Identification and Characterization of Highly Divergent Simian Foamy Viruses in a Wide Range of New World Primates from Brazil

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Abstract

Foamy viruses naturally infect a wide range of mammals, including Old World (OWP) and New World primates (NWP), which are collectively called simian foamy viruses (SFV). While NWP species in Central and South America are highly diverse, only SFV from captive marmoset, spider monkey, and squirrel monkey have been genetically characterized and the molecular epidemiology of SFV infection in NWP species remains unknown. We tested a large collection of genomic DNA (n = 332) comprising 14 genera of NWP species for the presence of SFV polymerase (pol) sequences using generic PCR primers. Further molecular characterization of positive samples was carried out by LTR-gag and larger pol sequence analysis. We identified novel SFVs infecting nine NWP genera. Prevalence rates varied between 14–30% in different species for which at least 10 specimens were tested. High SFV genetic diversity among NWP up to 50% in LTR-gag and 40% in pol was revealed by intragenus and intrafamilial comparisons. Two different SFV strains infecting two captive yellow-breasted capuchins did not group in species-specific lineages but rather clustered with SFVs from marmoset and spider monkeys, indicating independent cross-species transmission events. We describe the first SFV epidemiology study of NWP, and the first evidence of SFV infection in wild NWP. We also document a wide distribution of distinct SFVs in 14 NWP genera, including two novel co-speciating SFVs in capuchins and howler monkeys, suggestive of an ancient evolutionary history in NWP for at least 28 million years. A high SFV genetic diversity was seen among NWP, yet these viruses seem able to jump between NWP species and even genera. Our results raise concerns for the risk of zoonotic transmission of NWP SFV to humans as these primates are regularly hunted for food or kept as pets in forest regions of South America.

Introduction

Foamy viruses (FV) are complex retroviruses in the Spumavirus genus that naturally infect a wide range of mammals, including bovines, felines, equines, sheep, and nonhuman primates (NHPs). In NHPs, FV are referred to as simian foamy viruses (SFV). Their unusual name refers to the formation of syncytia of multicellulared giant cells with numerous vacuoles seen by electron microscopy with a foamy appearance upon virus infection in vitro [1]. Although highly cytopathic in tissue culture, FV is apparently nonpathogenic in vivo, but a clear causality between infection and disease has not been systematically investigated either in animals or in humans with zoonotic SFV infection [2].

While SFV isolates have been identified in numerous, diverse species of African and Asian NHPs [3–7], or Catarrhini, humans do not appear to be a natural host of these viruses. All SFVs detected in humans were acquired by zoonotic transmission from infected African and Asian NHPs and thus far are persistent but seemingly asymptomatic infections [2,8–12]. The zoonotic transmission of SFV to humans in the wild is not a rare event and the presence of FV ranges from approximately 1% in persons having contact with NHPs through hunting, butchering and keeping NHP pets, to as high as 19% in hunters with severe mucocutaneous exposures [13–17]. Persons infected with SFV while working with NHPs in biomedical facilities have also been reported with prevalence ranging from 3–4% [9,14,15].
Despite the wide distribution and diversity of SFV in different Old World primate species, studies of SFV in New World primates (NWP), or Platyrrhini, have been limited to very small numbers of captive animals. NWP are a diverse group of American primates, comprising over 110 different species in 15–19 genera and three families (Atelidae, Aotidae, Cebidae, and Pitheciidae) [18,19]. However, molecular characterization of SFV has been reported for only three species of captive NWP, *Ateles sp.* (spider monkey), *Saimiri sciureus* (common squirrel monkey) and *Callithrix jacchus* (common marmoset), representative of only two families (Atelidae and Cebidae), with complete genomes only available recently [20,21]. Thus, the prevalence of SFV in NWP, especially in wild animals remains largely unknown. NWP are commonly kept as pets in several countries because of their small size and are also hunted and butchered for consumption in South America, resulting in potential human exposure to SFV. Similarly, NWP are commonly used in biomedical research studies placing animal handlers, veterinarians, and scientists at increased risk of exposure to SFV. Determining the prevalence and geographical distribution of SFV in NWP is the first step to better understand the public health risk of infection with SFV in persons exposed to NWP.

Here we identify SFV infection in a wide range of NWP species from all three NWP families, including the molecular characterization of highly divergent SFV in capuchin (*Cebus*) species and howler (*Alouatta* sp.) monkeys. We also report for the first time SFV infection in wild NWP and evidence for independent cross-species transmission of SFV in two different captive capuchins (*Cebus xanthosternos*) likely originating from spider monkey (*Ateles* sp.) and marmoset (*Callithrix jacchus*). Our results highlight the need to further characterize the geographic distribution and evolutionary history of SFV in NWP from other South American countries. In addition, our results emphasize the need to define the risk of infection with SFV in persons exposed to NWP.

**Materials and Methods**

**Ethics Statement**

Original NWP samples have been previously collected by venous puncture (n = 462) or by liver biopsy of dead animals (n = 9) following the national guidelines and provisions of IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Brazil; permanent license number 11375–1), which included animal welfare standard operational procedures. All samples (wild and captivity) have been collected by some of the authors (EAS, MAM, AP, CRB and HNS) for previous host phylogenetic and cytogenetic studies [22–25]. All nine liver samples were from animals sacrificed during a survey of *Trypanosoma* reservoirs in the northern states of Pará and Rondônia (see Table 1 and Figure 1 for specific geographic locales). Sacrifice of these animals was conducted by anesthetic lethal dose injection following the guidelines of the Brazilian Council of Biology (http://www.cfbio.gov.br). Specimens from wild animals were collected during their rescue and relocation in forest areas to be flooded for construction of hydroelectric power houses in northern Brazil (see Table 1 and Figure 1 for specific collection locales). Animals were caught and subject to ketamine anesthesia for venous puncture, followed by relocation to another area of the same habitat.

**NWP Specimen Preparation and Species Confirmation**

Genomic DNA (gDNA) samples were extracted from 471 NWPDs comprising 14 different genera and were stored at −20°C at the Primate Genomic DNA Bank of the Instituto Nacional de Cáncer, located in Rio de Janeiro, Brazil. These specimens were previously collected from NWP in the wild (n = 65) and in captivity (n = 406) from the Brazilian National Primate Center, the Rio de Janeiro Primate Center, the Brazilian Primate Protection Center, the Center for Medical Education and Clinical Investigation Norberto Quirino and the Zoos of Rio de Janeiro and São Paulo, as described elsewhere [22–25] and comprised whole blood (n = 462) or liver (n = 9) biopsies. Locations of the five study sites in Brazil are shown in Fig. 1. DNA was extracted using the Qiagen DNA genomic DNA extraction kit (Qiagen, Chatsworth, CA), according to the manufacturer’s specifications, or by standard phenol-chloroform techniques.

Genomic DNA integrity (and suitability for SFV proviral detection) and primate host species taxonomic classification were determined by analysis of a 975-bp cytochrome B (cytB) mitochondrial sequence obtained by one-step PCR using primers L14724 (5′CGA AGC TTG ATA TGA AAA ACC ATC GTT G 3′) and Mus15398 (5′GAA TAT CAG CTT TGG GTG TTG RTG 3′) [26]. Through this analysis, 332 samples (70.5%) were further considered suitable for SFV detection (Table 2). cytB sequences from SFV-positive specimens (see below) were aligned with those available from NWP at GenBank and trees were inferred using the ML method with goodness of fit measured by the Bayesian information criterion in MEGA v.5.0. Cytochrome B sequences from a chimpanzee (*Pan troglodytes*, GenBank accession number EF660790) and an African green monkey (*Chlorocebus aethiops*, GenBank accession number AB495295) were used as outgroups for the phylogenetic analyses.

**SFV PCR and Sequence Analysis**

All DNA samples were first screened for 192-bp SFV sequences using a novel semi-nested PCR that utilizes generic polymerase gene (pol) primers (Table S1) and conditions previously reported for other generic SFV *pol* PCR [9]. These primers were designed using an alignment of sequences from the three complete NWP SFV genomes available at GenBank from marmoset, squirrel, and spider monkey (accession numbers GU356395, GU356394, and EU010385, respectively) [20,21].

To analyze the phylogenetic relationships with other previously described NWP SFV, two additional SFV subgenomic regions were PCR-amplified and sequenced. Generic primers for this additional testing were designed using conserved regions in an alignment of the three complete NWP SFV genomes [20,21] to amplify 398-bp LTR and *gag*-matrix (225-bp in LTR and 173-bp in *gag*) and 520-bp *pol* sequences using nested PCR (Table S1). Amplified products were purified, quantified, and sequenced on both strands using the Big Dye v.3.1 kit (Life Technologies, Carlsbad, USA) and an automated ABI 3130xL Genetic Analyzer and edited with SeqMan v.7.0 (DNASTAR, Madison, USA). New sequences were aligned with those available from NWP retrieved from GenBank. Nucleotide mean genetic distances were calculated within NWP and OWP SFV, within NWP families (Ateidae and Cebidae) and within different NWP genera using the pairwise distance tool in MEGA5 with Kimura’s 2-parameter model of nucleotide substitution.

Phylogenetic analysis of the LTR-*gag* and *pol* sequences was conducted using neighbor-joining (NJ), and ML methods implemented in MEGA v.5.0 using either the Tamura-Nei or Huy+I+G nucleotide substitution models inferred in MEGA v.5.0. SFV sequences from a chimpanzee (SFVcpz; GenBank accession number U04327) and an African green monkey (GenBank accession number NC_010820) were used as outgroups for the phylogenetic analyses. Phylogenetic signal in the align-
SFV and Simian Host Co-evolution Inference

Reconciliation analysis and comparison of branch lengths and coalescence times of the SFV and cytB Bayesian trees were performed with the TreeMap (v1.0) program in accordance with the author’s instructions [31]. The significance of the observed fit between the SFV and primate trees and branch lengths was determined by comparison with the distribution of the same measure of fit for 10,000 randomly generated trees or branch lengths by using the proportional-to-distinguishable model of the randomization test incorporated in TreeMap.

GenBank accession numbers. All SFV and cytB sequences generated herein have been deposited at GenBank with the accession numbers KC331071 to KC331109.

Results

High Prevalence and Broad Distribution of SFV in NWP

A novel PCR assay was developed to generically detect SFV pol sequences in a variety of NWPs using an alignment of complete SFV genomes from marmoset, squirrel, and spider monkeys. The assay was validated using peripheral blood lymphocyte DNA from 47 seronegative and 59 seropositive NWPs identified using a Western blot (WB) test that utilizes SFV antigens from marmoset and spider monkeys grown in Cf2Th cells. Details of the SFV WB test are similar to those previously published with the exception that the previous assay utilizes two antigens each from an SFV-infected OWM or ape [32]. The 105 NWPs used for the PCR assay validation were all housed at various US institutions. gDNA was available from seven genera of NWPs including Cebus, Alouatta, Callithrix, Aotus, Ateles, Saimiri, Cacajao and Pithecia. The PCR assay had a sensitivity of 100% (92–100%; 95% CI) and detected SFV sequences in all 59 WB-positive animals. Forty-three out of the forty-seven WB-negative animals were PCR negative, giving an assay specificity of 91% (79–97%; 95% CI). The lower assay specificity results from DNA samples from four Saimiri specimens that were repeatedly PCR-positive but WB-negative using samples collected 2 years apart, suggestive of latent infection. These data have been reported previously [33].

The shorter SFV pol sequences were detected in 80 of the 332 (24.1%) NWP DNA specimens using the generic PCR screening assay. As shown in Table 2, nine distinct genera and at least 19 different species, including representatives of all three NWP families according to the cytB phylogenetic relationships of the Platyrhini group [30], had detectable SFV integrated into their genomes. Phylogenetic comparison of new cytB sequences from 15

Table 1. SFV PCR prevalence in NWP living at primate centers and in the wilda.

| Species       | Originb | State/location in Brazil/South Americaa | # pos/total (%) |
|---------------|---------|----------------------------------------|-----------------|
| Alouatta belzebul | UHE Tucurui (wild) | Pará (PA) | 13/45 (29%) |
| Alouatta caraya      | UHE Manso (wild)  | Mato Grosso (MT) | 6/20 (30%) |
| Aotus azarai       | CNP       | Rondônia (RO) | 2/9 (22%) |
| Aotus azarai       | Pará (PA) | 2/9 (22%) |
| Cebus apella      | CPB       | Paraíba (PB) | 3/22 (14%) |
| Cebus apella      | CEMIC     | Frontier Brazil/Argentina (RS) | 3/19 (16%) |

aPCR testing using diagnostic primers to detect 192-bp polymerase sequences in DNA specimens from species listed;
bUHE, Usina Hidroelétrica; CNP, Centro Nacional de Primatologia; CPB, Centro de Proteção de Primatas Brasileiros; Instituto Chico Mendes de Conservação da Biodiversidade; CEMIC, Centro de Educação Médica e Investigaciones Clínicas Norberto Quirino;

N, north; SE, southeast; NE, northeast; S, south.
doi:10.1371/journal.pone.0067568.t001
### Table 2. Molecular detection and distribution of SFV in New World primates from Brazil.

| Family | Scientific name | Common name* | No. pos/No. total (%)b |
|--------|----------------|--------------|-------------------------|
| **Atelidae** | | | |
| | Alouatta belzebul | black-and-red howler monkey | 13/45 (29) c |
| | Alouatta belzebul | | 2/5 (40) |
| | Alouatta caraya | black howler monkey | 6/20 (30) |
| | Alouatta guariba | brown howler monkey | 2/3 (66.7) |
| | Alouatta seniculus | red howler monkey | 4/8 (50) |
| | Ateles paniscus | black spider monkey | 0/1 (0) |
| | Brachyteles arachnoides | wooly spider monkey | 0/1 (0) |
| | Lagothrix species | wooly monkey | 0/2 (0) |
| **Cebidae** | | | |
| | Aotus azarai | Azara’s owl monkey | 4/28 (14.3) |
| | Aotus species | owl monkey | 2/24 (8.3) |
| | Callimico goeldii | Goeldi’s marmoset | 0/2 (0) |
| | Callithrix argentata | silvery marmoset | 3/8 (37.5) |
| | Callithrix aurita | white-eared marmoset | 1/1 (100) |
| | Callithrix emiliae | Emilia’s marmoset | 3/9 (33.3) |
| | Callithrix geoffroyi | Geoffroy’s marmoset | 0/1 (0) |
| | Callithrix humeralifera | Santarem marmoset | 0/3 (0) |
| | Callithrix jacchus | white-tufted-ear marmoset | 1/1 (100) |
| | Callithrix kuhlii | Wied’s marmoset | 0/3 (0) |
| | Callithrix melanura | black-tailed marmoset | 1/2 (50) |
| | Callithrix penicillata | black-pencilled marmoset | 0/1 (0) |
| | Cebus albifrons | white-fronted capuchin | 3/13 (23.1) |
| | Cebus apella | tufted capuchin | 11/50 (22) |
| | Cebus cay | hooded capuchin | 0/7 (0) |
| | Cebus olivaceus | weeper capuchin monkey | 6/16 (37.5) |
| | Cebus xanthosternos | yellow-breasted capuchin | 5/9 (55.6) |
| | Cebus species | Capuchin | 0/3 (0) |
| | Leontopithecus chrysomelas | golden-and-black lion tamarin | 0/1 (0) |
| | Leontopithecus chrysopygus | golden-rumped lion tamarin | 0/1 (0) |
| | Leontopithecus rosalia | golden lion tamarin | 1/2 (50) |
| | Saguinus fuscicollis | brown-headed tamarin | 0/2 (0) |
| | Saguinus imperator | Emperor tamarin | 2/2 (100) |
| | Saguinus martinsi | Martin’s bare-face tamarin | 0/1 (0) |
| | Saguinus midas | Midas tamarin | 0/3 (0) |
| | Saguinus mystax | moustached tamarin | 0/1 (0) |
| | Saguinus niger | black-handed tamarin | 0/1 (0) |
| | Saimiri sciureus | common squirrel monkey | 3/17 (17.6) |
| | Saimiri ustus | bare-eared squirrel monkey | 4/16 (25) |
| | Saimiri species | squirrel monkey | 1/3 (33.3) |
| **Pitheciidae** | | | |
| | Callicebus moloch | red-bellied titi | 1/1 (100) |
| | Callicebus nigrifrons | black-fronted titi | 0/3 (0) |
| | Callicebus personatus | masked titi | 0/3 (0) |
| | Callicebus torquatus | yellow-handed titi | 0/2 (0) |
| | Callicebus species | titi monkey | 0/1 (0) |
| | Chiropotes species | bearded saki monkey | 1/3 (33.3) |
| | Pithecia irrorata | bald-faced saki | 0/3 (0) |
| **Total** | | | 80/332 (24.1) |

* NWP common names are as in [19].

** PCR testing using diagnostic primers to detect 192-bp polymerase sequences in DNA specimens from species listed.

* Underlined numbers refer to specimens from the wild.

[10.1371/journal.pone.0067568.t002]
Brazilian primates with those from 16 reference sequences identified five Cebus apella, three Cebus xanthosternos, one Cebus albifrons, three Alouatta seniculus, two Alouatta guariba, and one Alouatta belzebul (Fig. 2). Species classification for 36 specimens from NWPs of the Aotus (n = 24), Lagothrix (n = 2), Cebus (n = 3), Saimiri (n = 3), Callicebus (n = 1), and Chiropotes (n = 3) genera could not be molecularly confirmed due to the lack of representative cytB sequences from these species in GenBank for comparison. However, classification of NWPs in our study also included morphological characteristics typical of the species recorded by experienced taxonomists from our group. SFV prevalence ranged from 0–100% in the total study population but the extreme range may likely reflect the low numbers of samples from some species (Table 2). However, when species with less than 10 representatives are excluded from the analysis, the SFV prevalence is 23.1% (54/234) which remains similar to the rate for the total population. Two specimens were found to be SFV-positive from liver biopsies, suggesting that this virus can also be retrieved from that body compartment. Similar proportions of SFV-positive samples were found from whole blood and liver biopsies (2 and 2.5%)

Table 3. Intra- and inter-primate family and order SFV nucleotide diversity⁹⁺.¹⁺.²⁺.

| Intra Cebidae Family | Intra Atelidae Family | Intra NWP Order | Intra OWP Order | NWP X OWP |
|----------------------|-----------------------|-----------------|----------------|----------|
|                      | within SFVcap | SFVcap X SFVmar | within SFVhow | SFVhow X SFVspm | Cebidae X Atelidae | S. cap. | S. mar. | S. sp. | S. pm. | S. cap. | S. mar. | S. sp. | S. pm. |
| LTR (225-bp) | 0.077 (0.047) | 0.217 (0.012) | 0.150 (0.056) | 0.254 (0.013) | 0.503 (0.035) | 0.180 (0.044) | 0.581 (0.084) |
| gag (157-bp) | 0.091 (0.036) | 0.188 (0.009) | 0.192 (0.024) | 0.265 (0.017) | 0.409 (0.029) | 0.698 (0.177) | 0.802 (0.108) |
| pol (347-bp) | 0.063 (0.019) | 0.124 (0.007) | 0.172 (0.024) | 0.311 (0.010) | 0.410 (0.028) | 0.383 (0.067) | 0.544 (0.034) |

* Nucleotide diversity calculated using pairwise distances implemented in MEGAS, numbers in parentheses are standard deviations from the mean diversity;
  ¹ Cap, capuchin; mar, marmoset (GenBank accession number GU356395); spm, spider monkey (EU010385);
  ² NWP, New World primate; OWP, Old World primate sequences included.
  ³ For this analysis we used SFVgor [GenBank accession # HM245790], SFVora [AJ544579], SFVcpz [U04327], SFVmac [X54482] and SFVagm [M74895].

doi:10.1371/journal.pone.0067568.t003
respectively, providing evidence for no particular bias in SFV detection when comparing both compartments.

All specimens surveyed were adults, and similar numbers of males and females were found among the SFV-positive and SFV-negative animals (56.44% and 51.49%, respectively), as it has been seen in other studies. Ages at specimen collection were not available for the majority of animals limiting an assessment of age-related SFV restriction in our study.

To obtain a preliminary estimate of SFV prevalence in wild and captive NWP, we determined the presence of SFV in four NWM species groups for which we had specimens from at least 10 different animals housed at primate centers or living in the wild. Overall, the molecular prevalence ranged between 14 and 29% (Table 1). Prevalence rates were consistently similar in populations from the wild or in captivity.

Diversity and Co-evolution of SFV in NWPs

Although an alignment of the shorter pol sequences (192-bp PCR product, 138-bp final alignment) was shown to contain adequate phylogenetic signal by likelihood mapping and lacked substitution saturation by the method of Xia et al. [29], only 44/138 (32%) sites were fully resolved and all three codon positions were found to have substitution saturation in Ts and T versus divergence plots. Indeed, the inferred phylogenies were poorly resolved with weak support at most nodes which contained mixtures of SFV from multiple species atypical of SFV evolution (Fig. S1). One clear example is the clustering of SFV pol sequences from an African green monkey (agg) and chimpanzee (cpz) within a NWM clade which have been shown previously to form distinct phylogenetic clades [18] (Fig. S1). Thus, we amplified longer pol (520-bp) and LTR/gag sequences to better resolve the SFV phylogenies and compared these to that of the host cytB sequences to infer possible co-evolutionary histories.

Genetic diversity in NWP SFVs was investigated by comparing the divergence present in the new SFV sequences generated in our study to each other and to other SFV from OW and NWP (Table 3). The nucleotide diversity of SFVcap (within capuchins) was 6–9% in both regions analyzed. The nucleotide diversity within SFV infecting members of the Cebidae (SFVcap and SFVmar) and of the Atelidae (SFVhow and SFVspm) families was similar in the gag and pol fragments (41%) and slightly higher in the LTR region (50%). We observed that the genetic diversity in pol was almost three times that in SFV infecting the Atelidae family than SFV infecting Cebidae. The nucleotide diversity between NWP and OWF SFV was very high, 80% in gag, the most divergent major gene among SFV, and 54% in pol (Table 3).

Significant substitution saturation was found in the 3rd codon position (cdp) of the SFV pol but not the cytB alignments, confirming the high levels of diversity detected above. Thus, a shorter 276-bp alignment consisting of the 1st and 2nd cdp from 13 different animals was used for the SFV phylogenies. In addition, phylogenetic relationships of SFV/LTR sequences from 12 different animals was determined. All SFV pol and LTR/gag sequences were from captive capuchins (Cebus) and howler (Alouatta) monkeys. Phylogenetic analysis of both regions identified individual clades comprising two novel lineages of SFVs from capuchins (SFVcap) and howler monkeys (SFVhow) in addition to the marmoset (SFVmar), squirrel monkey (SFVspm) and spider monkey (SFVspm) lineages (Figures 3A and B). The SFV phylogenies strongly resembled those of the cytB host sequences with formation of NWM and OWM clades (Figs. 2 and 3). In addition, within the NWM (Platyrrhini) clade the Cebidae and Atelidae SFVs also clustered together as for the cytB phylogenies suggestive of SFV host co-evolution (Figs. 2 and 3). Within the howler monkey clades, further structuring of SFV phylogeny by species is also evident with three distinct lineages corresponding to individual howler monkey species (Alouatta guariba, A. belzebul and A. senicula) in both the LTR/gag and pol phylogenies. Similarly, SFVs from two capuchin species (Cebus apella and C. xanthosternos) formed separate lineages in the LTR/gag tree (Fig. 3A), but were mixed with SFVs from C. albifrons or spider monkeys in the pol tree (Fig. 3B). The LTR/gag phylogeny also showed two pairs of viruses from different specimens with no genetic variation in Cebus xanthosternos (F261 and F266) and Alouatta guariba (F37 and F38) (Fig. 3A). In both cases, these pairs represent animals that shared the same cage and are likely direct transmissions between cage mates. Longer pol sequences were not available from animals F261 and F38 for comparison with F266 and F37 to verify the high genetic identity in these pairs in another genomic region, but all four animals were PCR-positive using the generic screening pol primers. Similar LTR/gag and pol tree topologies were inferred using the NJ, ML and BI methods (data not shown).

Interestingly, SFV pol sequences from two specimens of Cebus xanthosternos (F15 and F266) did not cluster with viruses from the remaining Cebus (capuchin) monkeys. Whereas F15 clustered with an SFV from a spider monkey, F266 grouped with SFV from a marmoset (Fig. 3B). Moreover, the placement of F266 outside the SFVcap clade was restricted to the pol fragment, whereas this sequence clustered within the capuchin virus group in the LTR/gag fragment, indicative of viral recombination, and will require further studies. These two viral strains in animals F15 and F266 likely represent cross-species transmission events, suggesting Cebus as a particularly susceptible primate to these viruses. Analysis of host cytB sequences from these two animals confirmed they are yellow-breasted capuchins (Fig. 2), eliminating any possibility of their misclassification or other systematic errors.

To further explore the co-speciation hypothesis and evaluate possible cross-species infection of the two Cebus xanthosternos, tree reconciliation analyses were performed and which identified a single and nine optimal reconstructions using the heuristic and exact search options with inference of 11–12 co-speciation events, respectively. Of these, one reconstruction fit the phylogenetic results and specimen histories better with an estimated 12 co-speciation, one duplication, three host switches, and 15 sorting events (Fig. 4) and was strongly supported following randomization of both primate and SFV trees (P<0.00001). The analysis confirmed the SFV from a marmoset and spider monkey switched hosts in two Cebus xanthosternos (F15Cxa and F266Cxa) (Fig. 3B).
Figure 4. Co-evolutionary relationships of simian foamy virus (SFV) polymerase (pol) (green branches and text) and primate cytochrome B (cytB) (brown branches and text) Bayesian-inferred phylogenetic trees based on reconciliation analysis. One of nine potentially optimal reconciled trees with 12 cospeciations (black circles), three host switches (blue arrows with dashed lines), 1 duplication (black square), and 15 sorting events (truncated branches without corresponding taxa).

doi:10.1371/journal.pone.0067568.g004
The third host switch was inferred to have occurred from an SFV-infected *C. apella* to a *C. albifrons* (F70) ([Fig. 4](#fig4){ref}.). We also found strong linear relationships between the branch lengths \( r = 0.8985 \) and coalescence times \( r = 0.9866 \) for the host and SFV trees ([Fig. 5](#fig5){ref}), indicating that the accumulation of genetic diversity has occurred over an equivalent period in both data sets. Moreover,
we found high agreement between the internode divergence times of the SFV and cytB trees and the fossil record (Table 4). For example, the tMRCA ranges for the both the SFV and cytB Platyrrhini overlap those estimated by others and the fossil record estimate of 20.5–26.5 MYA, which in our analysis is also the Atelidae/Cebidae split since SFV sequences from Pithicidae have not yet been reported [30]. Although fossil record estimates are not available for the NWM families and subfamilies, the inferred divergence date ranges for the SFV and cytB are also in general agreement with each other and with those obtained by others (Table 4) [30]. Finally, although the strict molecular clock was strongly rejected (p<0.000001) for both SFV and cytB trees, we obtained mean nucleotide substitution rates (nucleotides/site/year) using a relaxed clock (7.79×10⁻⁹, 95% HPD 4.89×10⁻⁹–1.16×10⁻⁸) that were very similar to that previously reported by our group for OWM SFVs (1.7–1.8×10⁻⁹). The mean substitution rate for the cytB sequence was also similar to that of SFV (6.24×10⁻⁹, 95% HPD 4.0–9.0×10⁻⁹), which would be expected if both virus and host were co-speciating.

**Discussion**

FVs are the only exogenous retroviruses that have been identified in NWP. However, despite five decades of studying FVs only three SFV variants have been molecularly characterized from platyrrhinines [20,21]. In addition, the diversity of SFV infecting NWP, as well as their dissemination and geographic distribution in nature, is largely unknown. African and Asian primates have been repeatedly shown as sources of zoonotic introduction of SFV into humans [3–6,12], and NWP may also pose a similar risk for such transmission, as these animals are kept as pets around the world and are also hunted and butchered for meat consumption in South America. Herein, we expand significantly our understanding of SFV infection in NWP. We detected eight distinct SFV lineages in 23 different NWP species comprising all three families of neotropical primates (Atelidae, Cebidae, and Pitheciidae). Six of these eight SFV strains are reported for first time and we molecularly characterized in further detail two novel SFV phylogenetic lineages infecting the *Cebus* (capuchins) and *Alouatta* (howler monkeys) genera.

The average molecular prevalence of SFV in NWP estimated here was substantially yet lower (24%) than those reported in African primates which ranged from 60–83% in wild-living and captive mandrills [6], 86% in wild-living red colobus monkeys [34], to 44–100% in communities of wild chimpanzees [35]. In our study, both captive and wild-caught animals showed consistently similar lower prevalence which may be explained by testing of more juvenile animals in our study, which typically have lower SFV infection rates. Unfortunately, the age at collection was not recorded for the animals analyzed and thus we cannot accurately evaluate the effect of age on our PCR results. The lower prevalence may also be due to low proviral loads or lower sensitivity for detecting highly divergent viruses by screening specimens using our new generic PCR assay. However, this assay was found to be highly sensitive and detected a broad range of SFVs in monkeys within each of the three NWP families. In addition, infection with highly divergent SFVs was confirmed in two families using additional sequence analysis, suggesting that the lower prevalence may be from testing of young animals. Although serum and plasma specimens were not available from animals in this study, supplemental screening of NWP using serologic assays for detecting SFV infection will enhance the sensitivity to accurately measure the prevalence of SFV in NWP.

**Phylogenetic separation of SFV LTR/gag and pol sequences**

from six NWP species in two (Atelidae and Cebidae) of the three NWP families mirrored that of their hosts and is consistent with virus-host co-speciation which appears to be a common characteristic of SFV evolution [36,37]. Detailed reconciliation analyses strongly confirmed the co-evolution of SFVs and NWP in these two families with significant correlations between host and SFV branch lengths and divergence times. Although we identified SFV in additional genera in each NWP family, the *pol* sequences were too short for phylogenetic resolution within the Platyrrhini to further investigate the co-speciation hypothesis. Molecular dating in our study estimates that the NWP SFV are at least 15 million years old, with a mean of 28 MYA, consistent with fossil records [30] and host sequence divergence date estimates determined in our study. Combined with the finding of an endogenous SFV in the prosimian aye-aye genome [38], our findings are congruent with an ancient evolution of FVs in simians for over 85 million years.

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**Table 4.** Time to most recent common ancestor (tMRCA) mean estimates for Haplorrhini and simian foamy virus (SFV) polymerase 
(*pol*) and simian cytochrome B (*cytB*) sequences.

| Branch node | tMRCA SFV *pol* | tMRCA *cytB* | tMRCA simian phylogeny | Fossil estimate |
|-------------|-----------------|--------------|------------------------|-----------------|
| Haplorrhini  | 42.38 (33.86–51.11) | 42.37 (34.26–51.27) | 43.47 (38.55–48.36) | 43–4.5 |
| Catarrhini   | 24.33 (15.52–35.17) | 24.17 (15.1–35.3) | 31.56 (25.66–37.88) | 29–6.0 |
| Platyrrhini  | 28.11 (15.02–45.21) | 34.58 (20.43–49.7) | 24.82 (20.55–29.25) | 23.5±3.0 |
| Atelidae     | 15.55 (6.12–31.21) | 20.55 (8.21–37.14) | 16.13 (10.52–21.35) | NA^d |
| Atelinae     | 3.4 (0.75–9.27) | ND^c | 11.25 (7.25–15.46) | NA |
| Alouattinae  | 9.06 (3.64–18.47) | 7.89 (2.4–17.85) | 6.03 (3.74–8.57) | NA |
| Cebinae      | 3.89 (1.32–8.57) | 13.1 (5.96–23.24) | 6.00 (3.13–9.35) | NA |
| Saimiriinae  | 3.37 (0.75–9.27) | 5.4 (1.48–12.62) | ND^c | NA |
| Callitrichinae | 3.21 (0.62–7.8) | 2.79 (0.4–6.67) | 8.42 (5.72–11.38) | NA |

aUsing an alignment of 276-bp of 1st and 2nd codon positions for 18 SFV taxa and 500-bp of all codon positions for 31 DNA, not available. 
^bDating and fossil estimates from Perelman et al. 2011 [30].
^cND, not determined.
^dNA, not available.

doi:10.1371 Journal.pone.0067568.t004
We also identified evidence of cross-species transmission in in two captive *Cebus xanthosternos*, involving different genera or even different families. Cross-species transmission of SFV has also been reported between African primate species [34,39], but is considered a rare event. Although the different NWP species we analyzed herein are typically kept in separate vivariums, it is possible that these two capuchins were in contact with spider (*Ateles*) and marmoset (*Callithrix*) monkeys during transportation or other circumstances. Many of the animals analyzed herein were from Brazilian Wildlife Department confiscations before they were sent to zoos or primate centers, and therefore co-transportation with other species by wildlife dealers or by the law enforcement teams are not known. *Cebus xanthosternos* and *Ateles* habitats also do not overlap in South America. While the former is restricted to the Caatinga forest of northeastern Brazil, the latter lives in the Amazon region. Both groups inhabit distinct biomes, separated by major rivers and a large distance (compare Figs. 1A and B).

Conversely, the observed species-specific structure of the SFV in the *Alouatta* genus may reflect the limited habitat overlap of the three *Alouatta* species analyzed, also living in distinct biomes (Fig. 1C), or enhanced species-specific restriction of SFV infection.

Cross-species transmission may also explain the clustering of SFV sequences [GU356394] LTR/gag sequences with Atelidae SFV rather than other Cebid SFV, as in the congruent pol and env phylogenies. Although the provenance of this tamarin has not been reported in detail and was most likely born in captivity [21], it is possible that it was in contact with Atelidae monkeys or the host taxonomy is incorrect since host sequences are not available for this animal. More parsimonious explanations are long branch attraction due to homoplasy in the sequence and/or poor taxonomic sampling to accurately resolve the SFV phylogeny in the region [40]. The characterization of additional SFV sequences and genomes from other squirrel and spider monkeys will help to clarify the classification of these viruses. We also identified two cases of direct SFV transmission between monkeys living in the same cage, one between two *Cebus xanthosternos* (F261 and F266), and the other between two *Alouatta guariba* (F37 and F38). The SFV LTR/gag sequences from each transmission pair were identical. The low intrahost variability of SFV, the smallest seen among complex primate retroviruses [34], is likely a result of limited viral replication in blood [6,34,37], which may explain the low divergence observed in these pairs. Recent transmission would also explain such low diversity. The exact route of transmission in these cases is currently unknown but likely occurred from grooming or biting which is known to transmit SFV. Further controlled studies with cohabited, yet discordant specimens would shed additional light on SFV transmission routes in NWP and in primates in general.

None of the NWP studied here presented clinical symptoms typical of retroviral infections at the time of specimen collection, including immunodeficiency, inflammation, neurologic disorders, and malignancies. All screened animals were adults at sample collection, and no age-specific correlations can be drawn from our study. It is known that SFV infection increases with age and malignancies. All screened animals were adults at sample collection. We would also like to thank Dr. Albert Menezes and Miss Elisabeth Farias da Silva for helping with sample collection, extraction and PCR. Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are

**Supporting Information**

**Figure S1** Inferred phylogenetic relationships of 138-bp polymerase sequences generated by using the diagnostic PCR primers. The tree was built using Bayesian inference in the program BEAST and a relaxed molecular clock and a Yule tree prior. Posterior probabilities >0.7 are shown at branch nodes. Genera abbreviations are: Ao., *Aotus*; Al., *Alouatta*; C., *Cebus*; Ca., *Callithrix*; Cal., *Callcebus*; Sa., *Saimiri*; Sag., *Saguinus*; Ch., *Chiroptera*; Le., *Leontopithecus*; spm, spider monkey (*Ateles* species), sqw, squirrel monkey (*Saimiri* species), mar, marmoset (*Callithrix jacchus*), agm, African green monkey (*Chlorocebus* species), cpz, chimpanzee (*Pan troglodytes*).

**Table S1** PCR primers for detection of SFV LTR/gag matrix and polymerase (*pol*) sequences.

**Acknowledgments**

We are indebted to the animal keepers of the primate centers and zoos from which some samples were collected. We would also like to thank Dr. Albert Menezes and Miss Elisabeth Farias da Silva for helping with sample collection, extraction and PCR. Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are
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Author Contributions

Conceived and designed the experiments: AFS WMS MAS. Performed the experiments: CPM EAM BS HJ AS. Analyzed the data: CPM EAM HJ AS. Contributed reagents/materials/analysis tools: AP MAM CRB HNS WMS MAS. Wrote the paper: AFS WMS MAS.