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Short Communication

Genetic diversity of simian lentivirus in wild De Brazza's monkeys (*Cercopithecus neglectus*) in Equatorial Africa

Avelin F. Aghokeng,[1] Ahidjo Ayouba,[1] Steve Ahuka,[1,2] Florian Liegeois,[1] Placide Mbala,[2] Jean-Jacques Muyembe,[2] Eitel Mpoudi-Ngole,[3] Eric Delaporte[1] and Martine Peeters[1]

[1]Laboratoire Retrovirus, IRD – UMR 145 'VIH et Maladies Associées' and the Department of International Health, University of Montpellier I, Montpellier, France

[2]Institut National de Recherches Biomédicales (INRB), Kinshasa, Democratic Republic of Congo

[3]Project PRESICA (Prévention du Sida au Cameroun), Military Hospital, Yaoundé, Cameroon

Correspondence
Avelin F. Aghokeng
aghokeng@ird.fr

De Brazza's monkeys (*Cercopithecus neglectus*) are non-human primates (NHP) living in Equatorial Africa from South Cameroon through the Congo-Basin to Uganda. As most of the NHP living in sub-Saharan Africa, they are naturally infected with their own simian lentivirus, SIVdeb. Previous studies confirmed this infection for De Brazza's from East Cameroon and Uganda. In this report, we studied the genetic diversity of SIVdeb in De Brazza's monkeys from different geographical areas in South Cameroon and from the Democratic Republic of Congo (DRC). SIVdeb strains from east, central and western equatorial Africa form a species-specific monophyletic lineage. Phylogeographic clustering was observed among SIVdeb strains from Cameroon, the DRC and Uganda, but also among primates from distinct areas in Cameroon. These observations suggest a longstanding virus–host co-evolution. SIVdeb prevalence is high in wild De Brazza's populations and thus represents a current risk for humans exposed to these primates in central Africa.

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Simian immunodeficiency viruses (SIVs) are lentiviruses infecting a wide variety of non-human primate (NHP) species in sub-Saharan Africa (Aghokeng & Peeters, 2005). Overall, each primate species seems to be infected with a species-specific SIV lineage. Human immunodeficiency virus (HIV)-1 and HIV-2 in the human population are most probably the result of contact with infected blood or tissues through hunting and butchering of chimpanzees, gorillas and mangabeys (Hahn *et al.*, 2000; Peeters *et al.*, 2002; Van Heuverswyn *et al.*, 2006). Several studies illustrated the ongoing exposure of humans to a plethora of different SIVs and thus it cannot be excluded that simian lentiviruses from other primate species have been or will be transmitted to humans. To better assess the existing risks of such cross-transmissions, it is essential to study in more detail the epidemiology and the genetic diversity of SIVs infecting Old World monkeys, especially within the genus *Cercopithecus*, which represents one of the most hunted and consumed primate species in sub-Saharan Africa.

Primate lentivirus evolution is characterized by examples of virus–host co-evolution, but also by cross-species transmission and recombination between divergent SIV lineages (Aghokeng *et al.*, 2007; Bailes *et al.*, 2003; Souquiere *et al.*, 2001). Among the members of the genus *Cercopithecus*, SIV infection has been identified in 17 species, and confirmed by PCR and partial sequence analysis in 12 species. For eight species the full-length SIV genome has been genetically characterized, including *Cercopithecus albugolaris* (SIVsyk), *Cercopithecus cephus* (SIVmus), *Cercopithecus mona* (SIVmon), *Cercopithecus nictitans* (SIVgsn), *Cercopithecus denti* (SIVden), *Cercopithecus solatus* (SIVsun), *Cercopithecus lhoesti* (SIVlho) and *Cercopithecus neglectus* (SIVdeb) (Aghokeng *et al.*, 2007; Barlow *et al.*, 2003; Beer *et al.*, 2000; Bibollet-Ruche *et al.*, 2004; Courgnaud *et al.*, 2002). All the SIVs derived from the genus *Cercopithecus* form a separate cluster in the SIV phylogeny, suggesting that they are derived from a common ancestor. Within the *Cercopithecus* SIV lineage, subclustes of SIVs from closely related species are observed, like the SIVgsn/mus/mon lineage from greater spot-nosed, mustached and mona monkeys (Courgnaud *et al.*, 2003), or the SIVlho/sun from l'Hoest's and sun-tailed monkeys (Beer *et al.*, 1999). But cross-species transmissions and recombinations also occurred, as we recently described for mustached monkeys infected with two distinct SIV lineages (SIVmus-1 and SIVmus-2) (Aghokeng *et al.*, 2007).

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Despite the increasing number of SIV lineages that have been described recently, our knowledge of SIV in their natural hosts still remains limited, because only few viruses have been characterized for each species and there is a major bias in geographical sampling. Therefore, in order to understand better the evolution of SIVs in their natural hosts, it remains necessary to study more samples per species and especially from different geographical locations. We recently showed that about 40 % of De Brazza's monkeys (*C. neglectus*) hunted as primate bushmeat in Cameroon are infected with SIVdeb (Aghokeng *et al.*, 2010). Here, we report novel SIVdeb sequences in primate bushmeat in Cameroon and the Democratic Republic of Congo (DRC) and analysed the genetic diversity of SIVdeb in animals across the home range of this species in Equatorial Africa.

We recently developed and validated new serological and molecular tools to detect and characterize SIV infection among NHP and used these assays to test up to 1873 NHP dried blood spot (DBS) specimens collected through south-eastern Cameroon from 2001 to 2004 (Aghokeng *et al.*, 2010). During this survey, we collected new De Brazza's monkey samples from fifteen individuals living in four different sites across southern Cameroon. In addition, in 2009 we collected three De Brazza's samples during a survey conducted in the Kasai province of the DRC. Sampling was subjected to local regulations without increasing the demand for bushmeat and we collected the DBS as described previously (Aghokeng *et al.*, 2010). We confirmed primate species by DNA sequencing of the slowly evolving mitochondrial 12S rRNA gene (Van Der Kuyl *et al.*, 2000) and used molecular testing to confirm SIV infection. We extracted total DNA from NHP DBS samples as described previously (Aghokeng *et al.*, 2010). PCR analyses were performed using several sets of universal and highly sensitive primers as well as SIV lineage-specific primers described previously (Aghokeng *et al.*, 2007; Clewley *et al.*, 1998; Courgnaud *et al.*, 2001; Liegeois *et al.*, 2006). For one animal (04CMPF3061), the complete genome was obtained by amplification of overlapping PCR fragments. Amplification and sequencing methods were described previously (Aghokeng *et al.*, 2007). The full-length genome sequence was reconstructed by assembling overlapping sequences into contiguous ones by using SeqMan II software (DNASTAR). We aligned nucleotide and protein sequences using CLUSTAL_X 2.0 (Thompson *et al.*, 1997), and applied minor manual adjustment where necessary. To generate full-length proteome sequences, we joined the deduced Gag, Pol, Vif, Env and Nef amino acid sequences, and excluded the carboxyl termini of Gag, Pol and Env that overlapped with Pol, Vif and Nef. Phylogenetic trees were inferred by the Bayesian method (Yang & Rannala, 1997) implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) by using an amino acid substitution matrix for inference of retrovirus and reverse transcriptase phylogeny: rtREV model (Dimmic *et al.*, 2002) with rates equal to invgamma (Yang, 1994). This model was selected as the best-fit evolutionary model for our datasets using ProtTest, a model selection program for protein evolution (Abascal *et al.*, 2005). One to 10 million generations were applied to run MrBayes analyses and Bayesian likelihoods and parameters were examined with the Tracer program (http://tree.bio.ed.ac.uk/software/tracer/). To determine the per cent difference between a new sequence and selected groups of reference sequences, diversity plots were produced using Simplot 2.5 (Lole *et al.*, 1999), with windows of 300 aa moved in steps of 20 residues. Genetic distances were calculated with the Kimura's two-parameter method by using nucleotide sequences (Kimura, 1981). Newly studied SIV sequences were compared to reference SIV strains obtained from GenBank (http://www.ncbi.nlm.nih.gov/). The previously published SIVdeb strains used in this report include: SIVdeb99CMCNE1 (GenBank accession no. AF478605), SIVdebCM5 (GenBank accession no. AY523866), SIVdebUK39257 (GenBank accession no. AY523858), SIVdebUK32771 (GenBank accession no. AY523860), SIVdebUK32772 (GenBank accession no. AY523859), SIVdebUK39260 (GenBank accession no. AY523861), SIVdebCM40 (GenBank accession no. AY523865), SIVdeb01CM1368 (GenBank accession no. FJ919721), SIVdeb01CM3418 (GenBank accession no. FJ919722), SIVdeb04CMPF1122 (GenBank accession no. FJ919725), SIVdeb04CMPF3061 (GenBank accession no. FJ919724) and SIVdeb04CMPF3074 (GenBank accession no. FJ919723).

Four De Brazza's monkeys from the southern part of Cameroon, including the centre-east region of Nanga Eboko (04CMPF1122), the east region of Bertoua (04CMPF2149) and the south region of Ebolowa (04CMPF3061 and 04CMPF3074), and one sample from the DRC (09DRCPS8) reacted strongly with our SIV-specific ELISAs. Successful PCR amplification in the *pol* gene for individuals 04CMPF1122, 04CMPF3061, 04CMPF3074 and 09DRCPS8 and in the *env* region for 04CMPF2149 confirmed that all the five animals carried SIVdeb virus known as infecting De Brazza's monkeys (Aghokeng *et al.*, 2010; Bibollet-Ruche *et al.*, 2004; Peeters *et al.*, 2002). Interestingly, phylogenetic analysis of the initial 397 bp *pol* fragment showed that the isolate from the DRC, SIVdeb09DRCPS8, and one from Cameroon, SIVdeb04-CMPF1122, clustered strongly with previously published SIVdeb sequences, SIVdebCM5 and SIVdebCM40 (Bibollet-Ruche *et al.*, 2004), while two other isolates from Cameroon, SIVdeb04CMPF3061 and SIVdeb04CMPF3074 from south Cameroon formed a separate distant cluster within the SIVdeb lineage (not shown). Larger nucleotide *pol* fragments were further obtained by performing additional PCR analyses for isolates SIVdeb04CMPF3061 (1716 bp), SIVdeb04CMPF3074 (1716 bp) and SIVdeb09DRCPS8 (635 bp). Additional phylogenetic analyses confirmed that isolates SIVdeb04CMPF3061 and SIVdeb04CMPF3074 clustered within the SIVdeb lineage, but distantly from previously reported SIVdeb viruses (data not shown).

To characterize in more detail the new SIVdeb subclade represented by isolates SIVdeb04CMPF3061 and SIVdeb04CMPF3074, we fully characterized the entire genome of one of the two divergent isolates (SIVdeb04-CMPF3061). The generated full-length genome was 9265 bp long and the genomic organization corresponds to previously reported SIVdeb strains, i.e. absence of *vpu* and *vpx* genes, and presence of expected reading frames for *gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *env* and *nef*. We constructed evolutionary trees from Gag, Pol and Env amino acid sequences to estimate the phylogeny of the new SIVdeb subclade represented by isolate SIVdeb04CMPF3061 in relation to other primate lentiviruses in these three main genome domains, i.e. *gag*, *pol* and *env*. In all three regions, the new SIVdeb strain clustered with the previous SIVdeb sequences, more closely in the Gag and Env regions compared with the Pol region (Fig. 1), confirming results obtained with partial *pol* sequences.

Genetic distances between the newly characterized SIVdeb subclade represented by SIVdeb04CMPF3061 and known representatives of the SIVdeb lineage, SIVdebCM5 and SIVdebCM40, were plotted in windows across the concatenated proteome. No evidence of recombination between the new isolate SIVdebCMPF3061 and previously described SIVdeb sequences (CM5 and CM40) or any other SIV strain was observed. However, amino acid sequence difference between SIVdeb04CMPF3061 and SIVdebCM5/CM40 ranged from 15 to 35 % over the entire length of the proteome, except for the beginning of the Gag region, with the highest sequence difference observed in the Pol region (Fig. 1). This distance was two times higher than the known distance between previously described SIVdeb strains, i.e. CM5 and CM40, which ranged from 0 to 16 % (Bibollet-Ruche *et al.*, 2004), thus suggesting an important genetic distance variation among SIVs infecting

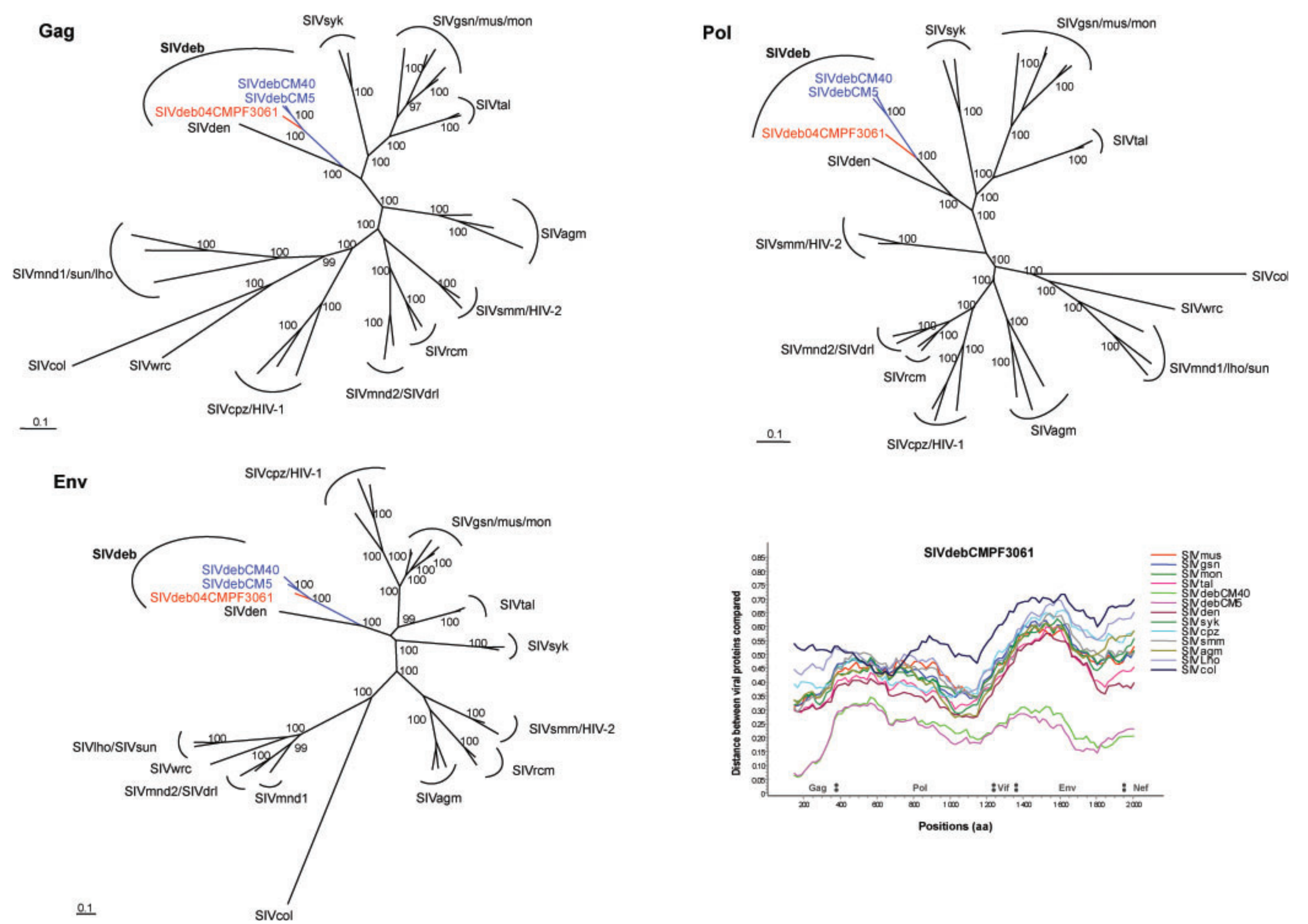

Fig. 1. Phylogenetic relationship of the newly derived SIVdeb strain (in red) to previously described SIVdeb viruses (blue) and other SIV lineages (black) in Gag, Pol and Env regions. The unrooted maximum-likelihood trees were inferred from protein sequence alignments. The numbers at nodes are the estimated posterior probabilities from the Bayesian method (values of 95 % and above are shown). Bars, 0.1 substitutions per site. The box shows diversity plots of concatenated protein sequences representing the extent of the genetic difference between SIVdeb04CMPF3061 and other primate lentiviruses. The *y*-axis indicates the distance between the viral proteins (0.1 = 10 % difference).

De Brazza's monkeys from Cameroon. To better assess the magnitude of the genetic diversity within the SIVdeb lineage, we calculated the genetic distances between SIVdeb viruses, and compared these distances with that observed within SIV lineages from different primate hosts and among SIV lineages that contain SIVs from closely related primate hosts. Distance calculations and comparison confirmed the results obtained with distant plot analyses since we found an average distance between SIVdeb-CMPF3061 and previously characterized SIVdeb viruses (CM5 and CM40) of 0.11, 0.27 and 0.23 in the *gag*, *pol* and *env* regions, respectively (Table 1). This genetic distance was about two times higher than that observed between SIVs derived from the same primate species (within SIV lineage and same primate host), and lower than that observed between SIVs derived from different primate species (between SIV lineage). The more interesting observation was that the distance between SIVdeb04-CMPF3061 and previously characterized SIVdeb viruses (CM5 and CM40) was to some extent similar to the distances observed between SIVs from the same lineage, but from different primate species within the lineage, e.g. as that observed between SIVgsn infecting *C. nictitans* and SIVmon infecting *C. mona*, or SIVlho infecting *C. lhoesti* and SIVsun infecting *C. solatus*, especially in the Pol and Env regions (Table 1). These findings together with distance plot results strongly suggest a high genetic diversity in the SIVdeb lineage.

Previous studies have shown that SIVdeb infection is frequent among wild De Brazza's monkeys and widespread throughout the species habitat (Bibollet-Ruche *et al.*, 2004) from south Cameroon through the Zaire basin to Uganda (Kingdon, 1997). Bibollet-Ruche *et al.* (2004) showed that SIVdeb strains from Uganda and Cameroon clustered distantly and reflected the existence of geographical sublineages, with genetic distances in the *pol* region reported as slightly higher than that observed within other host species SIVs (Bibollet-Ruche *et al.*, 2004). We performed a maximum-likelihood tree including Ugandan, the DRC and Cameroonian SIVdeb Pol sequences to assess the clustering pattern of these strains according to their geographical origin. Interestingly, the SIVdeb sequences clustered in four distinct sublineages: one sublineage represented by previously described SIVdeb strains, including CM5 and CM40 isolates from East Cameroon, a second sublineage represented by the DRC strain, Ugandan strains clustered together in a third group, and the fourth sublineage only involved the newly characterized SIVdeb sequences from south Cameroon, 04CMPF3061 and 04CMPF3074 (Fig. 2). Although we performed the analysis on short Pol sequences (164 aa), these data seem to support a geographical clustering of SIVdeb strains not only between Cameroonian, the DRC and Ugandan strains, but possibly also between strains from Cameroon since partial information accompanying samples from Cameroon suggested that isolates representing each of the two Cameroonian SIVdeb sublineages were collected in two separated areas in the southern part of the country. Previously described SIVdeb viruses (CM5 and CM40) were isolated from bushmeat sold in Yaoundé (capital city of Cameroon) bushmeat market known as mainly supplied with NHP hunted in the eastern part of Cameroon, while the new strains here identified (04CMPF3061 and 04CMPF3074) are from south Cameroon (Fig. 2). The phylogeographic clustering of SIVdeb strains from Cameroon, the DRC and Uganda, can be explained by the geographical barriers that separate the animals. However, to what extent the high genetic diversity among the Cameroonian SIVdeb strains is related to geographical separation has to be further examined. Although the two SIVdeb lineages were observed in two

Table 1. Comparison of nucleotide sequence distances between the new SIVdeb04CMPF3061 and previously characterized SIVdeb with the distance within and between host species SIV and SIV lineages

			gag	*pol*	*env*	
SIVdebCM5	vs	SIVdebCM40	0.04	0.12	0.17	Within SIVdeb
SIVdeb04CMPF3061	vs	SIVdebCM5	0.11	0.26	0.22	
SIVdeb04CMPF3061	vs	SIVdebCM40	0.10	0.27	0.23	
SIVgsnCM166	vs	SIVgsnCM71	0.09	0.06	0.15	Within SIV lineage and same primate host
SIVtalCM266	vs	SIVtalCM8023	0.05	0.09	0.13	
SIVgsn	vs	SIVmon	0.24	0.29	0.35	Within SIV lineages but different primate species*
SIVlho	vs	SIVsun	0.27	0.24	0.27	
SIVcpzTAN1	vs	SIVsmm239	0.40	0.39	0.59	Between SIV lineages
SIVsyk173	vs	SIVmnd14CG	0.37	0.42	0.64	

*SIVgsn and SIVmon are isolated from greater spot-nosed (*C. nictitans*) and mona (*C. mona*) monkeys infected with SIVgsn and SIVmon, respectively, belonging to the SIVgsn/mus/mon lineage (Courgnaud *et al.*, 2003); SIVlho and SIVsun are isolated from l'Hoest (*C. lhoesti*) and sun-tailed (*C. solatus*) monkeys infected with SIVlho and SIVsun, respectively, belonging to the SIVlho/sun lineage (Beer *et al.*, 1999).

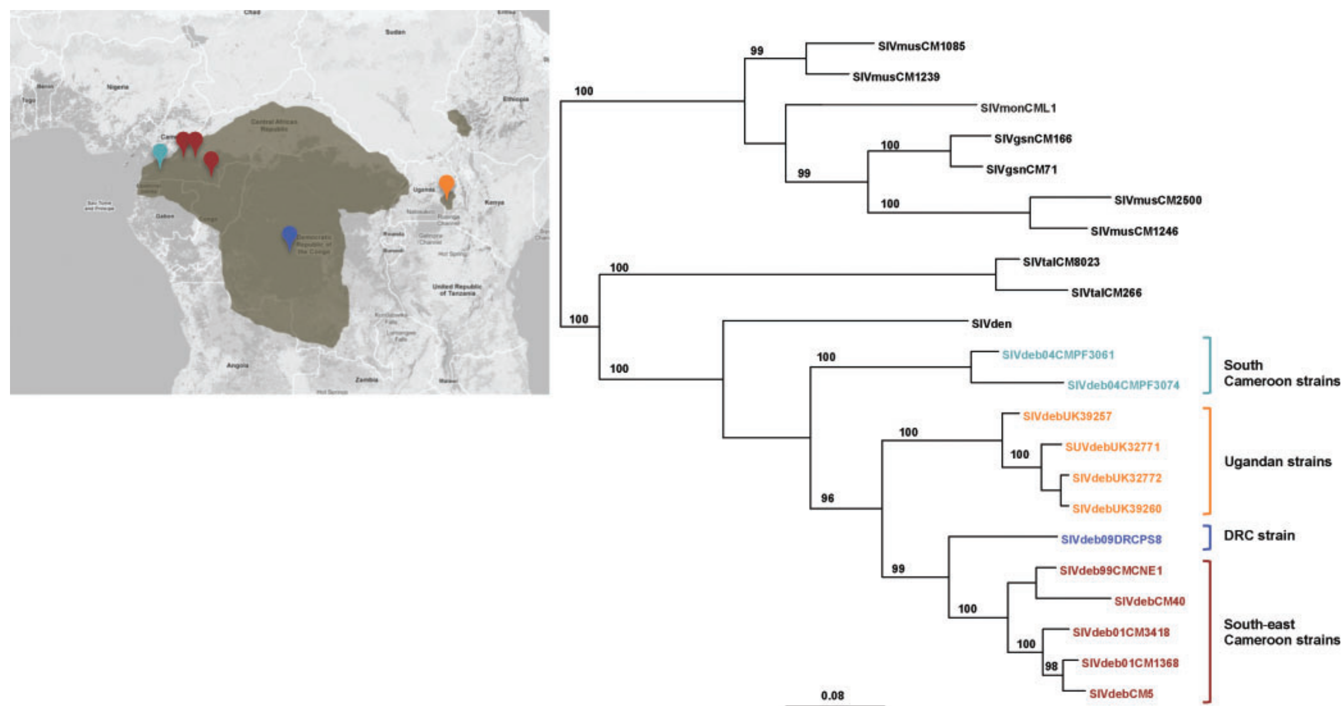

Fig. 2. Phylogenetic tree of SIVdeb from south-east and south Cameroon, the DRC and Uganda. Maximum-likelihood trees were inferred from a partial (164 aa) Pol protein sequence alignment. The numbers on the internal branches indicate estimated posterior probabilities (only values of 95 % or greater are shown). Bar, 0.08 substitutions per site. The map on the right shows the natural range of De Brazza's monkey in Equatorial Africa (dark) and locations of sample collection for each of the SIVdeb group represented on the phylogenetic tree.

different geographical areas of Cameroon, the geographical barriers that separate the animals are less important than the biogeographical barriers between the animals from Cameroon, the DRC and Uganda.

NHP infected with divergent SIVs are reported in sub-Saharan Africa. Mandrills from both sides of the Ogooué river in Gabon are infected with two divergent viruses: SIVmnd1 and SIVmnd2 (Souquiere *et al.*, 2001). Recently, we showed that mustached monkeys found in Cameroon are infected with two distinct SIVmus variants, that we designated SIVmus-1 and SIVmus-2, which differ in *gag* and *pol* (but not *env*) to a similar extent as SIVs from different host species. The key difference between SIVmnd and SIVmus epidemiology was that contrary to mandrills that are geographically separated by the Ogooué river, mustached monkeys infected with SIVmus-1 and SIVmus-2 shared approximately the same habitat, with no evidence of geographical separation (Aghokeng *et al.*, 2007). But in both cases, cross-species transmission followed by recombination were identified as playing an important role in the emergence of novel viruses. In the present study, although the collection sites in Cameroon are distant enough, about 300 km apart, to believe that the habitat of the two De Brazza's monkey populations do not overlap, there is also no evidence that these two populations are geographically isolated because the range of De Brazza's monkeys is continuous across central Africa. Also, we confirmed primate species by the DNA sequencing of the slowly evolving mitochondrial 12S rRNA gene and the analysis did not suggest the presence of De Brazza's subspecies among our study samples, although the sequenced fragments could be too short to reliably identify subspecies. In addition, no evidence of existing subspecies of De Brazza's monkeys has been reported to date (Groves, 2001), in contrast to mandrills for which mitochondrial haplotypes on each side of the Ogooué river have been reported (van der Kuyl *et al.*, 2004). Therefore, primate host evolution cannot currently be considered as being responsible for the genetic variation observed among SIVdeb strains circulating in Cameroon. Also, phylogenetic analyses did not suggest recombination as being responsible for the origin of any of the two SIVdeb subclades, but since ancient recombination events can be masked by further cross-transmission and primate–virus co-evolution, we cannot definitely exclude recombination as being involved in the evolution of these two SIVdeb subclades. But, looking for the phylogeny of SIVdeb from De Brazza's monkeys from distinct parts of Africa (Cameroon, the DRC and Uganda), it appears that geographical separation of these primates tends to create geographically distant SIVdeb sublineages. Whether this geographical differentiation of

SIVdeb is related to genetically distinct De Brazza's monkey subpopulations needs to be assessed.

Cross-species transmissions of SIVs from NHP to humans have given rise to HIV-1 and HIV-2 and it is likely that frequent contacts with infected animal through hunting and butchering of primates have facilitated these transmissions (Hahn *et al.*, 2000; Peeters *et al.*, 2002). In a recent survey, we showed that SIV prevalence among De Brazza's monkeys used as bushmeat in sub-Saharan Africa was very high, up to 40 % (Aghokeng *et al.*, 2010). Although we found during these surveys that De Brazza's monkeys account for a reduced proportion of bushmeat in Cameroon, this species represents a high risk for new SIV cross-transmission to humans because of its very high SIV prevalence (Aghokeng *et al.*, 2010). To date, all our attempts to confirm such transmissions of SIVdeb from *C. neglectus* to humans have been unsuccessful (Ndongmo *et al.*, 2004; C. F. Djoko and others, unpublished results). This lack of evidence can either reflect an absence of transmission of SIVdeb to humans, which means that other factors should be considered in addition to exposure to infected primates, or the screening tools and/or sample size are not reliable enough to allow the identification of such transmission and therefore should be improved.

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