian immunodeficiency virus, SIVwrcpbt from Temminck's red colobus (*Piliocolobus badius temminckii*) from Abuko Nature Reserve, The Gambia

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## Abstract

Simian immunodeficiency viruses (SIVs) are found in an extensive number of African primates, and humans continue to be exposed to these viruses by hunting and handling of primate bushmeat. The purpose of our study was to examine to what extent *Piliocolobus badius* subspecies are infected with SIV in order to better characterize SIVwrc in general and to gain further insight into the impact of geographic barriers and subspeciation on the evolution of SIVwrc. We analysed sixteen faecal samples and two tissue samples of the *P. b. temminckii* subspecies collected in the Abuko Nature Reserve (The Gambia, West Africa). SIV infection could only be identified in one tissue sample, and phylogenetic tree analyses of partial *pol* and *env* sequences showed that the new SIVwrcPbt virus is closely related to SIVwrcPbb strains from *P. b. badius* in the Taï forest (Côte d'Ivoire), thus suggesting that geographically separated subspecies are infected with a closely related virus. Molecular characterization and phylogenetic analysis of the full-length genome sequence confirmed that SIVwrcPbt is a species-specific SIV lineage, although it is distantly related to the SIVlho and SIVsun lineages across its entire genome. Characterization of additional SIVwrc viruses is needed to understand the ancestral phylogenetic relation to SIVs from l'Hoest and sun-tailed monkeys and whether recombination occurred between ancestors of the SIVwrc and SIVlho/sun lineages.

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**Keywords:** Red colobus; SIVwrc; Evolution; Genetic diversity; Cross-species transmission

## Introduction

It is now well established that the human immunodeficiency viruses, HIV-1 and HIV-2, are the results of cross-species transmissions of simian immunodeficiency viruses (SIV) naturally infecting non-human primates in sub-Saharan Africa. SIVsmm from sooty mangabeys (*Cercocebus atys atys*) is

recognised as the progenitor of HIV-2, whereas SIVcpz from chimpanzees (*Pan troglodytes troglodytes*) and SIVgor from gorillas (*Gorilla gorilla gorilla*) in West Central Africa are the ancestors of HIV-1 (Gao et al., 1999; Keele et al., 2006; Van Heuverswyn et al., 2006). Serological evidence of SIV infection has been shown for at least 39 of the 69 different primate species in Africa and has been confirmed by sequence analysis in 32. Complete SIV genome sequences are available for 19 species (Grubb et al., 2003; van de Woude and Apetrei, 2006). Interestingly, only Old World primates are infected with SIVs, and only those from the African continent. Although SIVs are called immunodeficiency viruses, they generally do not induce

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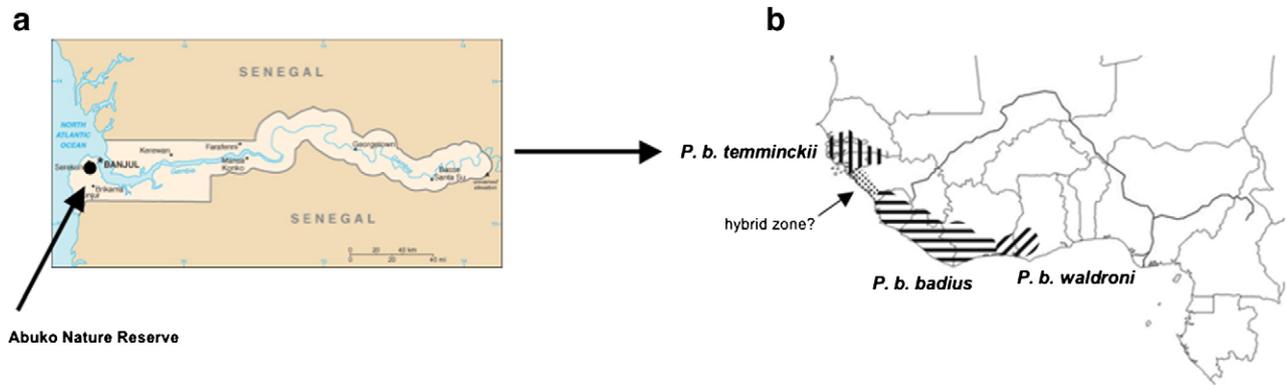


Fig. 1. Location of the Abuko Nature Reserve, The Gambia, where the SIVwrcPbt-05GM-X02 specimen was collected (a) and geographical distribution of the Western *Piliocolobus* subspecies (Grubb, 1999; Oates et al., 1994) (b).

an AIDS-like disease in their natural hosts, suggesting that they have been associated and evolved with their hosts over an extended period of time. However, recent reports have demonstrated that SIV infection in natural hosts can eventually lead to immunodeficiency, but this seems to occur only when animals have been infected over long periods of time. (Ling et al., 2004; Pandrea et al., 2001).

Phylogenetic analyses have revealed high levels of genetic diversity among the known SIVs, but generally each primate species is infected with a species-specific virus. Although for some primates, virus and host phylogenies seem to match, cross-species transmission followed by recombination in distantly related primate species has been frequently documented (Bibollet-Ruche et al., 2004; Jin et al., 1994). The most remarkable example is that of SIVcpz from chimpanzees, which is the result of the recombination between the ancestors of SIVs infecting red capped mangabeys (*Cercocebus torquatus*) and the ancestor of a clade of viruses (SIVgsn, SIVmon, and SIVmus) found in three *Cercopithecus* species, greater spot-nosed (*C. nictitans*), mona (*C. mona*), and moustached monkeys (*C. cephus*), all of which are hunted by chimpanzees in West Central Africa (Bailes et al., 2003; Mitani and Watts, 1999). A single species can also be infected with different viruses. For example, mandrills (*Mandrillus sphinx*) living in Gabon south of the Ogoûé river are infected with SIVmnd-1 but those spanning the regions of southern Cameroon and northern Gabon are infected with SIVmnd-2 (Onanga et al., 2006; Souquiere et al., 2001; Telfer et al., 2003). Even in the absence of rivers as geographical barriers, two viruses can co-circulate in one species within small geographic areas, as observed in moustached monkeys from Cameroon infected with SIVmus-1 and SIVmus-2 (Aghokeng et al., 2007).

SIV transmission from primates to humans is most likely the result of contact with infected blood and tissues from primates hunted for bushmeat (Hahn et al., 2000). The ancestors of HIV-1 and HIV-2 have crossed the species barrier to humans on multiple occasions, but the transmission potential of the other primate lentiviruses remains unknown (Hahn et al., 2000). However, we have shown that a substantial proportion of wild-living monkeys in Cameroon are infected with SIV and that humans are exposed to a plethora of genetically diverse viruses

through hunting and handling of bushmeat (Peeters et al., 2002), thus emphasizing the need to continue surveillance and characterization of SIVs in primates. With the exception of SIVcpz and SIVgor, all SIVs identified so far originate from primates belonging to the family Cercopithecidae, or Old World monkeys, which is subdivided into two subfamilies: the Colobinae and Cercopithecinae (Disotell, 1996; Groves, 2001). Due to their high species number and large distribution in sub-Saharan Africa, the Cercopithecini tribe of the Cercopithecinae represents today the largest reservoir for SIV. The African Colobinae instead are composed of only about 11 species which are classified in three genera, *Colobus*, *Procolobus* and *Piliocolobus* (Groves, 2001; Grubb et al., 2003). Their geographic distribution spans the regions in Africa where equatorial forests are still present, ranging from The Gambia in the west to Zanzibar in the east. In certain regions of Africa, colobines are heavily hunted by the human population (Caspary et al., 2001) and also by chimpanzees (Boesch, 1994; Stanford et al., 1994; Stern and Goldstone, 2005; Tappen and Wrangham, 2000), possibly exposing the human and the chimpanzee populations to additional retroviruses. SIV infection has been identified in at least one species of each African colobine genus but only SIVcol, from a mantled guereza (*Colobus guereza*) in Cameroon has been fully characterized (Courgnaud et al., 2003, 2001). Whereas SIVcol forms a separate divergent lineage, molecular characterization of a 2000 bp fragment in *pol* showed that SIVwrc from western red colobus (*Piliocolobus badius badius*) and SIVolc from olive colobus (*Procolobus verus*) in the Taï forest, Côte d'Ivoire, each forms a species-specific lineage unrelated to SIVcol (Courgnaud et al., 2003). The *P. badius* species can be further subdivided into three geographically isolated subspecies: *P. b. badius*, in Guinea, Sierra Leone, Liberia and Côte d'Ivoire; *P. b. waldroni*, formerly found in Ghana and Côte d'Ivoire east of the Bandama river, but nearly extinct today (McGraw, 2005) and *P. b. temminckii* which is found in Senegal, The Gambia, Guinea Bissau and NW Guinea.

The purpose of our study was to examine to what extent other *P. badius* subspecies are infected with SIV in order to better characterize SIVwrc in general and to gain further insight on the impact of geographic barriers on the evolution of SIVwrc. We analysed sixteen faecal samples and two tissue samples of the

Table 1  
Primers used to amplify the genome of sample SIVwrcPbt-05GM-X02

PCR	Fragment <sup>a</sup>	Size (kb)	Primers
First round	A	~0.25	GAGwrcF1 (5'-ATD <sup>b</sup> GAGGATAGAGGNTTTGGAGC-3')
			GAGwrcR1 (5'-GCCCTCCTACTCCTTGACATGC-3')
Second round			GAGwrcF2 (5'-CCAACAGGGTCAGATATAGCAG-3')
			GAGwrcR2 (5'-ACTTCTGGGGCTCCTTGTCTGCTC-3')
First round	B	~0.67	POLwrcolF1 (5'-TAGGGACAGAAAGTATAGTAATHHTGG-3')
			POLwrcolR1 (5'-GCCATWGCYAA TGCTGTTTC-3')
Second round			POLwrcolF2 (5'AGAGACAGTAAGGAAGGGAAAAGCAGG-3')
			POLwrcolR2 (5'-GTTCWATTCTTAACCACCAGCADA-3')
First round	C	~0.58	ENVwrcolF1 (5'-TGGC AGTGGGACAAAAATATAAAC-3')
			ENVwrcolR1 (5'-CTGGCAGTCCCTCTTCCA AGTT GT-3')
Second round			ENVwrcolF2 (5'TGATAGGGMTGGCTCCTGGTGATG3')
			ENVwrcolR2 (5'-AATCCCCATTTAACCAGTTCCA-3')
First round	D	~2.6	GAGwrcF1 (5'-ATD <sup>b</sup> GAGGATAGAGGNTTTGGAGC-3')
			POLpbtR1 (5'-GTATTTCTCCTATCCCTTTATGTGCTG-3')
Second round			GAGwrcF2 (5'-CCAACAGGGTCAGATATAGCAG-3')
			POLpbtR2 (5'-AGGGAGATTCACTTTGAGTTGGGTG-3')
First round	E	~3	POLpbtF1 (5'-GCACCCACTTGAAGGAAAAATCAT-3')
			ENVpbtR1 (5'-ACTGTTGATACCGTGCCCATG-3')
Second round			POLpbtF2 (5'-CCTATCAAACAGCACTTTTCACCCT-3')
			ENVpbtR2 (5'-GCTCCTCGTTTTTCTCTATGATGGT-3')
First round	F	~2.8	GAGwrcR1 (5'-GCCCTCCTACTCCTTGACATGC-3')
			ENVpbtF1 (5'-CACCTGCTTGAATAATGAAACA-3')
Second round			GAGwrcR2 (5'-ACTTCTGGGGCTCCTTGTCTGCTC-3')
			ENVpbtF2 (5'-TAAGAACAGACACCTTGATGAGTAAT-3')

<sup>a</sup> Letters correspond to the fragments labelled as such in Fig. 2.

<sup>b</sup> D=G or A.

*P. b. temminckii* subspecies collected in the Abuko Nature Reserve in The Gambia, West Africa. SIV infection could only be identified in one tissue sample, and the new SIVwrcPbt virus was closely related to SIVwrcPbb strains from the Taï forest in Côte d'Ivoire. Full genome sequence revealed that SIVwrc is a species-specific SIV lineage with a phylogenetic relation to SIVs from the l'Hoest lineage, thus suggesting an ancestral link of their respective genomes.

## Results

### *SIV infection in faecal and tissue samples from Temminck's red colobus (P. b. temminckii) in Abuko Nature Reserve, The Gambia*

Despite the positive results obtained in faecal samples from chimpanzees and gorillas (Keele et al., 2006; Van Heuverswyn et al., 2007, 2006), antibody detection in faecal samples from western red colobus was unsuccessful and samples were thus directly screened with molecular tools for SIV infection (Locatelli et al., 2008). Sixteen faecal samples and two tissue samples collected in Abuko Nature Reserve, The Gambia, were analysed for SIV infection using SIVwrc specific primers which amplified partial *pol* and *env* regions in samples from the *P. b. badius* subspecies collected in the Taï forest, Côte d'Ivoire (Locatelli et al., 2008) (Figs. 1a and b). None of the above mentioned fragments was amplified in the faecal samples, but in one out of two tissue samples 672 bp and 578 bp fragments in the *pol* and gp41 *env* regions, respectively, were amplified. This sample was named SIVwrcPbt-05GM-X02, with *Pbt* referring to the *P. b. temminckii* subspecies (Table 1 and Fig. 2).

### *Phylogenetic analysis of partial pol and env sequences of the new SIVwrcPbt isolate from Temminck's red colobus*

To determine the relationships between the newly identified SIVwrcPbt-05GM-X02 in a Temminck's red colobus (*P. b.*

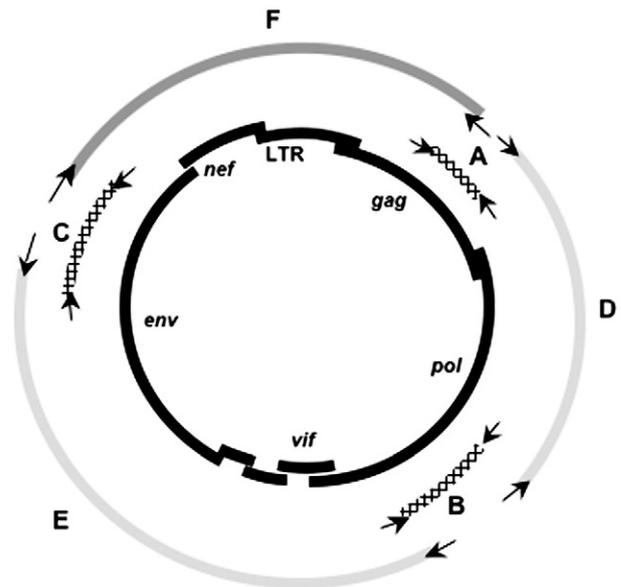


Fig. 2. Schematic representation of the PCR amplification of full-length SIVwrc sequences from uncultured liver cells. The positions of the various amplification products are shown in relation to an unintegrated circular intermediate of SIVwrcPbt-05GM-X02. Primer sets and fragment designations are identical to those in Table 1.

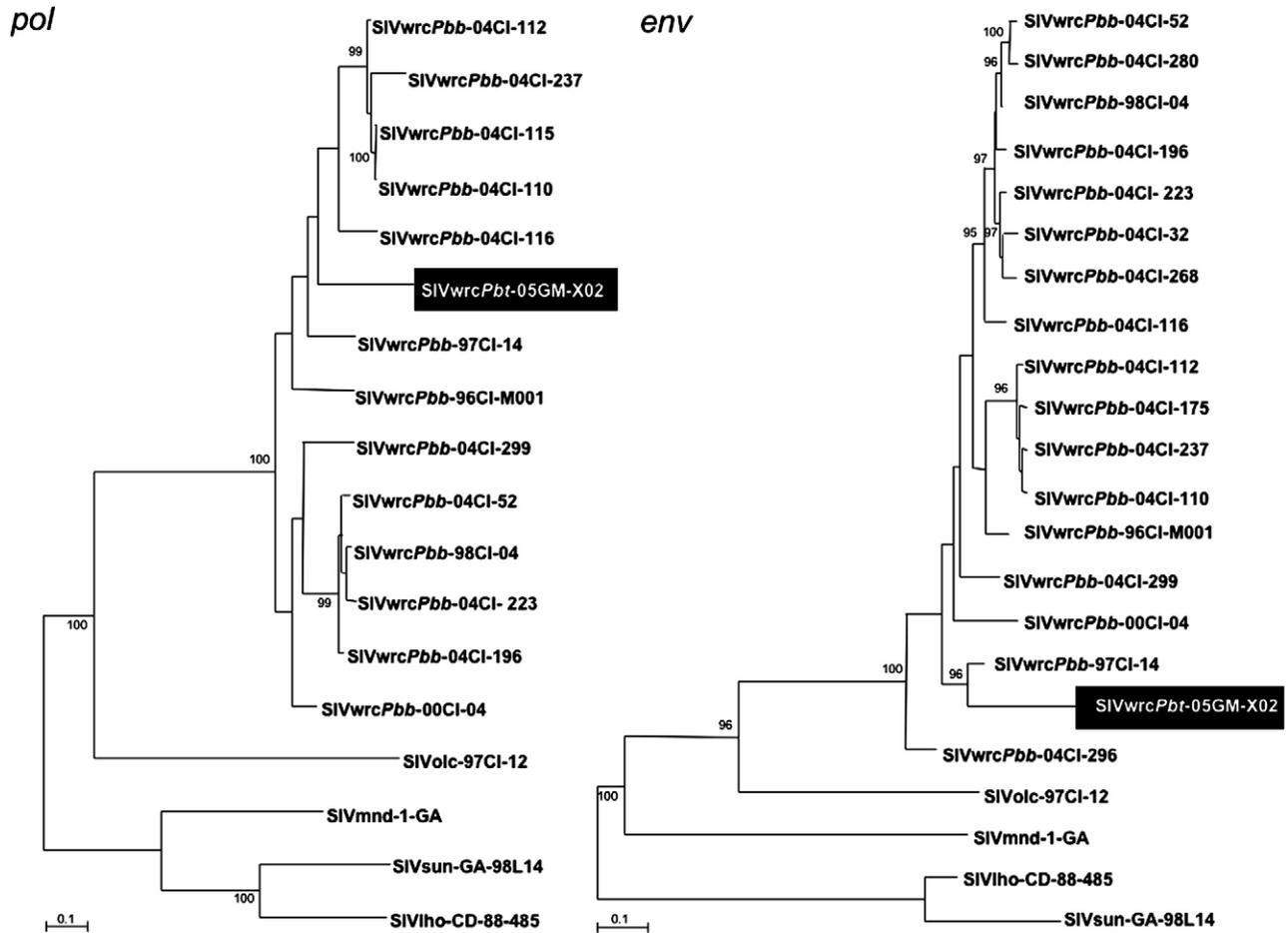


Fig. 3. Phylogenetic analysis of partial (a) *pol* (polymerase, ~670 bp) and (b) *env* (gp41, envelope transmembrane protein, ~570 bp) genomic regions. The sequence of the newly identified SIVwrcPbt strain is highlighted. Previously published SIVwrcPbb and SIVolc strains collected during the Ebola study conducted in the Taï forest (1997–2000) as well as SIVwrcPbb strains from red colobus samples collected in 2004 are also included in the analysis (Courgnaud et al., 2003; Locatelli et al., 2008). The trees were inferred by the Bayesian method. Numbers on branches are posterior percentage probabilities (only values above the significance level are shown). The scale bars indicate 0.1 substitutions per site.

*temminckii*) and previously characterized SIVwrc strains from the red colobus subspecies in Côte d'Ivoire (*P. b. badius*), we constructed phylogenetic trees from partial *pol* and *env* sequences using SIVolc and SIVs belonging to the SIVlho lineage (SIVmnd-1, SIVsun and SIVlho) as outgroups. In both phylogenies, SIVwrcPbt-05GM-X02 falls within the SIVwrcPbb cluster (Fig. 3).

#### Full-length genome sequence and genomic organisation of SIVwrcPbt-05GM-X02

In order to ascertain this result and to better characterize the SIVwrc virus, we sequenced the full-length genome of the SIVwrcPbt-05GM-X02 strain. Because of the apparent high degree of genetic similarity to SIVwrcPbb in *pol* and *env*, we designed consensus primers in *gag* from an alignment of two unpublished SIVwrcPbb *gag* sequences (SIVwrcPbb-97CI-14 and SIVwrcPbb-98CI-04) to amplify a 252 *gag* bp fragment for the new SIVwrcPbt-05GM-X02 sample. Based on the partial sequences obtained in *gag* and the 3' end of the *pol* and gp41, strain specific primers were designed to amplify the remainder of

the SIVwrc genome. The complete genome sequence of SIVwrcPbt-05GM-X02 was then obtained by successive nested PCRs as illustrated in Fig. 2 and using primers shown in Table 1. Briefly, we proceeded to PCR amplifications spanning the regions of *gag-pol* (fragment D), *pol-env* (fragment E) and *env-nef-LTR-gag* for the circular unintegrated form of the virus (fragment F). These amplified fragments corresponding to amplicons of 2.6 kb, 3.0 kb, and 2.8 kb, respectively, were gel purified and sequenced with specific primers. The concatenated linear complete genome contains 8709 bp, including the LTR fragment. The SIVwrc genome was compared to the other primate lentiviruses and displayed the expected reading frames for *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *env* and *nef*. As for most of the SIV lineages, SIVwrcPbt-05GM-X02 did not encode a *vpu* or *vpx* analogue. The genomic organisation of SIVwrc is thus similar to that of, e.g., SIVagm, SIVsyk, SIVmnd-1, SIVcol or the SIVlho lineage. The SIVwrc long terminal repeat (LTR) contained all the characteristic features of other primate lentivirus LTRs including TATA, 1 potential NF-κB site, and 1 potential SP-1 region (data not shown). The secondary structure prediction of the SIVwrc tar element showed an unusual

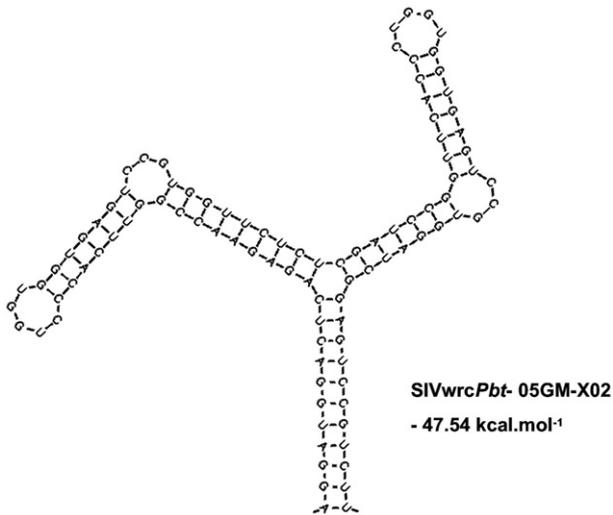


Fig. 4. Secondary structure prediction of the SIVwrcPbt-05GM-X02 TAR element with the lowest free energy value ( $-47.54 \text{ kcal mol}^{-1}$ ).

organisation with two identical stem-loops consisting of 3 nucleotides bulges (GCC) and 7-bp stems with a 5-bp terminal loop(5'-UGGUC-3'; Fig. 4).

#### Phylogenetic relationship between SIVwrcPbt-05GM-X02 and the other SIV lineages

To compare the SIVwrcPbt-05GM-X02 genome to previously characterized SIV strains, we performed similarity plots of concatenated Gag, Pol, Vif, Env and Nef protein sequences. Figs. 5a and b depicts the trend of amino acid similarities between SIVwrcPbt-05GM-X02 and representatives of SIV strains from the major lineages. In the Gag and the N-terminal part of Pol, SIVwrcPbt-05GM-X02 seems to be equidistantly related to the other SIV lineages. Interestingly, in the Pol1 fragment, SIVwrcPbt-05GM-X02 is closest to the SIVlho and SIVcol lineages (represented by strains SIVlho, SIVsun14, SIVmndGB1 and SIVcolCGU1). In the Env fragment, SIVwrcPbt-05GM-X02 is close to the SIVlho, SIVmnd-1 and SIVmnd-2/drl lineages (strains SIVdrl1FAO, SIVmnd14cg, and SIVlho, SIVsun14, SIVmndGB1). In that latter part of the genome, the SIVwrc Simplot analysis identifies two groups of similarity that correspond to the SIVlho/SIVmnd-1/SIVmnd-2/drl and to all other SIVs, respectively (Fig. 5).

We then constructed phylogenetic trees for Gag, Env as well as for two segments of Pol corresponding to the regions identified in the similarity analysis (Pol1 and Pol2) (Fig. 6). Overall, SIVwrcPbt-05GM-X02 appears to be more closely related to

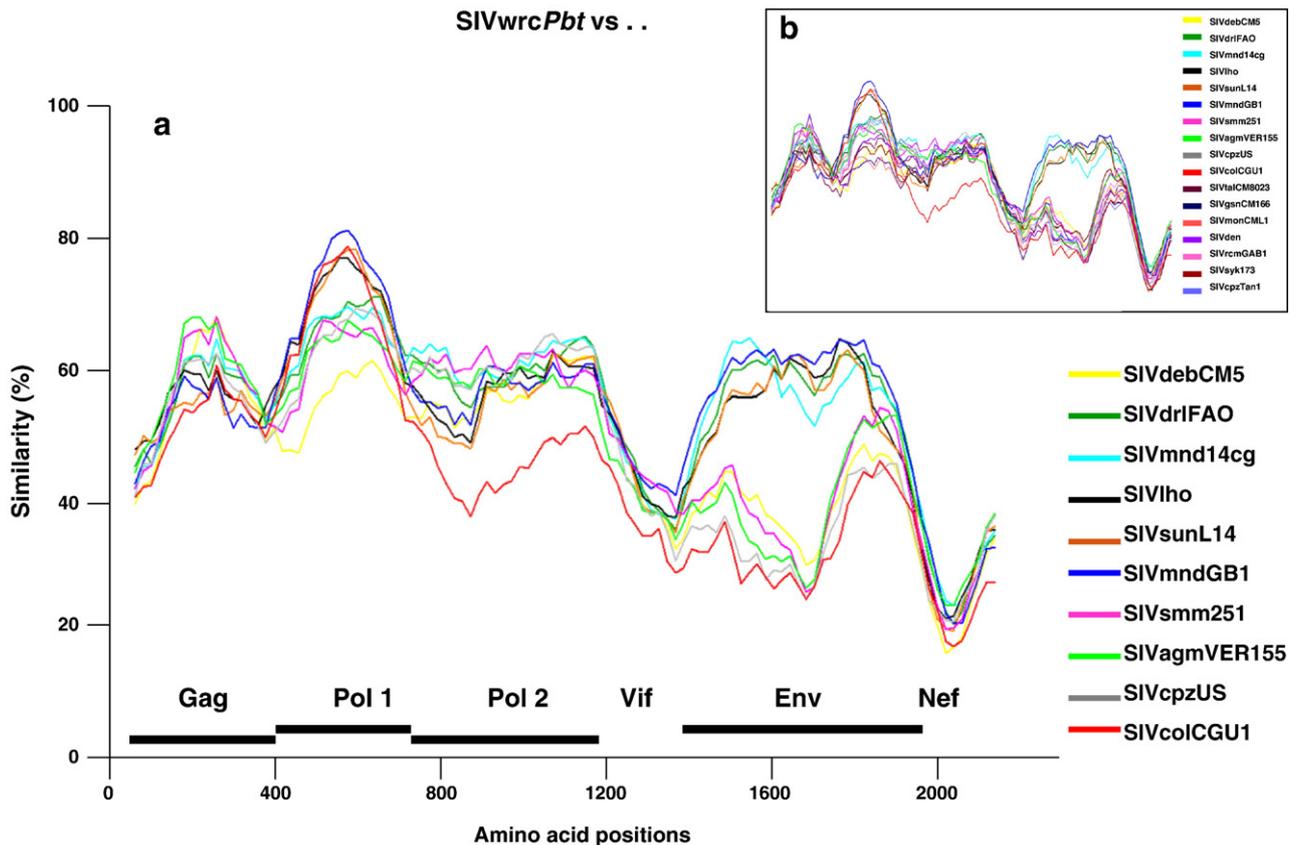
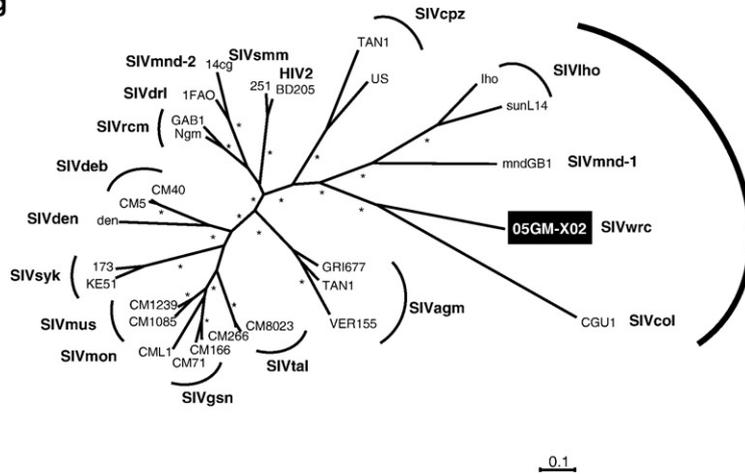
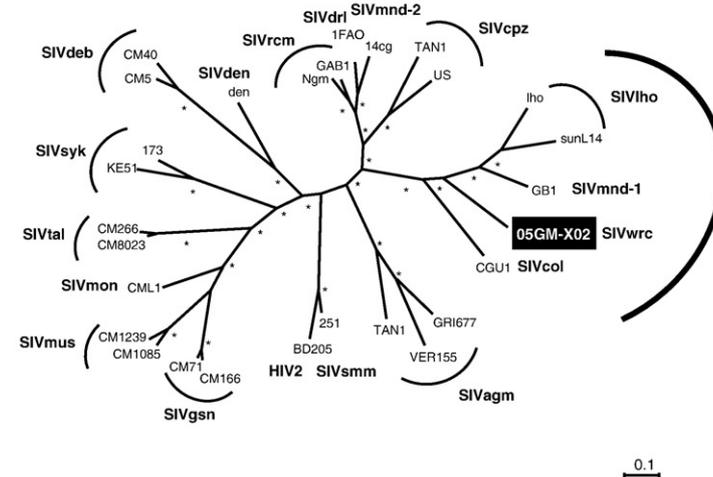


Fig. 5. Similarity plots of concatenated Gag, Pol1 (corresponding to residues 1–436 of Pol in SIVwrcPbt-05GM-X02), Pol2 (corresponding to residues 437–1013 of Pol in SIVwrcPbt-05GM-X02), Vif, Env and Nef protein sequences showing the extent of genetic similarity between SIVwrcPbt-05GM-X02 and 10 (a) versus 17 (b) strains from other SIV lineages. The proportion of amino acid sequence similarity per 200 residues window (vertical axis) is plotted against the midpoint of the sequence window (horizontal axis).

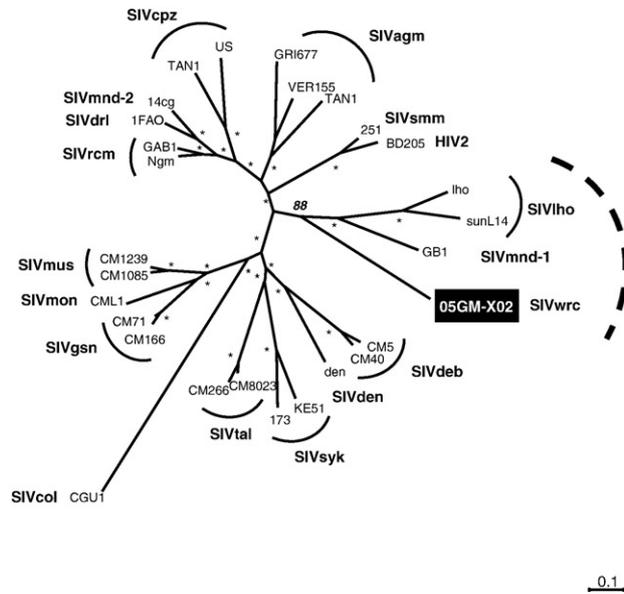
**gag**



**pol1**



**pol2**



**env**

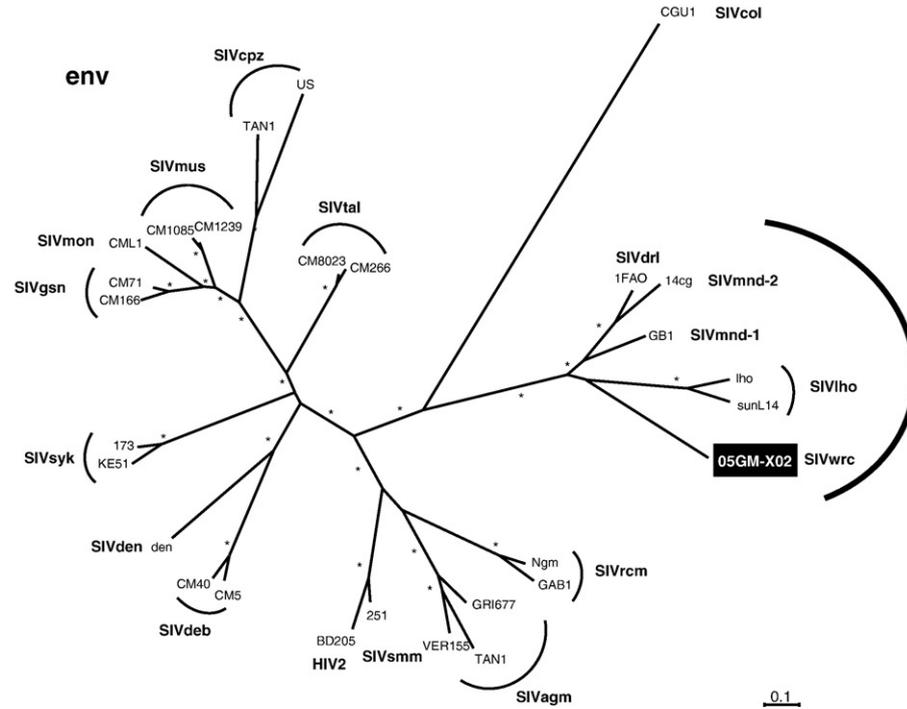


Fig. 6. Phylogenetic relationships of the newly derived *SIVwrcPbt* sequence to other SIV lineages. Trees were inferred from unambiguous parts of the protein sequence alignments from: Gag, Pol1, Pol2 and Env. The newly characterized *SIVwrcPbt* full-length genome is boxed. The numbers on nodes are estimated posterior probabilities, values greater than 92% are represented by an asterisk (\*). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 amino acid replacements per site.

Table 2  
gag P6 motifs after alignment of primate lentiviral gag p6 protein sequences

SIV strain	gag P6 motifs	
SIVtalCM8023	PTAP	YPSL
SIVtalCM266	PSAP	YPSL
SIVdebCM5	–	YDDL
SIVdebCM40	–	YDDL
SIVgsnCM166	PTAP	YPSL
SIVgsnCM71	PSAP	YPSL
SIVmonCML1	PSAP	YPSL
SIVmusCM1085	PTAP	YPSL
SIVden	–	YPSL
SIVmusCM1239	PTAP	YPSL
SIVrcmNgm	PTAP	–
SIVrcmGAB1	PSAP	–
SIVdr11FAO	PSAP	YNSL
SIVmnd14cg	PSAP	YNSL
SIVsykKE51	PTAP	YPSL
SIVsyk173	PSAP	YPSL
SIVcpzTAN1	PTAP	–
SIVlho	PSAP	–
SIVsunL14	PSAP	–
SIVmndGB1	PTAP	–
SIVsmm251	PTAP	–
SIVagmGRI677	PTAP	–
SIVagmTAN1	PTAP	–
SIVagmVER155	PSAP	–
SIVcpzUS	PTAP	–
HIV2BD205	PSAP	–
SIVcolCGU1	–	YPSL
SIVwrc-05GM-X02	PTAP	YPSL

SIVs from the SIVlho/sun and SIVmnd-1 lineages. The grouping of these taxa was supported by high posterior probabilities (above the 91% threshold; (Zander, 2004)) in Gag, Pol1 and Env trees and at a slightly lower (non significant) value in the Pol2 phylogeny. SIVcol seems also to cluster with this group of viruses in the Gag, Pol1 and Env trees, although generally with higher degrees of divergence. Only in the Pol2 fragment does the SIVcol strain form a separate branch. Interestingly, in accordance with the similarity plot data, the branching pattern of SIVwrc, SIVlho, SIVsun, SIVmnd-1 in the Env phylogeny (i.e. a long internal branch leading to shorter than expected ingroup branches relative to relationships among other taxa in the tree) suggest a more complex evolutionary history than for the rest of the genome in the SIVlho/sun/mnd-1/wrc lineages, possibly involving recombination.

#### Functional motifs in the SIVwrc genome

Among the African primates the genus *Cercopithecus* harbours the largest number of species known to be infected with SIVs, and recently a *Cercopithecus*-specific SIV lineage has been described (Bibollet-Ruche et al., 2004). SIVs derived from different *Cercopithecus* species consistently form one highly supported group in phylogenetic trees despite examples of co-evolution between viruses and hosts and cross-species transmission within this genus. In addition, they share functional motifs in gag and env that distinguish them from other primate lentiviruses.

Two different sites known to be critical for primate lentivirus budding have been identified in SIV Gag p6 protein sequences — PT/SAP and YPXL. All *Cercopithecus* SIVs have both motifs, except SIVdeb and SIVden (Bibollet-Ruche et al., 2004; Dazza et al., 2005) which, like SIVcol, only have a YPXL motif. On the other hand, SIVcpz, SIVlho, SIVsun, SIVmnd-1, SIVsmm, SIVagm, SIVrcm and HIV-2 encode only for a N-terminal PT/SAP Tsg101 binding motif. The presence of one motif compensates for the lack of the other, but to date the functional significance of the occurrence of both motifs remains unknown (Martin-Serrano et al., 2001; Puffer et al., 1997). The alignment of SIVwrcPbt-05GM-X02 with other SIV Gag p6 protein sequences revealed the presence of both PT/SAP and YPD/SL motifs (Table 2).

We then inspected the five variable regions designated V1 to V5 and the CD4 binding domain of the gp120 envelope glycoprotein. As in other primate lentiviruses, the V1 region of the Env protein of SIVwrcPbt-05GM-X02 revealed high levels of variability, insertion/deletion polymorphism, and the characteristic presence of threonine residues. The CD4 binding domain was conserved and 3 amino acid insertions were observed, which is similar to SIVdr1, SIVmnd-1, SIVlho, SIVsun, SIVmnd-2 and SIVcol (data not shown).

All primate lentiviruses, including HIV, have at least eighteen conserved cysteine residues in the extracellular envelope domain. They form paired disulfide bonds responsible for the conformational structure of the virus surface and for the envelope functions, including the interaction with the CD4 receptor on the host cell surface (Bibollet-Ruche et al., 2004). All SIVs derived from *Cercopithecus* species (SIVdeb/SIVsyk/SIVgsn/SIVmon/SIVmus), SIVcpz and SIVcol contain 18 conserved cysteine residues. SIVs belonging to the other lineages (SIVagm/SIVlho/SIVsmm/SIVrcm/SIVmnd/SIVdr1) encode for additional pairs of cysteine residues. SIVwrcPbt-05GM-X02 encodes for three additional cysteine pairs which fell within variable domain 2 and between variable domains 3 and 4 at similar positions as the additional cysteines observed in the SIVlho, SIVsun, SIVmnd-2 and SIVdr1 lineages.

#### Discussion

In this study we showed that geographically isolated subspecies of the western red colobus (*P. badius*) in The Gambia and Côte d'Ivoire are both infected with closely related species-specific SIVs, designated SIVwrc. We obtained the full-length genome sequence of an SIVwrc strain from an individual of Temminck's red colobus (*P. b. temminckii*) in The Gambia and showed that SIVwrc is a species-specific lineage, although distantly related to the SIVlho and SIVsun lineages across its entire genome. Interestingly, in gag and in the 5' end of pol, SIVwrc was also distantly related to SIVcol isolated from a mantled guereza (*C. guereza*) from Cameroon, the only other fully characterized SIV in the subfamily Colobinae.

The habitat of *P. b. temminckii* spans the regions of Senegal, The Gambia, Guinea Bissau and NW Guinea whereas the *P. b. badius* range is limited by the mountainous areas of Sierra Leone, the Niger river in north-east Guinea and the Sassandra

river in Côte d'Ivoire (Kingdon, 1997). However, the coastal forests of Guinea have not been extensively surveyed, therefore we cannot exclude the possibility that a red colobus population exists that connect these two subspecies. Pelage and vocalization data indicate a close relationship between *P. b. badius* and *P. b. temminckii*. Mitochondrial lineages are paraphyletic with respect to one another (Ting, in press). This reflects the results we obtained from the partial *pol* and *env* SIVwrc fragments and suggests that these two subspecies may either still be in contact or only recently diverged. A study conducted on red colobus monkeys in Taï National Park, Côte d'Ivoire, shows that they represent a substantial reservoir of SIVwrcPbb, with at least 25% of the adult population infected (Locatelli et al., 2008). None of the 16 faecal samples from Temminck's red colobus analysed in this study by RT-PCR resulted to be positive. However, the limited amount of material available allowing only a single RNA extraction for each sample, together with several thaw and freeze procedures and the low sensitivity of viral RNA detection previously reported, do not allow us to provide any significant information of infection rates in the Abuko population of Temminck's red colobus (Ling et al., 2003; Locatelli et al., 2008).

Phylogenetic analysis of the full-length genome showed that SIVwrc forms a distinct lineage and it is most closely related to the SIVlho/sun/mnd-1 lineage across the entire genome, although to a lower extent in the 3' end of *pol*. Interestingly, SIVcol clusters also with SIVwrc and the above mentioned SIVs in *gag* and especially in the 5' end of *pol*. Since their hosts (subfamily Colobinae) are phylogenetically related, the relationship between these SIV lineages may reflect a common ancestry of their genomes.

Overall, the phylogenetic relationships between the different SIVs and the presence of specific functional motifs, with the exception of the motifs in *gag* typical of the *Cercopithecus* lineage, suggest that SIVwrc has an ancestral link with the SIVs from l'Hoest and sun-tailed monkeys. This relationship is surprising because of the geographical separation of the different hosts of these SIV lineages. However, the African continent has been subjected to periodic episodes of climatic change that mediated alternating phases of tropical rainforest contraction and expansion throughout the Plio-Pleistocene (from about 2.9 to 0.8 Ma) (deMenocal, 2004), thereby creating montane and lowland forest remnants or 'refuges'. These events have certainly affected the speciation and current geographic distribution of primates, and they have possibly also influenced the spreading patterns of an SIV ancestor. The territories we inspect today may have been inhabited by different or more primates species in the distant past. For example, the olive colobus is a relict species confined to the forests of West Africa, whereas the widely distributed fragmented populations of red colobus show that these animals once ranged across the forested parts of Africa; the black-and-white colobus (genus *Colobus*) is also widely distributed across equatorial Africa.

In order to better understand the evolution of SIVs in the colobines, it will be important to characterize additional SIVs in the olive colobus (*P. verus*) and the remaining species of *Colobus* and *Piliocolobus* across Africa. Particular attention should

be paid to *Piliocolobus* species whose ranges overlap today with those of the *Cercopithecus* species harbouring SIVlho and SIVsun. This will help to determine whether the virus emerged before or after red colobus speciation events and will provide further insight into the importance of biogeographic barriers and cross-species transmission in SIV evolution. Geographic barriers can influence evolution of SIVs among different species but also within a single species, as is shown for mandrills (*M. sphinx*) in Gabon. Based on partial *pol* and *env* sequences, the two geographically separated western red colobus subspecies seem to be infected with a similar SIVwrc variant. However, full-length characterization of SIVwrcPbb is necessary to confirm this observation. Geographic isolation, human presence and ecological factors like vegetation type and distribution can shape or elicit new or different behaviours which can play a role in cross-species transmission and recombination of divergent SIVs. For example, contrary to most colobus species which are mainly arboreal, Temminck's red colobus has been observed to spend relatively long periods of time at ground level (Galat-Luong, 1988) partly because populations in these regions do not face human or chimpanzee predation pressure, which is in contrast to the red colobus population in Taï forest in Côte d'Ivoire (Boesch, 1994; Boesch and Boesch-Achermann, 1989; Refisch and Koné, 2005), but also because Temminck's red colobus has adapted to an increasingly dry and fragmented ecosystem (Galat-Luong and Galat, 2005). One of the consequences of this diminished predation is that Temminck's red colobus have been observed in polyspecific association with ground living primates like patas monkeys (*Erythrocebus patas*) and green monkeys (*Cercopithecus aethiops sabaesus*) (Galat-Luong, 1988; Starin, 1994). These polyspecific associations are thus different than those observed for the *P. b. badius* subspecies in the Taï forest.

Overall, our results confirm the complex evolutionary history of primate lentiviruses which has been driven by host–virus co-speciation, cross-species transmission and recombination events over an extended period of time. Characterization of additional SIVwrc viruses is needed to understand the ancestral phylogenetic relation to SIVs from l'Hoest and sun-tailed monkeys and whether recombination occurred between ancestors of the SIVwrc and SIVlho/sun lineages. In addition, western red colobus are heavily hunted by the human population and given the relatively high frequency of SIVwrc infection in the wild, we should keep this virus under investigation for potential cross-species transmissions.

## Materials and methods

### *Primate specimens and collection site*

Sixteen faecal samples and two tissue samples from carcasses found on the forest floor were collected between January and February 2005 in the Abuko Nature Reserve, The Gambia. The Abuko Nature Reserve is situated outside the village of Lamin in the Kombo North District, 25 km from Banjul (Figs. 1a and b). The reserve was officially declared a nature reserve in 1968. The 106.6 ha reserve is a mosaic habitat consisting mostly of tree and shrub savannah (56.6%),

woodland savannah (24.8%) and gallery forest (16%) (Starin, 2006). About 200 red colobus live there in 5 groups (Starin, 1994). Faecal samples were collected in the morning underneath sleeping trees from opposite ends of the red colobus range to ensure sampling from different individuals. Samples were between five minutes and two hours old at collection. They were put directly into RNAlater (1/1 vol) and were kept at room temperature for less than a week before storage at  $-20^{\circ}\text{C}$ .

#### Nucleic acid extraction from faecal and tissue samples

Viral RNA was extracted from faecal samples using the RNAqueous-Midi kit (Ambion, Austin, Texas, USA) as previously described (Keele et al., 2006; Santiago et al., 2003). Briefly, 6 ml of lysis-binding solution were added to 1.5 ml of faecal sample solution and vortexed vigorously until the sample was thoroughly homogenized. The suspension was clarified by centrifugation (at 4500 RPM for 5 min), and an equal volume of 64% ethanol was added. The solution was passed through a glass fibre filter unit to bind nucleic acids and washed three times with washing buffer. The nucleic acids were then eluted (1200  $\mu\text{l}$ ) and subsequently precipitated with LiCl and spun at 13,000 RPM for 30 min. The resulting pellet was washed once with cold 70% ethanol, air dried, resuspended in 50  $\mu\text{l}$  of RNase free-water and then stored at  $-80^{\circ}\text{C}$ . Total DNA was extracted from liver tissue using the Qiagen Qiaamp<sup>®</sup>DNA Micro Kit according to the manufacturer's instructions (Qiagen, Valencia, California, US); once extracted, RNA was stored at  $-80^{\circ}\text{C}$  and DNA at  $-20^{\circ}\text{C}$ .

#### Amplification of SIVwrc sequences from faecal RNA

RT-PCR amplification of faecal virion was performed using two sets of primers specific for SIVwrc *pol* and *env* sequences as previously described (Locatelli et al., 2008). cDNA was synthesized using the wrcpolR1/wrcenvR1 primers followed by nested PCRs using primers F1/R1 and F2/R2 as inner and outer primers, respectively. The *pol* primers included wrcpolF1 (5'-TAGGGACAGAAAGTATAGTAATHTGG-3') and wrcpolR1 (5'-GCCATWGCYAA TGCTGTTTC-3') as outer primers and wrcpolF2 (5'-AGAGACAGTAAGG AAGGGAA-AGCAGG-3') and wrcpolR2 (5'-GTTCWATTCCTAACCAC-CAGCADA-3') as inner primers for the second PCR round. The *env* primers included wrcenvF1 (5'-TGGCAGTGGGACAA-AAATATAAAC-3'), wrcenvR1 (5'-CTGGCAGTCTCTTCCA AGTTGT-3'), wrcenvF2 (5'-TGATAGGGMTGGCTCCT GGTGATG-3') and wrcenvR2 (5'-AATCCCCATTTAAAC-CAGTTCCA-3'). The amplified regions correspond to the 3' end of the *pol* gene and the gp41 region of the *env* gene. PCRs were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Mannheim, Germany) under the conditions previously described (Locatelli et al., 2008). Briefly, a hot start at  $94^{\circ}\text{C}$  for 2 min was followed by 10 cycles of denaturation at  $92^{\circ}\text{C}$  for 20 s, annealing at  $45^{\circ}\text{C}$  for 45 s, extension at  $72^{\circ}\text{C}$  for 1.5 min, and 20 cycles with the annealing temperature increased to  $50^{\circ}\text{C}$  with extension at  $72^{\circ}\text{C}$  for 1.5 min. Amplification was completed by a final extension at  $72^{\circ}\text{C}$  for 5 min. PCR conditions for the second PCR round were the same except that

the extension time during cycling was 45 s. RT-PCR products from *pol* (~650 bp) and *env* (~570 bp) regions were purified (Q-Biogene, Illkirch, France), and directly sequenced using the inner (F2/R2) primers on an ABI 3130xl Genetic Analyser (Applied Biosystem, Courtaboeuf, France). Sequences were then checked and assembled using the software package Lasergene (DNASTAR Inc. Madison, USA).

#### PCR amplification and sequencing of full-length SIVwrcPbt genome

In addition to the partial *pol* and *env* fragments, a small *gag* fragment was also amplified using degenerate consensus primers designed from the alignment of the *gag* region of two SIVwrc samples from *P. b. badius* (SIVwrc-98CI-04 and SIVwrc-97CI-14, collected in Côte d'Ivoire in the context of an Ebola study (Courgnaud et al., 2003).

The complete SIVwrc genome was then obtained by amplifying overlapping PCR fragments of integrated genomic as well as unintegrated circular DNA using a combination of specific SIVwrcPbt-05GM-X02 primers and consensus primers. More precisely, three fragments spanning the *gag-pol*, *pol-vif-env* and *env-nef-LTR-gag* regions were amplified and specific primers were designed to sequence these fragments. The primers used are shown in Table 1, and the corresponding amplification strategies are depicted in Fig. 2. PCR amplifications were performed using the Long Expand PCR kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Each amplification reaction included a manual hot-start at  $94^{\circ}\text{C}$  followed by 35 cycles with a denaturation step at  $94^{\circ}\text{C}$  for 20 s, an annealing temperature set according to the primer melting temperatures and a variable extension time depending on the size of the expected fragment (1 min/kb). PCR products were purified on agarose gel and directly sequenced 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 3130XL, Applied Biosystems, Courtaboeuf, France) using a primer walking approach. To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences using SEQMAN DNASTAR software (Lasergene, DNASTAR, Inc., Madison, WI).

#### Phylogenetic analysis of partial *pol* and *env* SIVwrc sequences

The new *pol* and *env* sequences were compared to previously published SIVwrc sequences identified in western red colobus from the Taï Forest (Courgnaud et al., 2003; Locatelli et al., 2008). The model of evolution for *pol* and *env* (general time reversible model of evolution with a gamma distribution of substitution rates) was selected under the Akaike information criterion using Modeltest v3.7 (Posada and Crandall, 1998). Bayesian inferences were performed using MrBayes v3.1 (Ronquist and Huelsenbeck, 2003). The tree-space was explored using four chains over 30,000,000 generations sampled every 100 generations. The burn-in value was fixed at 10% of the

total generation number after empirical determination of the convergence. Bayesian parameters were examined with the Tracer program (<http://evolve.zoo.ox.ac.uk/software.html?id=tracer>). *Pol* and *env* phylogenies of red colobus monkeys' SIV were rooted using sequences of corresponding regions in SIVlho from l'Hoest monkeys (*Cercopithecus lhoesti*) (AF188114, SIVsun from sun-tailed monkeys (*Cercopithecus solatus*) (AF131870) and SIVmnd-1 from mandrills (*M. sphinx*) (M27470) as well as SIVolc from olive colobus (*P. verus*) (accession and manuscript in preparation).

#### Sequence and phylogenetic analyses of the full-length SIVwrcPbt-05GM-X02 genome

The new SIVwrc nucleotide sequence was compared to various previously published SIV sequences (see below). Predicted protein sequences were aligned using ClustalW (Thompson et al., 1994); where necessary, minor manual adjustments were performed using SEAVIEW (Galtier et al., 1996). Sites that could not be unambiguously aligned were excluded from the analyses. Proteome sequences were generated by joining deduced Gag, Pol, Vif, Env and Nef amino acid sequences; the carboxy terminal Gag, Pol and Env amino acid sequences that overlapped with Pol, Vif, and Nef amino acid sequences, respectively, were excluded. In order to study whether the newly characterized SIVwrc sequence was recombinant with any of the other SIV lineages, a similarity plot analysis was performed on the proteome sequence alignment with SIMPLOT package version 2.5 (Lole et al., 1999), using a sliding window of 200 amino acids (aa) moved in steps of 20 aa.

Phylogenies were inferred by the Bayesian method (Yang and Rannala, 1997), implemented in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003), run for 2,000,000 to 4,000,000 generations with a 10% burn-in. Parameters were examined with the Tracer program (<http://evolve.zoo.ox.ac.uk/software.html?id=tracer>). Four major regions of the proteome, Gag, Pol1, Pol2 and Env, were selected on the basis of the Simplot analysis. Using the mixed model in MrBayes indicated that the rtREV model of amino acid change (Dimmic et al., 2002) was most appropriate; this model was thus used with gamma distributed rates of substitution across sites.

#### Secondary structure prediction

The transactivation response element (TAR) secondary structure was predicted using the software GENEQUEST DNASTAR (Lasergene, Madison, US).

#### Nucleotide sequence accession numbers

The virus strain whose full length sequence was determined in this study can be found under the GenBank accession number AM937062.

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