Cocirculation of Two env Molecular Variants, of Possible Recombinant Origin, in Gorilla and Chimpanzee Simian Foamy Virus Strains from Central Africa

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Running Head: Genetic variability of Apes-foamy virus *envelope* gene.

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Abstract

Simian foamy virus (SFV) is a ubiquitous retrovirus in non-human primates (NHPs) that can be transmitted to humans, mostly through severe bites. In the past few years, our laboratory has identified more than 50 hunters from Central Africa infected with zoonotic SFVs. Analysis of the complete sequences of five SFVs obtained from these individuals revealed that *env* was the most variable gene. Furthermore, recombinant SFV strains, some of which involve sequences in the *env* gene, have been recently identified. Here, we investigated the variability of the *env* gene of zoonotic SFV strains, and searched for possible recombinants. We sequenced the complete *env* gene or its surface glycoprotein region (SU) from DNA amplified from the blood of: 1) a series of 40 individuals from Cameroon or Gabon infected with a gorilla or chimpanzee FV strain; and 2) one gorilla and three infected chimpanzees living in the same areas as these hunters. Phylogenetic analyses revealed the existence of two *env* variants among both the gorilla and chimpanzee FV strains that also were present in zoonotic and NHP strains. These variants differ greatly (more than 30% variability) in a 753 bp long region located in the receptor-binding domain of the SU whereas the rest of the gene is very conserved. Although the organization of Env protein sequences is similar, the potential glycosylation patterns differ between variants. The analysis of recombination suggests that the variants emerged through recombination events between different strains, although all parental strains could not be identified.

Importance

SFV infection in humans is a great example of a zoonotic retroviral infection that has not spread among human populations, in contrast to human immunodeficiency viruses (HIV)

and human T-lymphotropic viruses (HTLV). Recombination was a major mechanism leading to the emergence of HIV. Here, we show that two SFV molecular *envelope* variants circulate among ape populations in Central Africa and that both can be transmitted to humans. These variants differ greatly in the SU region that corresponds to the part of the Env protein in contact with the environment. These variants may have emerged through recombination events between SFV strains infecting different NHP species.

INTRODUCTION

The emergence of zoonotic viruses is a multi-step process involving the transmission of viruses from domestic or wild animals to humans and their subsequent spread among human populations. Such viral infections are frequent (1). In particular, several viruses originating from non-human primates (NHPs) have had a major impact on human health. Among them, the retroviruses, simian immunodeficiency virus (SIV) and simian T-lymphotropic virus (STLV), have crossed the species barrier from NHPs to humans, leading to the emergence of human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV), respectively, in human populations (2).

Another retrovirus that can be transmitted from NHPs to humans is the simian foamy virus (SFV). SFVs are complex retroviruses from the *Spumaretrovirinae* subfamily that are ubiquitous in both Old and New World NHPs, with a seroprevalence of up to 75–100% in adult NHPs. Phylogenetic studies suggest that SFV have evolved by co-speciation with Old World primates over more than 85 million years (3, 4). Consequently, host-specific groups have emerged. Recombination can also be involved in the genetic diversity of SFV. Indeed, NHPs can become co-infected with strains either from the same group (5-7), or from different groups, as shown by some chimpanzees, which were found to be co-infected with SFVcpz strains and with SFV from colobus monkeys, or Cercopithecus monkeys (5, 8). Co-infection may create recombinant strains, as suggested within the *env, gag* and *pol* genes (5, 7, 9). Of note, both co-speciation and host switching may have contributed to the evolutionary history of SFVs infecting New World prosimians (10).

Humans are not considered to be natural SFV hosts; however, more than 100 cases of human SFV infection have been reported mostly among individuals exposed to NHPs either

in a professional context (e.g. veterinarians or zookeepers) (11-15) or natural settings (e.g. hunters in Africa, and monkey temple workers or visitors, pet owners, or people living around free-ranging macaques in Asia) (16-22). The clinical significance of zoonotic SFV infection remains unknown. This may be due to the very small number of persons yet studied with a limited follow-up (23) and also to a possible recruitment bias (among healthy individuals). SFV is mainly transmitted through biting (22). Indeed, in African green monkeys and macaques, the oral mucosa is a major site for viral replication (24-26) and SFV viral RNA accumulates at high concentrations in saliva (25, 27). In humans, SFV infection is persistent. SFV DNA is detectable in peripheral blood and saliva cells. However, cell-associated viral RNA has not been detected (28), (21) and secondary human-to-human transmission (22, 29) has not been reported. Hence, human infection with zoonotic SFVs represents a unique natural model to study the role of viral and immunological factors in the restriction of viral transmission.

In the past few years, we have undertaken a large epidemiological and molecular study involving hunters of monkeys and apes, living in Central Africa, to obtain insight into the natural history of SFV emergence in humans. We identified a large series (more than 50) of hunters that had been infected with SFVs, mostly following severe bites from NHPs (17, 22). We previously showed that the virus mostly localizes to CD8+, CD4+ T, and B lymphocytes in the blood of infected humans (30). Our study also revealed natural polymorphisms in *gag* and *bet* between SFV strains originating from different chimpanzee subspecies and polymorphisms in U3 and *tas* at the inter-individuals level (31). Based on the complete sequence of five replication-competent strains, we found no evidence of viral adaptation among SFV strains isolated in humans (31).

Our previous study revealed some genetic diversity in the *env* gene of zoonotic strains of SFV from Apes. The *env* gene is involved in different viral cycle steps such as receptor binding (32, 33), fusion (34-36), budding (37-39) and site of particle formation (40, 41). The broad tropism of SFV suggests an ubiquitous cellular receptor (42, 43). While all SFVs seem to use the same cellular receptor (44-46), only heparin sulfate has been identified as an attachment factor so far (47, 48).

Here, we aimed to investigate the genetic variability of *env* among SFVs infecting humans or NHPs living in Cameroon and Gabon. We found that viral strains from both gorilla and chimpanzee FVs segregate into two *env* variants. These variants co-circulate among humans and NHPs, and may have arisen by recombination.

MATERIALS AND METHODS

Population

Human population: In three previous studies, we identified 48 individuals from Cameroon who were infected either with a SFV from a *Gorilla gorilla gorilla* (*Ggo*) or from a *Pan troglodytes troglodytes* (*Ptr*) as defined by the analysis of a 465 bp-long *pol-integrase* fragment (17, 22, 49), In a few cases, SFV was detected by analyzing a smaller LTR fragment. These individuals live in villages or settlements located in the rain forest of South and East Cameroon. They belong to different Bantu tribes or the Baka and Bakola Pygmy tribes. Fourteen *Ggo*- or *Ptr*-FV infected individuals from Gabon, all of whom were Bantus, were also included in this study (20).

This study was approved by the research division of the Ministry of Public Health and the National Committee of Ethics in Cameroon, the Ministry of Health and the Ethics Committee

of the CIRMF in Gabon, and the Comité de Protection des Personnes and the Commission Nationale de l'Informatique et des Libertés in France. All individuals provided written informed consent.

NHP population: One *Ggo* from Cameroon and three *Ptr* (one from Cameroon and two from Gabon) were included in this study. NHPs from Cameroon were born and caught in the wild in the Southern rain forest area (50, 51). NHPs from Gabon were all born in the wild (20). Samples were collected in accordance with the rules of animal care committees.

Sampling and DNA preparation

Blood samples were collected from all individuals and NHPs in EDTA tubes. The buffy coat (BC) was obtained after centrifugation and genomic DNA was extracted using the Qiamp DNA Blood minikit (Qiagen, Courtaboeuf, France). DNA samples were quantified using a Nanodrop instrument (ThermoScientific).

PCR amplification of *env* from genomic DNA

Regarding *Ggo*-FVs, three different overlapping *env* fragments (720, 1102 and 1490 bp-long) were amplified from BC-DNA using specific primers (Table 1). Nested PCR was performed as follows: 500 ng of DNA was mixed in the enzyme buffer with the external primers (0.25 µM of each), $MgCl_2$ (3.5 mM), dNTP (200 µM of each) and 0.5 µL of HotStarTaq polymerase (Qiagen) in a final volume of 50 µL. The external PCR consisted of a 15' long denaturation step at 95°C, followed by 40 amplification cycles (45'' at 95°C, 45'' at 57°C and 1' per kb at 72°C) and a 7' long extension step at 72°C. The product (5 µL) was then used as template for a second internal PCR in the same conditions, but using the internal primers (table 1).

Regarding *Ptr*-FVs, two different overlapping *env* fragments (1645 and 1802 bp) were amplified from BC-DNA using specific primers (Table 1). The PCR parameters were the same

as those used for the *Ggo*-FV strains, except for the annealing temperature of the PCR of the second fragment (60°C instead of 57°C).

The *env* gene of the prototypic *Pan troglodytes verus* SFVpvr.SFV7 strain (52) was amplified from SFVpvr.SFV7-infected BHK-21 cells (a gift from A. Rethwilm) using the CPZENVF1 and CPZENVR2b primers (Table 1) with an annealing temperature of 57°C and an extension time of 3'30''.

PCR products were directly sequenced by MWG Operon (Ebersberg, Germany). Both sense and antisense sequences were obtained for each fragment and were found to be identical. To obtain complete *env* sequences, the different *env* fragments were concatenated (the overlapping regions were identical in all samples).

Phylogenetic analyses

Multiple sequence alignments on the previously known (Table 2) and the newly generated sequences were performed using the DAMBE program (v4.2.13 [http://www.dambe.bio.uottawa.ca]). Modeltest v3.6 was used to select the most appropriate nucleotide substitution model, based on the Akaike information criterion (AIC). GTR was found to be the best-fitting model. Phylogenetic trees were constructed using the Neighbor Joining method and bootstrap values were calculated on 1,000 replicates. Phylogenetic tree topologies were confirmed using the maximum likelihood method with the PAUP program (v4.0 [http://www.paup.csit.fsu.edu]). Percentage identity was calculated by CLC software (CLC DNA Workbench 6 [http://www.clcbio.com]) on the alignment obtained with DAMBE.

By definition, a clade is a monophyletic group supported by a strong bootstrap value. Herein, we used "group" as a host-specific clade, and "subgroup" as a clade defined on the central region of *env*.

Recombinant analyses

To search for potential recombination events, similarity plot and bootscanning analyses were performed with Simplot software (v3.5.1 [http://www.simplot.com]). These analyses were performed with default parameters, except for the bootscan repetitions (set to 1,000) and the evolution model (Kimura 2-parameter). The similarity plot depicts a similarity score between a group of interest (query) and other groups (defined based on the phylogenetic tree) over a 200 bp-long region and the overall *env* gene was analysed by 20 bp-long steps. The bootscan analysis reflects the phylogenetic relationship (bootstrap value) between a group of interest (query) and the other groups (window 200 bp, step 20 bp).

The Recombination Detection Program (RDP4 (53)) was also used to investigate putative recombination. Unlike the Simplot derived studies, the RDP method looks for recombination by analyzing every possible sequence triplet (instead of considering groups). A window is moved along the genome and a percentage of identity between each of the three possible pairs is calculated at each position. A *p*-value is calculated to determine the likelihood that the potential recombination event is due to chance.

Protein analysis

Percentage identity was calculated by CLC software (CLC DNA Workbench 6) on the alignment obtained with DAMBE after the translation of the sequences into amino acids. Putative glycosylation sites were identified by the NetGlyc 1.0 program and consisted of

189 NXS/T sites, where X denotes any amino acid except proline [http://www.cbs.dtu.dk/
190 services/NetNGlyc].

191 **Nucleotide sequence accession number**

192 All complete and partials *env* sequences generated in this work are published in GenBank.
193 Complete sequences are published through accession numbers KT211246 to KT211269, the
194 latter corresponding to SFVpvr.SFV7 sequence; partial sequences through accession
195 numbers KT211270 to 211290.

196

197 **RESULTS**

198 We previously identified 48 SFV-infected individuals in Cameroon (17, 22, 49) and 14
199 SFV-infected individuals in Gabon (20). The strains belonged to the *Gorilla gorilla gorilla*
200 (*Ggo*)- or *Pan troglodytes troglodytes* (*Ptr*)-FV groups according to their *pol* and/or LTR
201 sequences. In addition, we previously obtained complete SFV sequences (Bad468, Bak74,
202 AG15 and Bad327) from four Cameroonian individuals (31). Buffy coat or DNA samples were
203 available for 51 of the remaining 58 individuals. From these samples, 40 new *env* sequences
204 were generated by PCR and direct sequencing (19 *env* sequences were complete and 21
205 partial). Low viral load may explain the absence of amplification in the 11 remaining
206 samples. Thus, overall, our study population yielded 44 *env* sequences from zoonotic SFVs
207 (33 *Ggo*-FV and 4 *Ptr*-FV from Cameroon, five *Ggo*-FV and two *Ptr*-FV from Gabon) (Table 3,
208 Figure 1).

209 In addition, we also obtained complete *env* sequences from four wild-born NHPs
210 living in the same region as the infected individuals (one gorilla, and three chimpanzees) and

211 we sequenced the *env* gene of a prototypic SFVpvr.SFV7 *Pan troglodytes verus* strain (Table
212 3).

213 All newly obtained sequences contained an Open Reading Frame of 2958 to 2970 bp
214 and were unique.

215

216 ***Complete env sequences define host-specific SFV groups***

217 We analyzed phylogenetically the complete *env* sequences using the neighbor joining
218 (Figure 2) and the maximum likelihood methods (data not shown). The newly generated
219 complete *env* sequences were included together with the available SFV *env* sequences in
220 Genbank (Table 2). The topologies of both phylogenetic trees were comparable. Host-
221 specific groups (Gorilla, Chimpanzee, Cercopithecus, Macaque) were identifiable and
222 supported by strong bootstraps (>90). Moreover, among the strains from the chimpanzee-FV
223 group, we identified three different clades corresponding to different subspecies: *Pan*
224 *troglodytes troglodytes*, *P. t. schweinfurthii* and *P. t. verus*.

225 Interestingly, the gorilla-specific FV group was composed of two monophyletic
226 subgroups supported by strong bootstraps. These subgroups did not correspond to
227 geographic segregation. Indeed, each subgroup contained sequences from both Cameroon
228 and Gabon (underlined strains on the tree; Figure 2), as well as both zoonotic and NHP
229 strains (SFVggo or SFVggo.Cam7). We named the subgroup containing the previously
230 described Bad468 *env* FV sequence, SFV-GorI, and the one containing the Bak74 *env* FV and
231 the prototypic SFVggo sequences, SFV-GorII.

Similarly, two phylogenetic subgroups were identifiable within the *Ptr*-FV group. We named the one containing the previously described Bad327 and AG15 *env* sequences, SFV-CpzII, and the other one, SFV-CpzI.

Definition of a central variant region within the surface env sequence

We performed sequence alignments to understand better the origins of the two subgroups present in the *Ggo*-FV and *Ptr*-FV groups.

Concerning the *Ggo*-FV strains, SFV-GorI *env* sequences were 2970 bp long, whereas SFV-GorII sequences were 2964 bp long. Complete sequences within a subgroup were 96.4 to 99.8% identical, whereas those belonging to different subgroups were only 88.2 to 90.4% identical (Figure 3.A). SFV-GorI and SFV-GorII sequences differed the most in a central region beginning at position 769 bp (red line) and ending at position 1473 bp (green line; the positions were defined on the *env* PFV strain) (Figure 3.B). In the *Ptr*-FV strains, SFV-CpzI sequences were 2967 bp long whereas SFV-CpzII sequences were 2958 bp long. These two *Ptr*-FV subgroups differed the most between positions 721 (red line) and 1440 (green line) (Figure 3.C). Interestingly, this corresponds closely to the same variable region defined for *Ggo*-FV strains.

Thus, we defined two regions in the *env* sequence (Figure 3.D): a conserved region, which comprises nucleotides 1 to 720 and 1474 to 2967 (positions defined in the PFV strain), and a variant region, from 721 to 1473. We confirmed a high sequence similarity (96.4 to 99.3% identity) in the conserved region between the SFV-GorI and SFV-GorII subgroups, and a very important divergence (62.2-64.7% identity) in the variant region (Figure 3.D). Similarity of the variant region was very high between strains from the same

subgroup (94.8-100% identity). This was also true for the two *Ptr*-FV subgroups (SFV-CpzI and SFV-CpzII). Strikingly, in the variant region, SFV-GorI and SFV-CpzI showed an identity score of 71.7-73% which is higher than that between SFV-GorI and SFV-GorII (62.2-64.7%) (Figure 3.D).

Two variants are present within SFV-groups

Given that a conserved and a variant region were defined, we performed phylogenetic analyses on each segment separately.

In the conserved region (Figure 4.A), sequences segregated following host-species and subspecies. The four major monophyletic and highly supported groups corresponded to SFV strains of gorilla, chimpanzee, macaque or Cercopithecus origin. Moreover, *Ggo*-FV strains were now subdivided into groups reflecting geographic origin (two Cameroonian clades and one Gabonese clade, underlined in Figure 4.A). This distribution resembles that of the *pol*-based phylogenetic tree established in previous studies on the same individuals (20, 22).

The phylogenetic tree based on the variant region (Figure 4.B) was quite different. The *Ggo*- and *Ptr*-FV segregated according to the subgroups identified previously (SFV-GorI, SFV-GorII, SFV-CpzI and SFV-CpzII,). In this phylogenetic tree, we could define two clades for African Ape SFVs (clade 1 and clade 2), each comprising both *Ggo*-FV and *Ptr*-FV strains. Of note, *P.t.verus*-FV sequences (SFV7 and SFVcpz) were also split: the SFV7 *env* sequence was closer to the SFV-CpzII sequences, whereas the SFVcpz *env* was closer to the *P.t.schweinfurthii* PFV and the SFV-CpzI strains. Finally, macaque- and cercopithecus-FV isolates were also divided into two distinct subgroups.

To confirm these findings, in the analysis of the variant region, we included the 21 partial *env* sequences obtained from other individuals infected by a *Ggo*-FV strain (Table 3) and 21 previously published African Green Monkey (AGM, cercopithecus group) *env* SU sequences (Table 2)(54). Phylogenetic analysis of the variant region (Figure 4.C) confirmed the robustness of the two major African Ape clades (clade 1 and clade 2) and of the different subgroups SFV-GorI, SFV-GorII, SFV-CpzI and SFV-CpzII. Interestingly, cercopithecus strains separated into two major subgroups: SFV-CercI and SFV-CercII. Macaque-FV strains were also split into two distinct subgroups (SFV-MacI and SFV-MacII). Of note, the unrooted phylogenetic tree was star-shaped (Figure 4.C), suggesting that, although SFV-GorI and SFV-CpzI are the closest groups, the sequences are still genetically distant and do not share a recent common ancestor.

Thus, the strains from four simian species (gorilla, chimpanzee, macaque, Cercopithecus) systematically segregate into two subgroups that differ regarding the *env* central region.

Genomic and functional organization of the envelope gene

All protein sequences generated from the new *env* sequences showed typical Env organization with the leader peptide (LP), the surface glycoprotein (SU) and transmembrane glycoprotein (TM) regions separated by RXXR cleavage signals (data not shown). Other important domains such as the fusion peptide (FP) or the membrane-spanning domain (MSD) were also present (figure 5.A, data not shown). Every cysteine residue (essential for proper folding) described for PFV Env (32) was also conserved.

The variant region was located within the SU domain, more specifically within the Receptor Binding Domain (RBD) identified on PFV (32). Given that glycosylation is important for RBD function, we examined the conservation of the putative glycosylation sites. Although amino acid sequences diverge in the central region between variants (figure 5.B), 13 of the potential glycosylation sites (previously defined on PFV) were conserved among *Ggo*- and *Ptr-FV* strains (55)(figure 5.C). *Ggo*-FV subgroups differed at position of N11 and chimpanzee-FV subgroups differed at positions of N11, N5 and N8. Thus, the different subgroups might have different glycosylation patterns.

Could variants be generated by recombination?

We performed bootscan analysis on the *env* sequences grouped by host specific subgroups to search for potential recombinants among *Ggo*- and *Ptr*-FV strains (Figure 6). Consistent with the phylogenetic trees, the conserved region of SFV-GorII *env* sequences was closely related to SFV-GorI (more than 99% of permuted trees grouped them together), whereas the variable region was closer to sequences from SFV-CpzII (Figure 6.A). Similarly, the conserved region of SFV-CpzI strains was closer to that of SFV-CpzII strains whereas the variable region of SFV-CpzI strains was closer to that of SFV-GorI strains (Figure 6.C).

Such a pattern suggests that strains from the SFV-GorII subgroup originated from a recombination event between SFV-GorI and SFV-CpzII strains. However, similarity plot analysis argues against this conclusion. Indeed, the similarity score between SFV-GorII and SFV-CpzII strains is low throughout the whole gene, and is lowest in the variant region (between 52 and 62% similarity)(Figure 6.B). Thus, if the strains from the SFV-GorII

subgroup did indeed appear by recombination, one of the parental strains remains unknown.

To investigate further this hypothesis, we performed a Recombinant Detection Program (RDP) analysis to detect recombinant sequences. This analysis strongly suggests that the strains from the SFV-GorI and SFV-GorII subgroups diverged upon recombination (*p*-value=$4.745.10^{-12}$). Similarly, the SFV-CpzI and SFV-CpzII subgroups probably recombined (*p*-value=$2.074.10^{-12}$). However, in both cases, the parental strain from which the central region was obtained could not be identified.

We conclude that the two variants observed among *Ggo*- and *Ptr*-FV strains probably arose by a recombination event, and that one of the parental strain is still unknown.

DISCUSSION

Here, we studied the variability of the *envelope* gene of SFVs infecting Apes (gorillas and chimpanzees) and humans living in Cameroon or Gabon. We demonstrate the co-circulation of two SFV *env* molecular variants in both the gorilla- and chimpanzee-FV groups. In a given group, the complete nucleotide sequence of *env* differs by more than 10% between the variants. These differences are mostly located in a central region of the *envelope* that we have called the "variant" region, in contrast with the "conserved" region that corresponds to the rest of the *env* gene. The genetic diversity of *env* may have arisen from recombination. These findings raise several important issues and questions:

1) Genetic variability in the conserved region reflects host-species origin and geographical clustering.

In the conserved region, the nucleotide variability among *Ggo*-FV strains was low, ranging from 0.2% to 3.6%. This genetic stability is consistent with the low *in vivo* variation rate estimated to be around $1.7.10^{-8}$ substitutions per site per year (3). The genetic variability of the conserved regions of the different *Ggo*-FV reflects geographic location. Indeed, we defined two clusters of Cameroonian sequences and a cluster from strains isolated in Gabon by phylogenetic analysis. Interestingly, these clusters were observed previously when the same strains were divided according to their *pol* sequence (20, 22). Similarly, geographic segregation was also observed in SFV strains in macaque populations in Bangladesh (7), and in mandrill populations in Gabon (56). Indeed, geographic segregation and consequent isolation lead to the accumulation of distinct mutations and speciation.

By contrast, Cameroonian and Gabonese *Ptr*-FV sequences did not segregate together, consistent with the findings of studies on the *pol-integrase* gene (20, 22). This may suggest that, unlike Gorillas, *Ptr* populations are not genetically or virologically isolated, despite being geographically distant. However such results are based on few sequences.

In the central variant region, we found two subgroups for each FV host-specific group with more than 35% nucleotide variability. This variability did not reflect geographic location. Strains from every *Ggo*- and *Ptr*-FV subgroup were present in both humans and NHPs.

2) What is the mechanism leading to the emergence of *env* diversity among the SFV variants?

We first examined whether recombination, which is a frequent evolutionary event in retroviruses, could explain the diversity of *env* sequences. (57-60). Indeed, recombination is also frequent among SFVs. *In vitro* studies show that it can occur between PFV-based vectors and reveal that the probability of a template-switching event within a 1 kb-long region is 27% (61).

Our analyses suggested that a *Ggo*-FV variant originated from recombination between *Ggo*-FV strains and unknown FV strains (the closest known strain being the chimpanzee strains). Similarly one of the *Ptr*-FV variants may have arisen from recombination between chimpanzee- and gorilla-like FV strains. Until the parental strains are found, the recombination origin of these sequences might be debated. However this hypothesis is the most realistic.

Such a scenario implies that NHPs can be co-infected with SFV strains of different host-species, and that these strains have recombined. Co-infection has been documented in chimpanzees (5, 6) and rhesus macaques (7). However, in these cases, co-infection occurred with strains from the same host-specific group. Interestingly, in these populations, recombination in FVs has been observed in the *pol* or *gag* gene (5, 7). Furthermore, co-infection with strains belonging to different NHP-related groups has been documented in wild chimpanzees. These animals were infected with both Colobus monkey- and chimpanzee-related FVs (5, 8) but no recombinant strain was detected. Recently, recombination between macaque- and Cercopithecus-FV strains has been reported (9, 62). However, macaques and Cercopithecus live in different continents, suggesting that the recombination event occurred in captivity. Indeed, animals from various species and/or geographical areas are frequently mixed in primate centers. The variants we report would

be the first recombinants detected between SFVs of different primate hosts found in a natural setting.

Interestingly, the putative recombination sites were quite similar between the gorilla- and the chimpanzee-FV variants. This site also corresponds closely to the same region described in macaque (9) and African Green Monkey FV sequences (54). A similar variant region, with similar recombination points, has been described in feline FVs (63). Together, this suggests that a recombination hot-spot may exist in the *env* region of foamy viruses.

Our data suggest the different *env*-based subgroups arose by recombination, but many questions remain unsolved. First, the identity of one of the parental strains is still undetermined. This second parental strain, which purportedly introduced the central variable region into the *env* gene, may be either an existing but yet undescribed FV, or a strain that has disappeared. Second, the date of the recombination events has not been addressed. If the recombination event was ancient, genetic drift would have segregated SFV-GorI and SFV-GorII even in the conserved region. Given that the conserved region is very similar between SFV-GorI and SFV-GorII strains and that they do not form separate monophyletic groups in the conserved region, the recombination event probably occurred fairly recently.

3) Why are only two *env* genetic variants present?

Strikingly, we observed two *env* molecular variants for each NHP species studied (i.e. gorilla, chimpanzee, macaque and Cercopitecus). If the defined sites were hot spots of recombination, we would expect many more variants to be generated. Strong functional

412 constraints may be an effective source of purifying selection that would only allow for the
413 emergence of two subgroups per host.

414 The variant central region is located in the putative receptor-binding domain of the
415 SU domain, as identified on PFV (32). SFV uses heparin sulfate to attach to target cells (47,
416 48), but the receptor for SFV is still unknown (64). Interference studies showed that one
417 cellular receptor is used by all SFV (44 , 45 , 46). Binding studies of recombinant envelope
418 protein suggest that SFVcpz interacts with two receptors, one low affinity and one high
419 affinity (65). Although most glycosylation sites are conserved, we found that the pattern of
420 glycosylation may differ between the *Ggo*- and *Ptr*-FV subgroups. Consequently, it is possible
421 that the two FV variants correspond to viruses that use different receptor complexes, as this
422 is the case for Murine Leukemia Virus (66). Alternatively, the SFV receptor may be expressed
423 in different conformations at the cell surface, allowing two modes of engagement by the two
424 Env variants. Indeed, such conformational heterogeneity was described for the CCR5
425 molecule and its interaction with the envelope of HIV and chemokines (67).

426 Finally, *env* molecular variants will probably induce distinct immune responses. The
427 Env SU is indeed the target of neutralizing antibodies (65). Two *env* genotypes
428 corresponding to two serotypes have been described among feline FVs (63, 68). The two
429 genotypes differ in a central region corresponding nearly to the one described in our study.
430 SFV strains from macaques and chimpanzees also segregate into two serotypes as defined by
431 neutralization assay with immune plasma or sera, serotype 1 and 2 for macaques (69),
432 serotype 6 and 7 for chimpanzees (52, 70, 71). We show here two cases in which strains
433 belonging to different serotypes belong to distinct genetic subgroups. Indeed SFV-mcy1 and
434 SFV-mcy2 are from serotype 1 and 2 respectively while belonging to SFV-MacI and SFV-

MacII respectively (fig. 4C); PFV/SFVcpz and SFVpvr.SFV7 are from serotype 6 and 7 respectively while segregating with SFV-CpzI or SFV-CpzII respectively (fig. 4C). Preliminary results on neutralizing antibodies present in the plasma of infected persons support the correspondence between serotype and genotype for SFV from the chimpanzee group (72). Studies are currently ongoing to characterize immune responses induced by SFV strains from the gorilla group.

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Figure 1. Geographic location of the 44 individuals infected with *Ggo*- or *Ptr*-foamy virus strains. Individuals infected with *Ggo*-FV strains are indicated by a circle: purple for SFV-GorI strains and blue for SFV-GorII strains. Individuals infected with a *Ptr*-FV strain are indicated by a square: yellow for SFV-CpzI strains and orange for SFV-CpzII strains. These colors will be used in all figures.

Figure 2. Phylogenetic analysis of complete simian foamy virus *envelope* sequences. Phylogenetic analysis of complete *env* sequences (2967 bp long for the PFV strain) from 43 SFV isolates including the 24 complete sequences generated in this study (in bold) and 19 previously published sequences. SFVmarm, a marmoset strain from South America, was the outgroup. Gabon isolates are underlined and asterisks indicate FVs isolated from non-human primates. The phylogenic tree was derived by the neighbor-joining method using the GTR model (gamma=0.6749). Horizontal branch lengths are drawn to scale, with the

bar indicating 0.1 nucleotide replacements per site. Numbers on each node indicate the bootstrap value (calculated on 1,000 replicates) supporting the group.

Figure 3. Definition of a conserved and a variant region in the *envelope* gene of *Ggo*- and *Ptr*-foamy virus. A, D: (%) nucleotide identity between the different subgroups of *Ggo*- and *Ptr*-FV in the complete (A), the conserved (D) or the variant region (D) of the *envelope* gene. Percentage identity was determined after alignment by the DAMBE program and using CLC software. B, C: Gorilla and chimpanzee foamy virus sequences were aligned using DAMBE software. The region from 698-798 bp and 1398-1498 bp (positions defined according to the PFV *env* sequence) are shown for *Ggo*-FV sequences (B) and *Ptr*-FV sequences (C), respectively. Dots indicate identity. The red vertical lines indicate the position between two codons where the *env* sequences diverge into separate variants (defined on the right of the alignment) and the green vertical lines show the position where the variants become very similar again.

Figure 4. Phylogenetic analysis of the *envelope* conserved and variant regions. Phylogenetic trees corresponding to (A) the conserved region (2214 bp for PFV) or (B) the variant region (753 bp for PFV) were derived from the neighbor joining method (GTR; gamma = 0.6749). Horizontal branch lengths are drawn to scale, with the bar indicating 0.1 nucleotide replacements per site. Bootstrap values were calculated on 1,000 replicates. The SFVmarm strain was used as an outgroup; Gabon isolates are underlined and asterisks indicate FVs isolated from non-human primates.

C- Unrooted phylogenetic tree of the variant region. Closely related strains that formed a robust cluster (as seen on B) are represented with black triangles. AGM A, B, C and D are clusters of African Green Monkey (Cercopithecus) sequences previously defined by Schweizer (54).

Figure 5. Comparison of Env protein of *Ggo*-FV and *Ptr*-FV strains. A: Structure and important domains of Env FV protein present in all sequences of this study. LP: leader peptide, SU: Surface glycoprotein, TM: Transmembrane glycoprotein; WXXW motif: Trp-X-X-Trp motif, interaction site with Gag protein; RXXR: Arg-X-X-Arg cleavage sites; RBD: Receptor Binding Site; FP: fusion peptide (i, i+3/4,i+7 pattern); MSD: Membrane Spanning Domain; ERRS: Endoplasmic Reticulum Retrieval Signal (Lys at -3, Lys or Arg at -4 or/and -5 relative to C-terminus). B: Amino acid identity in the conserved and variant regions was investigated using CLC software based on the alignment performed with DAMBE. C: Location of N-glycosylation sites of the different *Ggo*- and *Ptr*-FV subgroups.

Figure 6. Recombination analysis of the SFV-GorII and SFV-CpzI *envelope* sequences. A, B, C, D: Bootscan (A,C) analyses and (B,D) Simplot analyses using the SFV-GorII (A,B) or SFV-CpzI (C,D) *env* sequences as a query against the other groups of SFV isolates were performed.

Gorilla FV	Length			Name	Sequence 5'->3'	Site of hybridization /SFVggo (bp)
Fragment 1	720 bp	External PCR	F	AENVF1b	GAACTGTGGTAATTGTGGACC	6566-6586
			R	AENVR1b	CCACGAGACCAAGAACAATA	7292-7311
		Internal PCR	F	AENVF1	GGTAATTGTGGACCATCTTGG	6573-6593
			R	AENVR1	GGATCCACGAGACCAAGAAC	7288-7307
Fragment 2	1102 bp	External PCR	F	BF3	CATCCACCCCTCCTGCCT	6431-6448
			R	RR3	CCTGTAAATGAAATGCCTAAT	8228-8248
		Internal PCR	F	DF3	CTATAATACACACGGAGAGG	7113-7132
			R	RR3	CCTGTAAATGAAATGCCTAAT	8228-8248
Fragment 3	1490 bp	External PCR	F	AENVF2	TACGACAACAAGATTATGAAG	8109-8129
			R	AENVR3	CTGAGTGAGCTTGTTGGTCC	9640-9659
		Internal PCR	F	AENVF2b	ATATCAAGAATGTAAGTTGG	8140-8159
			R	AENVR3b	TCTGCAAACTCTGAGTGAGC	9630-9649
Chimpanzee FV	**Length**			**Name**	**Sequence 5'->3'**	**Site of hybridization /PFV (bp)**
Fragment 1	1645 bp	External PCR	F	CPZENVF1b	ACTGTTGTTATTTTGGACCA	7066-7085
			R	CPZENVR1	CCTTTGTAGGCCTAGTAGAT	8763-8782
		Internal PCR	F	CPZENVF1	GGCAACAACAGAACTGTAAG	7090-7109
			R	CPZENVR1b	CCTGTAAATGAAATGCCTAA	8735-8754
Fragment 2	1802 bp	External PCR	F	CPZENVF2	TTCTCTTTGTGGGAAGGAG	8330-8349
			R	CPZENVR2	CTTAGTGAGCTTGTTGGTCC	10141-10160
		Internal PCR	F	CPZENVF2b	TCTTTGTGGGAAGGAGATTG	8333-8352
			R	CPZENVR2b	CAGACTCTTAGTGAGCTTGT	10135-10154

Table 1. Polymerase chain reaction primers used to amplify the *envelope* gene of gorilla and chimpanzee foamy virus strains.

F: Forward; R: Reverse; bp: base pair. Position / SFVggo or PFV complete sequences.

Isolate	Strain	Origin	Type of sequence	Genbank Accession no.
PFV	Chimpanzee (*P. t. schweinfurthii*)	human	complete genome	Y07725
SFVcpz	Chimpanzee (*P. t. verus*)	NHP	complete genome	U04327
AG15	Chimpanzee (*P. t. troglodytes*)	human	complete genome	JQ867462
Bad327	Chimpanzee (*P. t. troglodytes*)	human	complete genome	JQ867463
SFVggo	Gorilla (*G. g. gorilla*)	NHP	complete genome	HM245790
Bak74	Gorilla (*G. g. gorilla*)	human	complete genome	JQ867464
Bad468	Gorilla (*G. g. gorilla*)	human	complete genome	JQ867465
SFVora	Orangutan	NHP	complete genome	AJ544579
SFVAGMhu	African Green Monkey (*Chlorocebus aethiops*)	human	complete genome	AX575326
SFV-agm3	African Green Monkey (*Chlorocebus aethiops*)	NHP	complete genome	NC_010820
SFVka	African Green Monkey (*Chlorocebus aethiops*)	human	partial *env*	AJ244092
SFV3	African Green Monkey (*Chlorocebus aethiops*)	NHP	partial *env*	AJ244094
agm1 to agm37 (19 isolates)	African Green Monkey (*Chlorocebus aethiops*)	NHP	partial *env*	AJ244067 to AJ244091
AG16	Cercopithecus	NHP	complete genome	JQ867466
SFV-mcy1	Macaque *(Macaca cyclopis)*	NHP	complete genome	NC_010819
SFV-mcy2	Macaque *(Macaca cyclopis)*	NHP	complete genome	KF026286
SFVR289HybAGM	Macaque *(Macaca mulatta)*	NHP	complete genome	JN801175
SFVmmu-K3T	Macaque *(Macaca mulatta)*	NHP	*env*	KF026287
SFVmmu-A4W	Macaque *(Macaca mulatta)*	NHP	*env*	KF026288
SFVsp	Spider monkey	NHP	complete genome	EU010385
SFVmar	Marmoset	NHP	complete genome	GU356395
SFVsq	Squirrel monkey	human	complete genome	GU356394

Table 2. Genbank accession numbers and origin of other SFV isolates used in the study.

NHP: Non Human Primate.

Human individuals	Country	Ethnicity	Individual	Sex	Age at cont/samp	FV strain	Type of *env* FV sequence	FV variant	FV isolate (accession number)
	Cameroon	Pygmy	Pyl106	M	15/60	Chimpanzee	Complete	SFV-CpzI	SFVptr-hu.Pyl106 (KT211263)
			BAK46	M	26/50	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bak46 (KT211255)
			BAK74	M	26/47	Gorilla	Complete	SFV-GorII	**Bak74 (JQ86746)**
			BAK82	M	46/50	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bak82 (KT211247)
			BAK228	M	29/70	Gorilla	Complete	SFV-GorII	SFVggo-hu.Bak228 (KT211246)
			BAK242	M	30/49	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bak242 (KT211254)
			Sabak36	M	40/68	Gorilla	Complete	SFV-GorII	SFVggo-hu.Sabak36 (KT211249)
			BAK33	M	25/45	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak33 (KT211277)
			Bak55	M	30/65	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak55 (KT211273)
			BAK56	M	40/65	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak56 (KT211274)
			BAK132	M	30/64	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak132 (KT211276)
			BAK177	M	26/36	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak177 (KT211281)
			BAK224	M	19/38	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak224 (KT211280)
			BAK232	M	40/60	Gorilla	Partial	SFV-GorII	SFVggo-hu.Bak232 (KT211278)
			BAK270	M	25/60	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bak270 (KT211272)
			Bobak153	M	53/59	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bobak153 (KT211279)
			Bobak237	M	?/68	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bobak237 (KT211275)
			Lobak2	M	37/57	Gorilla	Partial	SFV-GorI	SFVggo-hu.Lobak2 (KT211282)
			Lobak89	M	20/50	Gorilla	Partial	SFV-GorI	SFVggo-hu.Lobak89 (KT211287)
			Mebak65	M	20/40	Gorilla	Partial	SFV-GorI	SFVggo-hu.Mebak65 (KT211283)
			801001	M	35/60	Gorilla	Partial	SFV-GorI	SFVggo-hu.801001 (KT211284)
			CH29	M	49/50	Gorilla	Partial	SFV-GorI	SFVggo-hu.CH29 (KT211289)
		Bantu	**AG15**	M	28/71	Chimpanzee	Complete	SFV-CpzII	**AG15 (JQ867462)**
			Bad316	M	35/51	Chimpanzee	Complete	SFV-CpzII	SFVptr-hu.Bad316 (KT211262)
			Bad327	M	30/33	Chimpanzee	Complete	SFV-CpzII	**Bad327 (JQ867463)**
			BAD348	M	19/27	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bad348 (KT211252)
			BAD456	M	24/30	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bad456 (KT211251)
			BAD463	M	37/43	Gorilla	Complete	SFV-GorI	SFVggo-hu.Bad463 (KT211253)
			BAD468	M	25/35	Gorilla	Complete	SFV-GorI	**Bad468 (JQ867465)**
			BAD551	M	37/38	Gorilla	Complete	SFV-GorII	SFVggo-hu.Bad551 (KT211248)
			CH101	M	65/76	Gorilla	Complete	SFV-GorII	SFVggo-hu.CH101 (KT211256)
			BAD332	M	25/37	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bad332 (KT211288)
			BAD349	M	32/40	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bad349 (KT211270)
			BAD350	M	40/68	Gorilla	Partial	SFV-GorII	SFVggo-hu.Bad350 (KT211290)
			BAD447	M	40/56	Gorilla	Partial	SFV-GorI	SFVggo-hu.Bad447 (KT211271)
			AKO394	M	53/53	Gorilla	Partial	SFV-GorII	SFVggo-hu.AKO394 (KT211286)
			CH61	M	52/65	Gorilla	Partial	SFV-GorI	SFVggo-hu.CH61 (KT211285)
	Gabon	Bantu	H3Gab56	M	47/48	Chimpanzee	Complete	SFV-CpzII	SFVptr-hu.H3Gab56 (KT211267)
			H4Gab59	M	50/51	Chimpanzee	Complete	SFV-CpzII	SFVptr-hu.H4Gab59 (KT211268)
			H2Gab54	M	52/53	Gorilla	Complete	SFV-GorI	SFVggo-hu.H2Gab54 (KT211257)
			H5Gab27	M	53/80	Gorilla	Complete	SFV-GorI	SFVggo-hu.H5Gab27 (KT211258)
			H6Gab51	M	28/56	Gorilla	Complete	SFV-GorI	SFVggo-hu.H6Gab51 (KT211259)
			H7Gab42	M	20/65	Gorilla	Complete	SFV-GorII	SFVggo-hu.H7Gab42 (KT211261)
			H12Gab69	M	22/38	Gorilla	Complete	SFV-GorI	SFVggo-hu.H12Gab69 (KT211260)
Non Human Primates	**Country**	**Species**	**Name**	**Sex**	**Age at sampling**	**Situation**	**Type of *env* FV sequence**	**FV variant**	
	Cameroon	Gorilla	GgoCam7SFV	F	7	wild-born, zoo	Complete	SFV-GorI	SFVggo.Cam7 (KT211250)
		Chimpanzee	CpzCam15SFV	M	6	wild-born, zoo	Complete	SFV-CpzI	SFVptr.Cam15 (KT211264)
	Gabon	Chimpanzee	CpzJudWd	F	adult	wild-born, zoo	Complete	SFV-CpzII	SFVptr.JudWd (KT211266)
			Cpz133Wd	?	adult	wild-born, pet	Complete	SFV-CpzII	SFVptr.133Wd (KT211265)

745

746 **Table 3. Epidemiological data of the 44 individuals and four non-human primates**

747 **infected with simian foamy virus included in this study.**

748 The *env* of FVs from individuals in bold (BAK74, AG15, BAD327, BAD468) was sequenced
749 by our laboratory in a previous study (31). Cont : contact; samp : sampling; FV : Foamy
750 Virus.

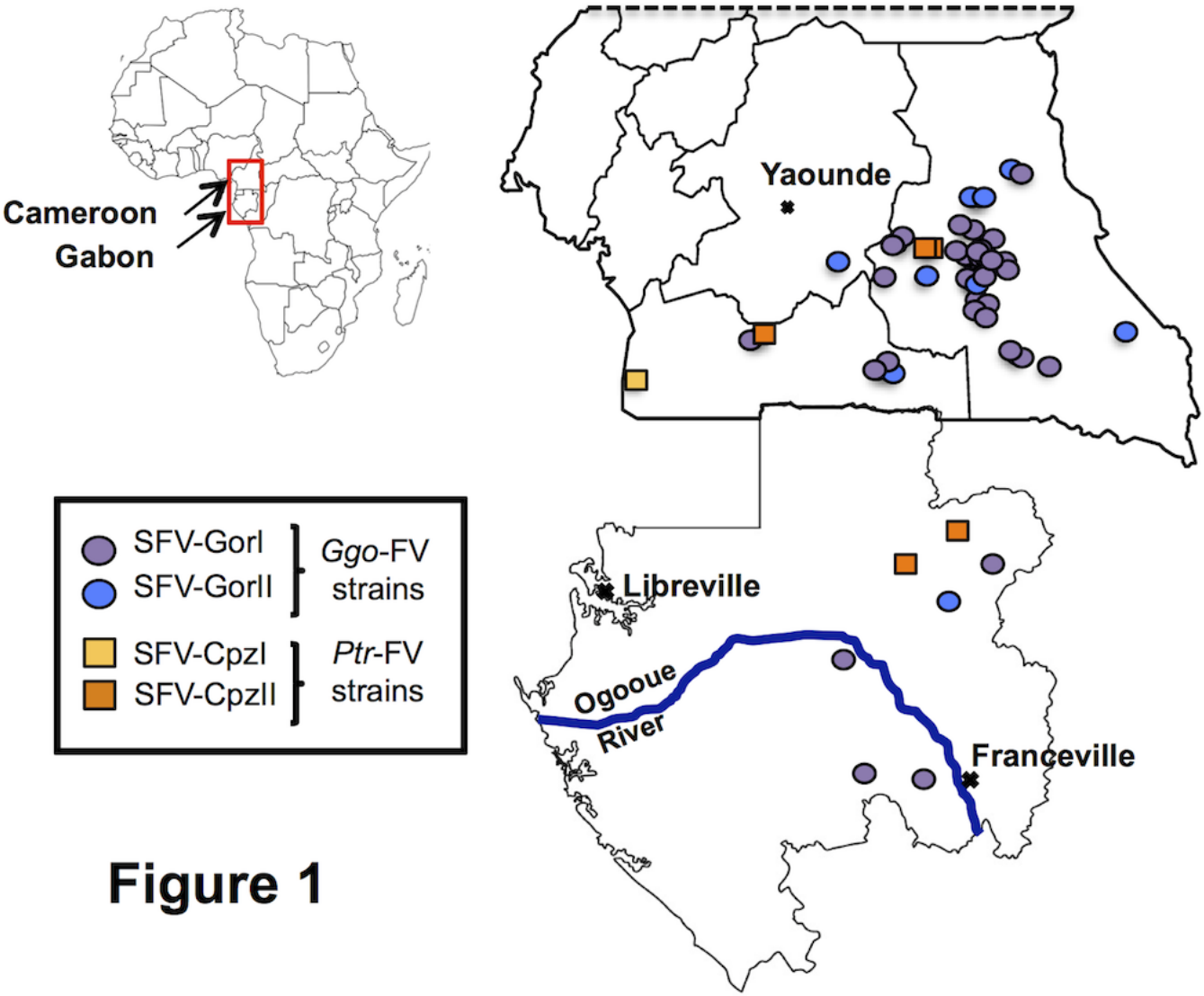
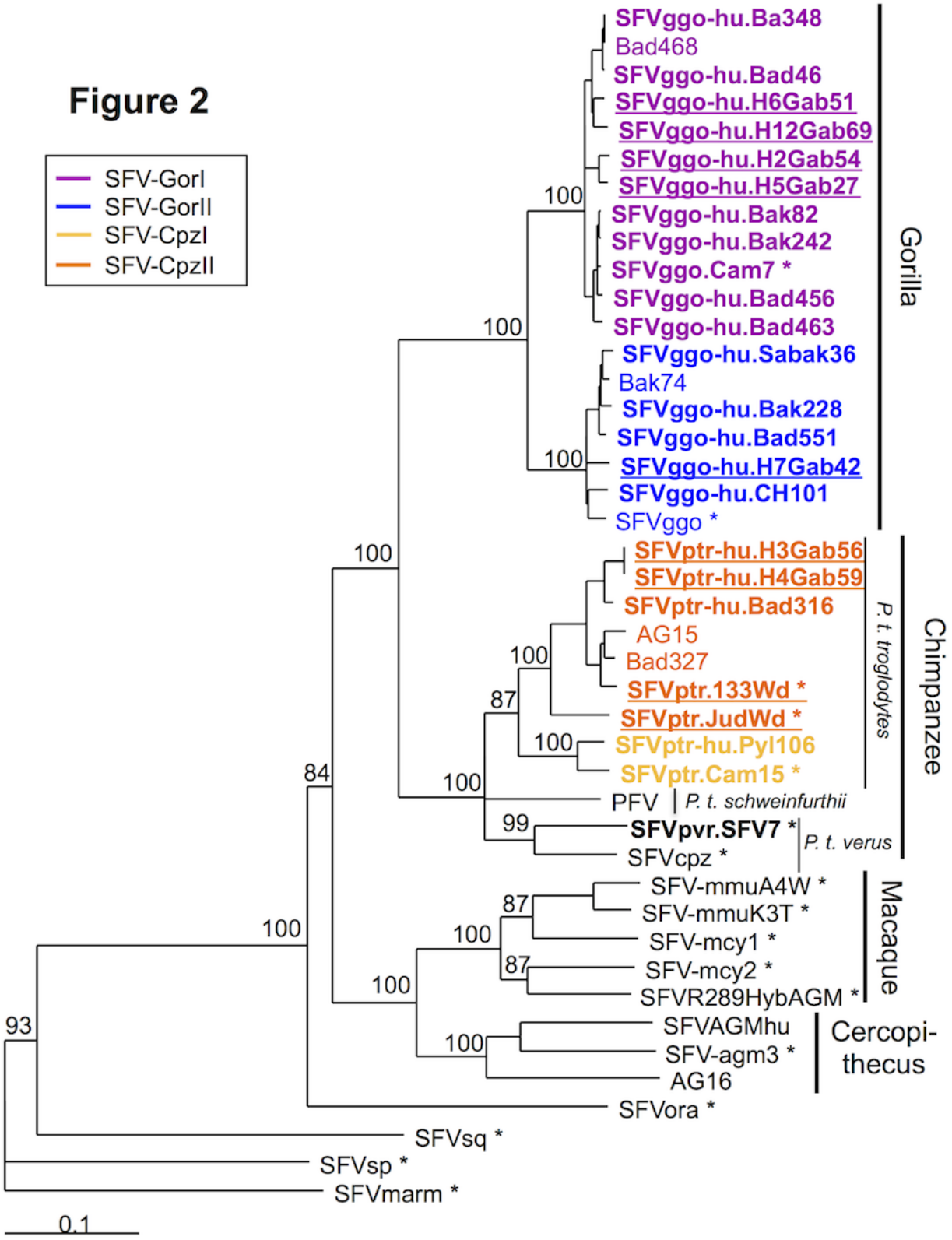


Figure 1

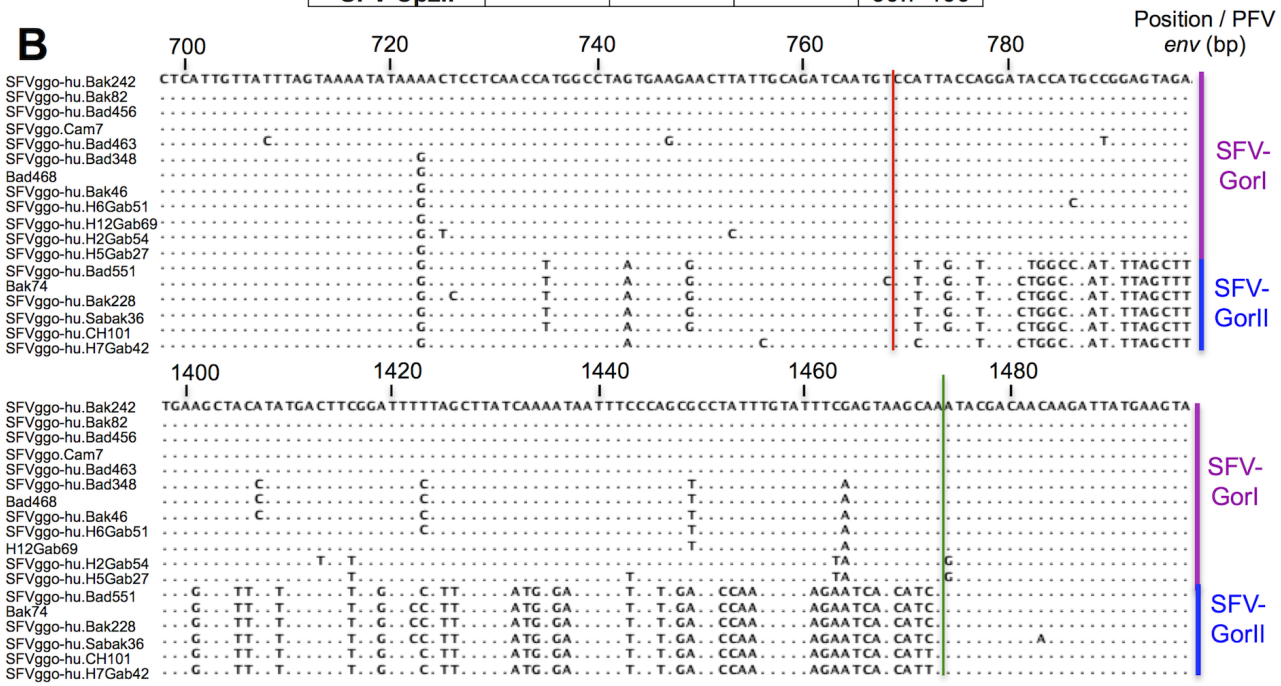
Figure 2



A Complete *env*

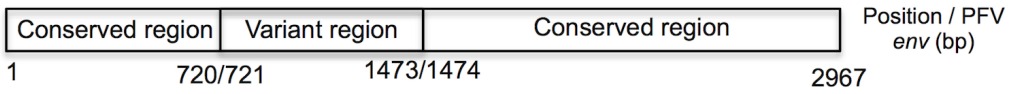
nt identity (%)	SFV-GorI	SFV-GorII	SFV-CpzI	SFV-CpzII
SFV-GorI	96.7-99.8	88.2-90.4	76.5-77.2	74.2-75.4
SFV-GorII		96.4-98.9	74.1-74.7	75.3-77
SFV-CpzI			95.9	86.5-89.4
SFV-CpzII				90.7-100

B



C

D



nt identity (%)	SFV-GorI	SFV-GorII	SFV-CpzI	SFV-CpzII
SFV-GorI	96.8-99.8	96.4-99.3	78-78.8	77.6-78.9
SFV-GorII		96.5-99	77.8-78.6	77.4-78.9
SFV-CpzI			95.9	91.4-97.6
SFV-CpzII				91.7-98

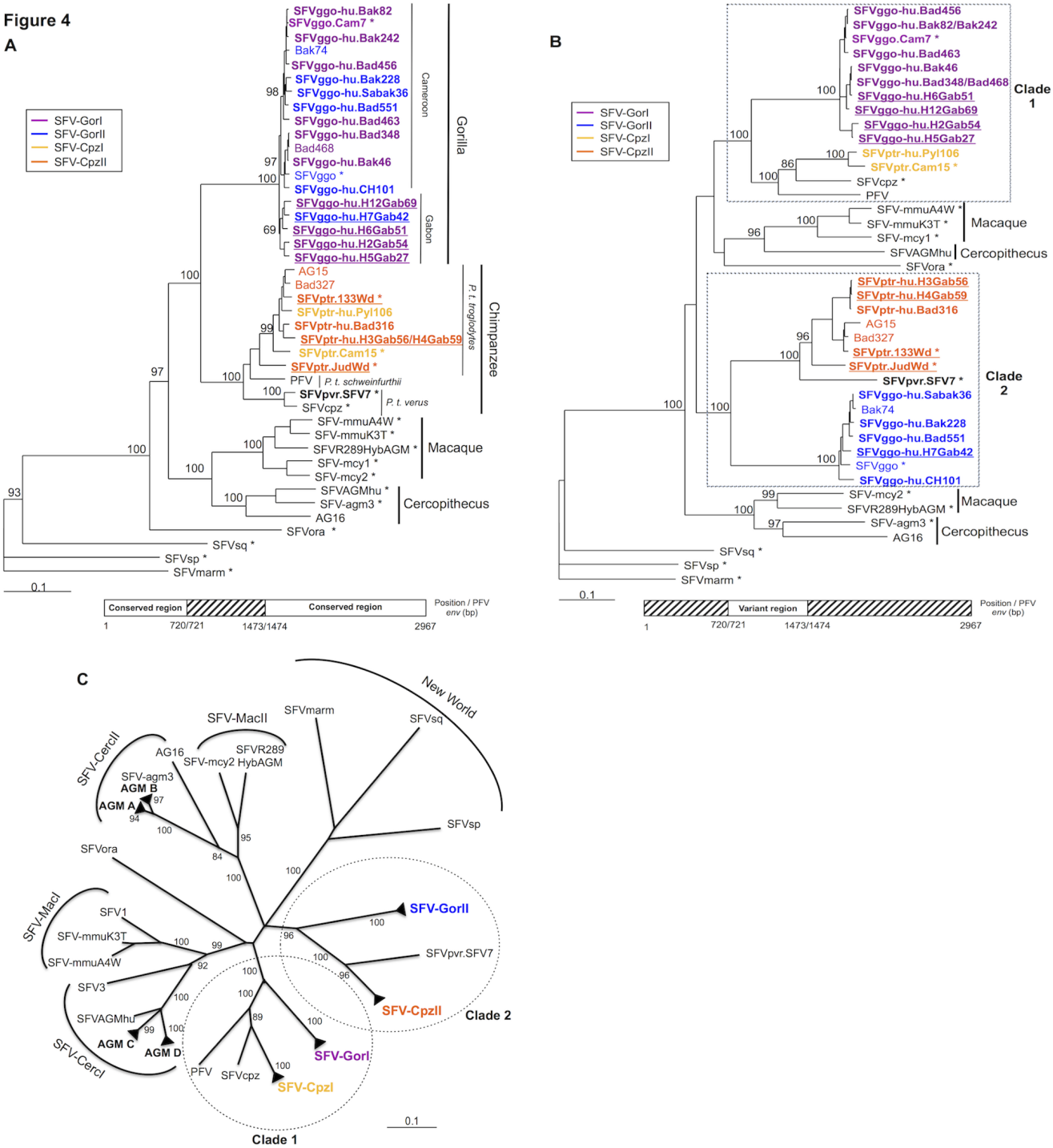
Conserved *env* region

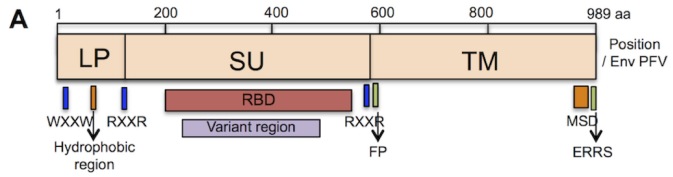
nt identity (%)	SFV-GorI	SFV-GorII	SFV-CpzI	SFV-CpzII
SFV-GorI	94.8-100	62.2-64.7	71.7-73	62.2-64.3
SFV-GorII		95-99.5	62.2-63.4	68.3-71.2
SFV-CpzI			95.8	62.3-63.9
SFV-CpzII				87.6-99.9

Variant *env* region

Figure 3

Figure 4





B

Conserved region	aa identity (%)	SFV-GorI	SFV-GorII	SFV-CpzI	SFV-CpzII
	SFV-GorI	97.8-99.9	98.1-99.5	83.6-84.6	82.8-84.6
	SFV-GorII		98.9-99.7	84-84.4	83.2-84.4
	SFV-CpzI			98.9	96.9-99
	SFV-CpzII				96-99.2

Variant region	aa identity (%)	SFV-GorI	SFV-GorII	SFV-CpzI	SFV-CpzII
	SFV-GorI	96.8-100	57.4-59	68.1-70.1	57.8-59.4
	SFV-GorII		96.8-99.6	53-54.2	68.6-71
	SFV-CpzI			97.6	55.4-56.6
	SFV-CpzII				91.9-100

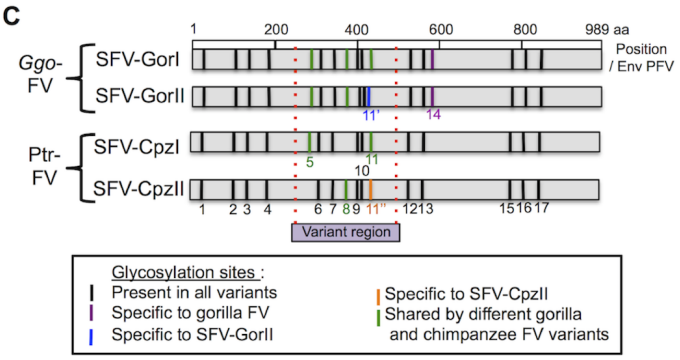


Figure 5

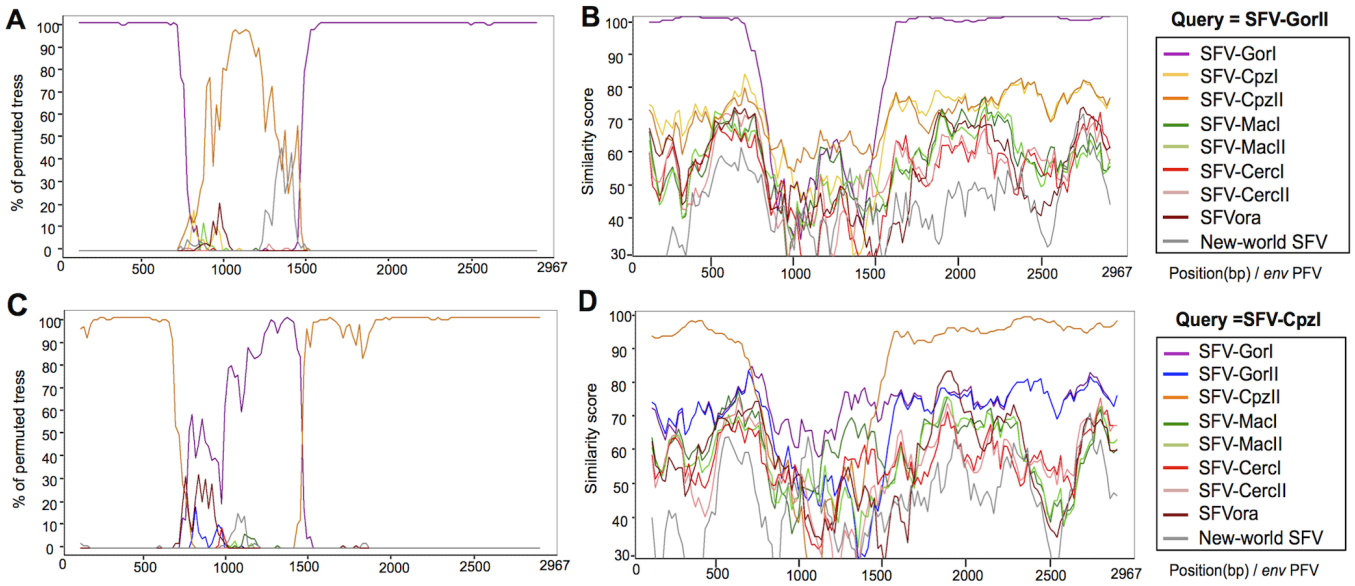


Figure 6