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Simian Foamy Virus Prevalence and Evolutionary Relationships in Two Free-Living Lion Tamarin Populations from Rio de Janeiro, Brazil

Déa Luiza Girardi [1], Thamiris dos Santos Miranda [1], Matheus Augusto Calvano Cosentino [1], Talitha Mayumi Francisco [2,3], Bianca Cardozo Afonso [3], Flávio Landim Soffiati [2,3], Suelen Sanches Ferreira [2], Silvia Bahadian Moreira [4], Alcides Pissinatti [4], Carlos Ramon Ruiz-Miranda [2,3], Marcelo Alves Soares [5], Mirela D'arc [1,*] and André Felipe Andrade dos Santos [1,*]

[1] Departamento de Genética, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-617, Brazil
[2] Associação Mico-Leão-Dourado, Silva Jardim, Rio de Janeiro 28820-000, Brazil
[3] Universidade Estadual do Norte Fluminense Darcy Ribeiro-UENF, Centro de Biociências e Biotecnologia, Laboratório de Ciências Ambientais, Campos dos Goytacazes, Rio de Janeiro 28013-602, Brazil
[4] Centro de Primatologia do Rio de Janeiro, Instituto Estadual do Ambiente, Guapimirim 25948-395, Brazil
[5] Programa de Oncovirologia, Instituto Nacional de Câncer, Rio de Janeiro 20231-050, Brazil
* Correspondence: mireladarc@gmail.com (M.D.); afsantos.ufrj@gmail.com (A.F.d.S.)

Abstract: Simian foamy virus (SFV) is a retrovirus that infects primates, including American primates (AP), but epidemiological studies are often limited to captive animals. The state of Rio de Janeiro (RJ) is home to an endemic AP species, *Leontopithecus rosalia*, and an invasive species, *Leontopithecus chrysomelas*, both endangered. This study assessed the molecular prevalence of SFV in these species. Genomic DNA was extracted from 48 oral swab samples of *L. chrysomelas* (Niterói/RJ) and 102 of *L. rosalia* (Silva Jardim/RJ). qPCR was performed to diagnose and evaluate proviral load (pVL). SFV prevalence was 23% in *L. chrysomelas* and 33% in *L. rosalia*. No age-related differences were observed, but *L. rosalia* showed a higher average pVL (3.27 log10/10^6 cells) compared to *L. chrysomelas* (3.03 log10/10^6 cells) (p=0.005). The viral sequence of *L. rosalia* clustered within a monophyletic SFVlro clade, distinct from two SFVlchrysom lineages. Origin of SFVlro dates back to 79.6 thousands of years ago. This was the first study to determine the molecular prevalence of SFV in free-living populations of *Leontopithecus*, and may be of great importance for elucidating the complex evolutionary history of SFV in AP.

Keywords: spumavirus; neotropical primates; South America; prevalence; retroviruses

1. Introduction

Simian foamy virus (SFV) is a complex retrovirus classified within the *Simiispumavirus* genus under the subfamily Spumaretrovirinae [1,2]. SFV was first described in 1954 [3] and isolated in 1955 [4] and, since then, numerous non-human primates have been described as hosts, including prosimians, Old World primates (OWP) [5–7] and American primates (AP) [8–11]. Among AP, the first SFV was detected in 1973, in a culture of brain cells from *Ateles* sp. [12]. Nevertheless, it was only after 34 years that the first complete genome of this virus was obtained [13–15]. AP are highly diverse, with approximately 187 species distributed within 23 genera under five families (Aotidae, Atelidae, Callitrichidae, Cebidae and Pitheciidae) according to molecular analyses [16,17]. Among AP, 41 species (~22%) have molecular evidence of SFV infection, however only five of them have complete viral genomes sequenced, a small number considering the wide diversity of AP. The available sequences include, in addition to the one that infects *Ateles* sp. (SFVasp) [10,15], the SFV that infects *Callithrix jacchus* (SFVcja) [18], *Sapajus xanthosternos* (SFVsxa) [13], *Brachyteles arachnoides* (SFVbar) [14] and *Saimiri sciureus* (SFVssc) [18].

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Little is known about the prevalence of SFV in AP. It is estimated that the average prevalence of SFV in captive AP is 23 - 61% [8,11,12,19,20] and among the free-living AP is 16 - 29% [8,9,20]. The scarce studies on natural SFV infections are a limiting factor in our understanding of the epidemiology of this virus [10]. Furthermore, although there are few complete and partial SFV genomes from AP available in the literature, it is possible to observe through phylogenetic analyses that in general, as in OWP, SFV follows the co-speciation theory [9,11,20]. AP arrived in the Americas approximately 40 million years ago [21], diverging between 41.1 - 22.7 million years ago [21]. As the AP speciation occurred recently and many of the existing species occupy the same environment and have similar habits, there is evidence of cross-species transmission events of SFV between AP species and genera [7,20,22,23].

Over 40% of AP species are endangered, including lion tamarins (genus *Leontopithecus*) [24]. The *Leontopithecus* genus is composed of four species: *L. rosalia* (golden-lion-tamarins), *L. chrysomelas* (golden-faced-lion-tamarins), *L. chrysopygus* (black-lion-tamarins) and *L. caissara* (black-faced-lion-tamarins) [25]. *L. rosalia* is endemic to the Atlantic Forest in Rio de Janeiro, Brazil [26]. They are arboreal and territorial primates, living in small familiar groups. Classified as endangered by the International Union for Conservation of Nature in 2022 [27], the non-governmental organization Associação Mico-Leão-Dourado (AMLD) has been working since 1992 on the conservation of this species in Rio de Janeiro [28]. *L. chrysomelas* also inhabits the Atlantic Forest, being natural of the state of Bahia, Brazil. Despite that, some *L. chrysomelas* have been found in the city of Niterói, Rio de Janeiro, Brazil, as a result of an introduction by a collector in the middle 90s [29]. Therefore, these primates have been established in fragments of the Atlantic Forest in this city. Also considered endangered, *L. chrysomelas* are monitored, captured, and transported for centers of preservation in their natural habitat in Bahia by institutions such as Centro de Primatologia do Rio de Janeiro (CPRJ), Fundação Pri-Matas, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Instituto Estadual do Ambiente (Inea) [29].

Both species have ecological importance and are threatened by deforestation, habitat fragmentation, illegal traffic and diseases. They also face threats in competition for territory and resources or exposure to pathogens due to interaction with other primate species, like those from the *Callithrix* genus [30]. Although there are studies demonstrating the infection and prevalence of SFV in lion tamarins, they are limited to a few individuals and/or captive animals [8,11,20,31]. Herein, we describe for the first time the prevalence of SFV in free-living *L. rosalia* and *L. chrysomelas*.

2. Materials and Methods

2.1. Sample Collection

Oral swabs were collected from *L. rosalia* in Silva Jardim (state of Rio de Janeiro, Brazil) by Associação Mico Leão Dourado (AMLD), and from *L. chrysomelas* in Niterói (state of Rio de Janeiro, Brazil) by Centro de Primatologia do Rio de Janeiro (CPRJ). Sample collection occurred between February and September 2021 and the animals were habituated to human contact, being constantly monitored by veterinarians of both centers. Animals were captured individually with Tomahawk® traps with banana baits as described in [32]. All captured tamarins from Silva Jardim/RJ were taken to AMLD field laboratory for routine veterinary examinations before release and the animals from Niterói/RJ were allocated in appropriate enclosures at CPRJ. Animals were anesthetized with an injection of ketamine (10-15 mg/Kg) in the caudal region and general information was collected for all sampled animals, such as identification number, group, species, age, sex, weight and clinical conditions. While the animals were still anesthetized, samples of oral swabs were collected. Animals aged four to nine months were considered juveniles, nine to 12 months as subadults, and adults aged < 12 months. For estimating age from animals whose birth date were unknown, measurements such as body size and weight, identification pattern and postnatal ossification were also used [33–35]. This project was approved by the Ethics Committee on the Use of Animals (CEUA) of UFRJ (reference number 037/14). All procedures were conducted in full compliance with Federal permits issued by

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the Brazilian Ministry of the Environment (SISBIO 75941-4) and samples were collected following the national guidelines and provisions of IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Brazil; permanent license number 11375–1). The geographic coordinates of samples collected in Silva Jardim are available in Supplementary Table S1. We did not have the coordinates of the collections made in Niterói.

2.2. Sample Processing and Analysis of Genomic DNA Integrity

Collected swabs were placed in 1.5 mL eppendorf tubes containing 500 µL of RNAlater (Invitrogen, Thermo Fisher Scientific, USA). All samples were stored at room temperature and sent to the Laboratory of Diversity and Viral Diseases (LDDV) at the Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil, to be stored at -80°C until processing. Genomic DNA (gDNA) was extracted from oral swabs using the PureLink® Genomic DNA kit (ThermoFisher Scientific, Grand Island, NY, USA) according to the manufacturer's specifications. After extraction, samples had their contaminants (PCR inhibitors) removed with the PureLink™ PCR Purification Kit (Invitrogen). Samples were then quantified using Nanodrop ND-1000 (ThermoFisher Scientific) and stored at −20 °C. gDNA integrity for PCR analysis was checked by PCR amplification of the mitochondrial constitutive gene cytochrome B (cytB), as previously described [8]. *cytB*-positive samples were considered suitable for performing quantitative real-time PCR (qPCR) diagnostic for AP SFV.

2.3. Diagnostic qPCR

As described by Muniz and collaborators [36], a qPCR assay was used to simultaneously detect and quantify AP SFV integrated into host gDNA of the oral mucosa epithelium cells, obtained through oral swabs. TaqMan (Thermo Fisher Scientific) was used with primers and a probe for the amplification of a 124 bp fragment located in the virus *pol* gene (integrase region) [36]. The construction of the standard curve was performed through serial dilutions of 10^8 down to 10^1 copies of a pCR4-TOPO plasmid containing an insert corresponding to a 6,400 bp genomic fragment of the Saimiri SFV (SFVsqu) containing the *pol* gene. The plasmid was purified with the QIAGEN™ Plasmid Midi Kit (QIAGEN, Chatsworth, CA), according to the manufacturer's protocol.

All reactions were set up in a total volume of 21 µl containing 10 µl of GoTaq® Probe qPCR Master Mix 20X (Promega, São Paulo, Brazil), 1 µl of primers and probe and 10 µl of gDNA. Reactions were carried out in 96-well optical microtiter plates on a 7500 Real-Time PCR platform (Applied Biosystems, Foster City, CA), following uniform cycling parameters previously described [30]. The sensitivity of the assay was 100 copies of SFV/reaction, as determined previously [30]. Samples with more than 100 viral copies per 10^6 cells were considered positive for SFV infection in the qPCR. To calculate the integrated proviral load in 10^6 cells, the amount of DNA was converted from nanograms to picograms (pg) and multiplied by 1 million cells, with a cell having an average of 6 pg of DNA [37].

2.4. PCR of Pol Region

A nested PCR was performed to amplify a larger fragment of SFV *pol* in *Leontopithecus* for subsequent sequencing and phylogenetic analysis. The first PCR round aimed to amplify a 486-bp fragment of the *pol* gene and the second round amplified a 404-bp fragment internal to the first round. The primers used were specific for SFV *pol* infecting the Cebidae and Callitrichidae families and conditions were the same as those previously reported for other generic PCRs for SFV *pol* [11]. Successfully amplified fragments were purified with the E-Gel CloneWellTM (Invitrogen), according to manufacturer instructions.

Purified samples were submitted to sequencing using the Big Dye Terminator Cycle Sequencing kit v3.1 (Life Technologies, Washington, USA). The plate was precipitated and stored at -20°C protected from light until sequencing in an 3130XL genetic analyzer (Life Technologies, Foster City, USA). The obtained sequences were assembled and edited in SeqMan 7.0 (DNASTAR, Madison, WI, USA).

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2.5. Phylogenetic and Timescale Analyses

The assembled sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) against the complete GenBank dataset available as of September 1st, 2024. Up to 100 SFV *pol* sequences were retrieved and, with the removal of identical sequences, a dataset of 90 sequences was built to further evolutionary contextualization. The SFV *pol* sequence of *Prosimiispumavirus otocrafo* (accession number: NC_03902.1) was added to this dataset to root the phylogeny. Sequences were aligned with sequences representing different SFV strains, comprising a comprehensive dataset of 92 *pol* sequences with 213 nucleotide positions (Supplementary Table S2).

Sequences were aligned with MAFFT v7.505 [38] and subsequently trimmed with TrimAl v.1.4 [39] with a gap threshold of 0.9, removing sites from alignments composed of gaps in 10% or more sequences. Maximum likelihood trees were inferred with IQ-Tree v.2.1.4 [40] with the best fit model suggested by ModelFinder [41]. Node supports were estimated using the SH-type approximate likelihood ratio test (SH-aLRT) [42] and ultrafast bootstrap [43] with 10,000 replicates.

As the phylogenetic pattern for the American SFV observed at the golden-lions tree was not consistent with the ancient within-species diversity model expected, we inferred a time-scaled phylogenetic tree by RelTime-ML [44–46] to obtain chronological information. This analysis was performed in MEGA11 and estimated host divergence dates were used to calibrate internal nodes of the viral tree, following [21].

A Jukes-Cantor substitution model with a Gamma distribution and invariable rate variation model (JC+G4+I) and six calibration constraints were used to build the timetree. The ancestral node of the *Macaca* SFV was calibrated with a normal distribution centered at 11.2 million years ago (Mya) and a standard deviation of 0.35. Similarly, the ancestral clade of the *Pongo* SFV was calibrated with a normal distribution of mean 1.55 Mya and a standard deviation of 0.15. For the *Pan* SFV ancestral node, a calibration of 8.01 Mya with a standard deviation of 0.45 was applied. The shared ancestral node of the *Pan* and *Pongo* SFV was calibrated at 20.32 Mya with a standard deviation of 0.85, while the shared ancestral node of the *Pan*, *Pongo*, and *Macaca* SFV was set to 31.08 Mya with a standard deviation of 0.95. Finally, the ancestral node of Platyrrhini and Catarrhini SFV was calibrated at 39.03 Mya with a standard deviation of 1.1 , following [21].

The phylogenetic tree and timetree were visualised using ggtree v.3.10 [47] in R v.4.3.2. The original nucleotide dataset, final alignment file, generated tree files, and timescale files are provided in Supplementary File S1.

2.6. Statistical Analyses

For statistical analysis and graphing, RStudio version 4.3.1 [48] and GraphPad Prism 8 [49] (GraphPad Software, Boston, Massachusetts USA) were used. To compare the prevalence and associations in relation to the analyzed species, sex, or age group of individuals, Pearson's chi-square test [50] was used, a test indicated to compare two independent categorical variables. To compare mean proviral loads, the Welch t-test was used, or t-test for unequal variances, a two-sample location test used to test the hypothesis that two populations have equal means.

3. Results

3.1. Study Population

Oral swab samples were collected from 150 *Leontopithecus* specimens, 48 of which were *L. chrysomelas* housed at CPRJ and 102 *L. rosalia* captured at AMLD. While for *L. rosalia* the distribution of samples was similar between sexes (48 females and 54 males), for *L. chrysomelas* there was a predominance of samples from male animals (n: 29, 60%). There was no additional information on the *L. chrysomelas* specimens, but for *L. rosalia* the age categories were obtained, where a predominance of adults was observed (n: 48, 47%), as well as morphological information and field data, all described in Table 1.

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The *L. rosalia* were in good body condition, with a median weight of 521g, with an average weight of 83.1 millimeters and no changes were observed in the physical examination. Such information was not available for *L. chrysomelas*. Geospatial coordinates and group composition were also available. The median collection per point was 5 individuals, and per group was 3. All information is summarized in Table 1.

Table 1. Demographic data of *Leontopithecus* population.

Individuals	*L. rosalia*	*L. chrysomelas*
All individuals	102	48
Males	54 (53%)	29 (60%)
Females	48 (7%)	19 (40%)
Adults	48 (47%)	N/A*
Subadults	23 (23%)	N/A
Juveniles	31 (30%)	N/A
Median weight (grams)	521 (259 - 754) g	N/A
Average knee-heel distance	83 (65 -97) cm	N/A
Median collection per site	5 (2-26)	N/A

* N/A - not available.

3.2. SFV Prevalence

All samples had the constitutive *cytB* gene PCR amplified successfully and were deemed suitable for SFVdiagnosis by qPCR. The overall prevalence of SFV infection in the *Leontopithecus* genus was 30% (45/150). In *L. chrysomelas*, the overall prevalence was 23% (11/48) (Figure 1A), with 33% of females (6/18) and 17% of males (5/29) positive for SFV (p=0.18) (Figure 1B). The overall SFV prevalence of *L. rosalia* was 33% (34/102) (Figure 1A). The prevalence in females was 33% (16/48), as well as in males (18/54) (p= 0.58) (Figure 1B). There was no statistical difference between SFV prevalence in *L. rosalia* and *L. chrysomelas* (p = 0.5).

For the *L. rosalia* population, it was possible to estimate the SFV prevalence according to the age of the individuals. Juveniles had a prevalence of 29% (9/31), subadults had 22% (5/23), and adults had 42% (20/48) (Figure 1C). There were no significant differences in SFV prevalence between the age groups. *L. rosalia* individuals are organized into groups at different geographic locations and, in this study, animals belonging to 12 locations and 31 groups were sampled (Figure 2; Tables 1 and 2). Thus, it was possible to indicate the SFV prevalence at each of the collection points, as well as in each of the groups, while the median prevalence was 25% (0-100%). Table 2 shows the SFV prevalence according to the location and Table 3 shows the prevalence according to the groups at each of these collection points.

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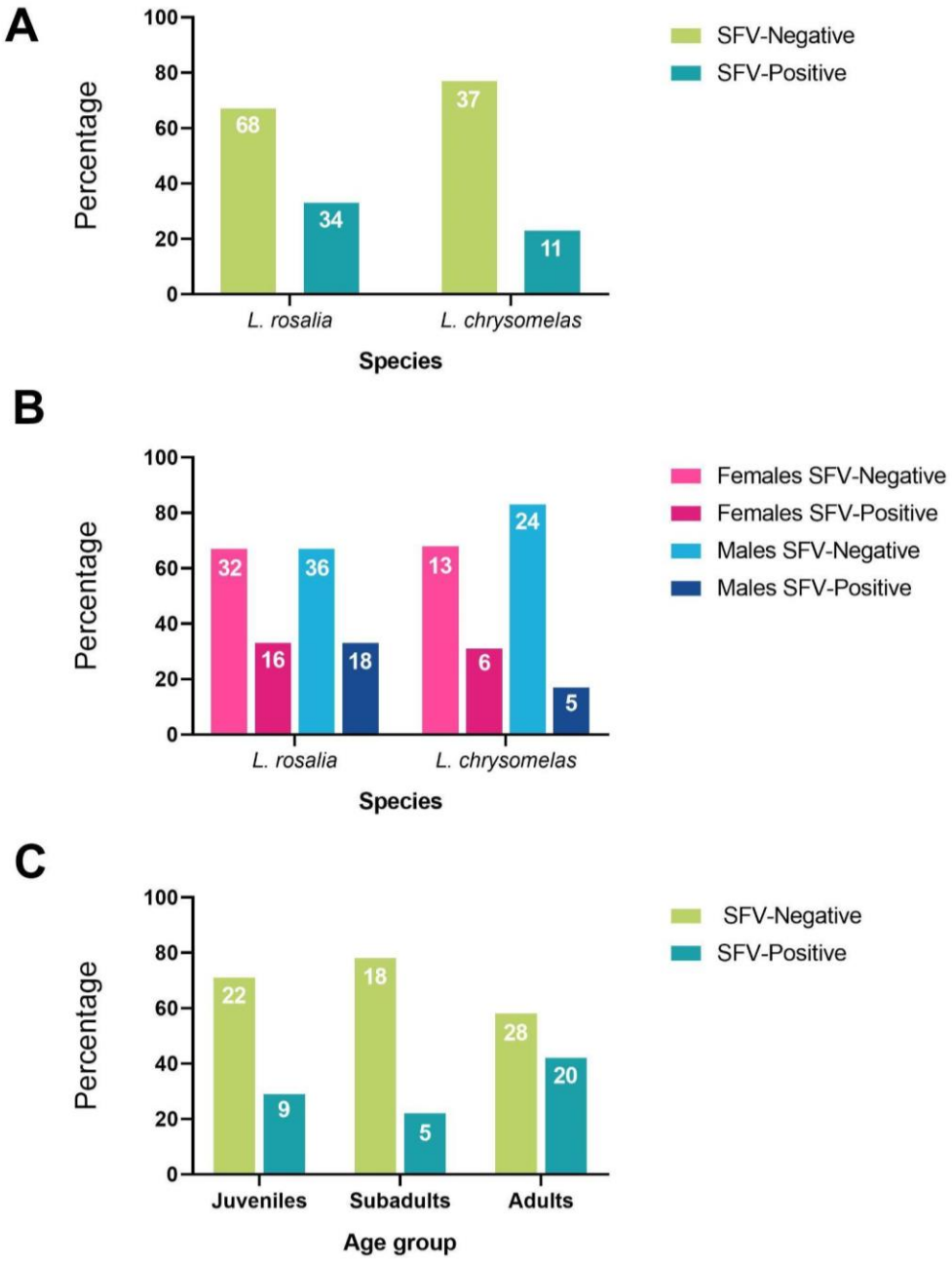

Figure 1. Prevalence of SFV infection. **1A**. Prevalence according to *Leontopithecus* species. Of the 102 *L. rosalia*, 34 (33%) were infected. Of the 48 *L. chrysomelas*, 11 (23%) were infected. The green bars represent SFV-positive individuals, while blue represents SFV-negative specimens. No significant difference was observed ($p = 0.500$). **1B.** Prevalence according to sex in *L. rosalia* and in *L. chrysomelas*. The dark pink bars represent SFV-positive females, while light pink bars represent SFV-negative females. The dark blue bars represent SFV-positive males, while light blue bars represent the SFV-negative males. No significant difference was observed ($p = 0.180$). **1C.** Prevalence according to the age group in *L. rosalia*. The green bars represent SFV-positive individuals, while blue bars represent SFV-negative individuals. SFV prevalence was split into juveniles (4 to 9 months), subadults (9 to 12 months) and adults (over 12 months). No statistically significant difference was observed between the comparison of the age classes. In all three panels, the number of individuals is found within the bars.

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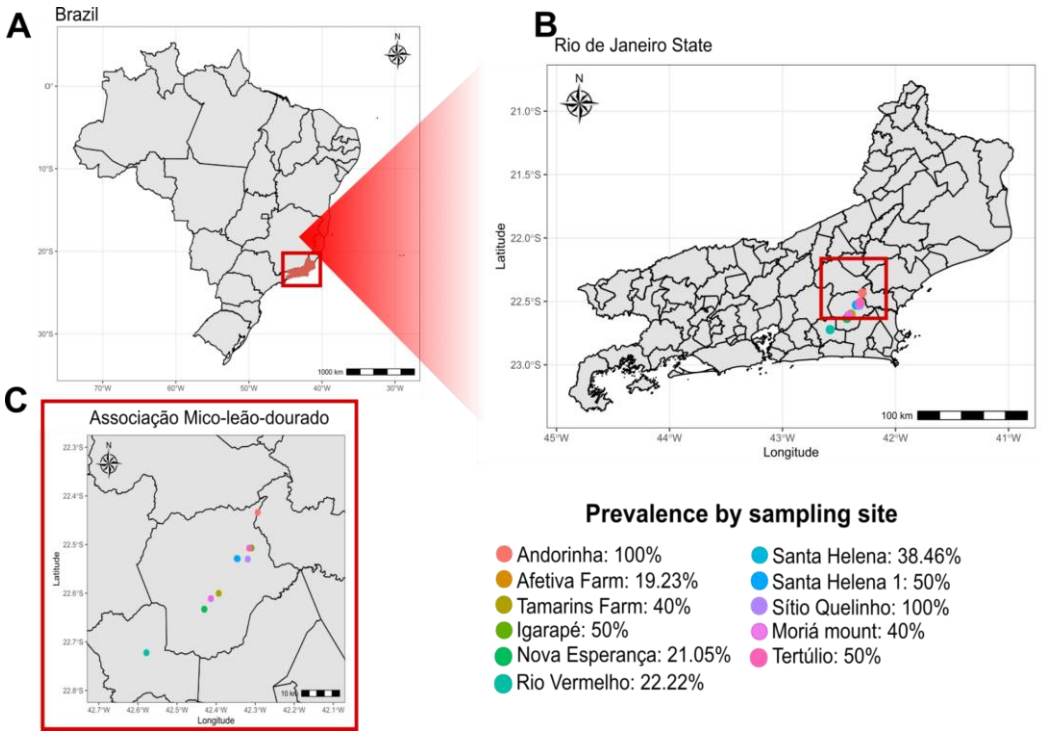

Figure 2. Geographic distribution of the groups sampled in the AMLD in the state of Rio de Janeiro (RJ), in the municipalities of Silva Jardim and Rio Bonito. In (A) the state of RJ is highlighted on the map of Brazil. In (B), the collection points of AMLD in the state of RJ are highlighted. In (C), we highlight the municipalities where the collections were carried out, with Silva Jardim being the most representative, with 10 collection points, and Rio Bonito with only one. Each color represents a different collection point, as can be seen in the image. SFV prevalence in each of the collection points can be seen in the legend.

Table 2. SFV prevalence and proviral load according to age group in *Leontopithecus rosalia.*

Collection point	Animals sampled	Juveniles	Subadults	Adults	Prevalence (%)	Average proviral load*
Afetiva Farm	26	12	9	5	19	03.04
Tamarins Farm	5	0	2	3	40	3.81
Igarapé	12	3	3	6	50	3.30
Nova esperança	19	4	4	11	21	3.14
Rio Vermelho	9	4	0	5	22	2.74
Ribeirão	2	0	0	2	0	N/A
Santa Helena	13	1	4	8	35	3.73
Santa Helena I	4	1	1	2	50	4.56
Sítio Quelinho	2	1	0	1	100	3.91
Tertúlio	2	1	0	1	50	3.93
Monte Moriá	5	4	0	1	40	3.63
Andorinha	3	0	0	3	100	4.22

*Average proviral load (log_{10}) per 10^6 cells.

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Table 3. SFV prevalence and proviral load according to location of collection in *Leontopithecus rosalia.*

Collection point	Group	Animals	Prevalence (%)	Average proviral load*
Afetiva	Afetiva 1	2	50%%	2.05
Afetiva	Afetiva 2/ AF2	12	25%	2.19
Afetiva	Afetiva 3/ AF3	2	0%	N/A*
Afetiva	UR	4	0%	N/A
Afetiva	FP	5	20%	2.33
Afetiva	FP3	1	0%	N/A
Andorinha	CH2	3	100%	4.22
Tamarins Farm	Sidney 3	1	100%	3.49
Tamarins Farm	TM2	4	25,00%	4.12
Igarapé	IG	8	62%	2.81
Igarapé	ph2	4	25%	1.88
Moriá Mount	Ronaldo Machado (RM)	2	40%	6.63
Nova Esperança	GM2	3	0%	N/A
Nova Esperança	GM3	7	29%	3.02
Nova Esperança	GM4	2	100%	3.05
Nova Esperança	GM5	4	0%	N/A
Nova Esperança	GM7	3	0%	N/A
Rio vermelho	M6	1	100%	2.51
Rio vermelho	Mistura fina	3	0%	N/A
Rio vermelho	RV	4	25%	2.96
Rio vermelho	RT	1	0%	N/A
Ribeirão	ZN	2	0%	N/A
Santa Helena	FN	2	100%	2.65
Santa Helena	JA	5	20%	4.34
Santa Helena	JN	2	100%	4.30
Santa Helena	JR	4	0%	N/A
Santa Helena 1	SH	1	0%	N/A
Santa Helena 1	SS2	3	67%	4.56
Sítio Quelinho	q1	2	100%	3.91
Tertulio	JD	2	50%	3.93

*Average proviral load (log_{10}) per 10^6 cells.

3.3. SFV Proviral Load

Upon qPCR analysis, it was possible to measure the SFV proviral load of the infected animals. The average proviral load was 2.36 x 10^4 copies per 10^6 cells (log_{10} 3.03; min 1.29 log - max 5.89 log). In *L. rosalia*, the average SFV proviral load was 3.04 x 10^4 per 10^6 cells (log_{10} 3.17; min 1.43 log - max 5.89 log), while in *L. chrysomelas*, the average SFV proviral load was 2.36 x 10^4 copies per 10^6 cells (log_{10} 3.03) (min 1.29 log - max 3.30 log) (Figure 3A). We observed a significant difference in the SFV proviral load between *L. chrysomelas* and *L. rosalia* (p =0.0017). There was no statistical significance between sexes when comparing the two different species or between the age groups in *L.rosalia* (Figures 3B and C, respectively).

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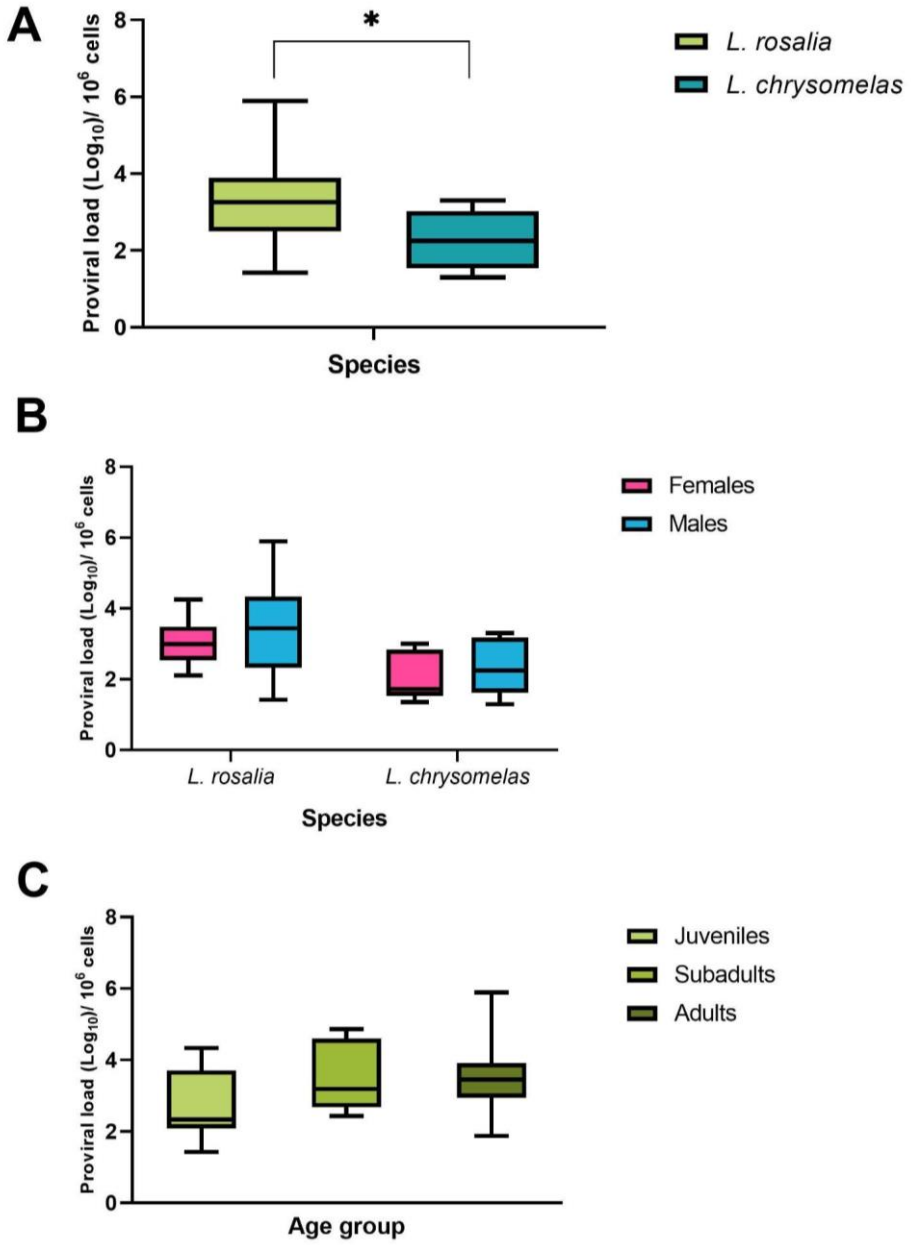

Figure 3. SFV proviral load among the animals studied. (A) SFV proviral load according to species. *L. rosalia* is represented in green and *L. chrysomelas* in blue; *p*-value = 0.0051. (B) SFV proviral load according to sex. *L. rosalia* is represented in light green and *L. chrysomelas* in dark green. No statistically significant difference was observed according to the sex in *L. rosalia* (p = 0.2266) nor in *L. chrysomelas* (p = 0.5809). (C) SFV proviral load according to age group in *L.rosalia*. No significant differences were observed between age groups.

3.4. Phylogenetic and Timescale Analysis

To sequence the SFV infecting free-living populations of the two *Leontopithecus* species, a PCR was performed to amplify a larger fragment of the SFV pol gene, a 404-bp fragment of the integrase region. All samples positive for SFV infection in the qPCR diagnosis (n = 50), 34 from *L. rosalia* and 16 from *L. chrysomelas*, were subjected to PCR of the larger fragment of *pol*. However, only three samples of *L. chrysomelas* and one sample of *L. rosalia* had that fragment successfully amplified and were thus directed to sequencing. Only one of the positive samples analyzed, a sample from *L. rosalia*, had the sequence successfully determined (MP261), generating a 213-bp DNA fragment (GenBank accession number: PP960560).

The maximum likelihood phylogeny inferred recovered a topology consistent with previously proposed SFV, with most host specific viral lineages inferred with high support values (SH-aLRT >

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75, UFBoot > 75). The *L. rosalia* generated sequence grouped with SFV of the Cebidae and Callitrichidae families, close to another sequence from *L. rosalia* (SFVlro; GenBank accession number: PP960560) as expected, forming a monophyletic clade of SFVlro with strong support (SH-aLRT > 98.8, UFBoot > 100). However, it did not form a sister clade with either of the two circulating *L. chrysomelas* SFV lineages (SFVlchrysom) (Figure 4A). The phylogeny of SFV infecting the Cebidae and Callitrichidae families can be further visualized in Figure 4B.

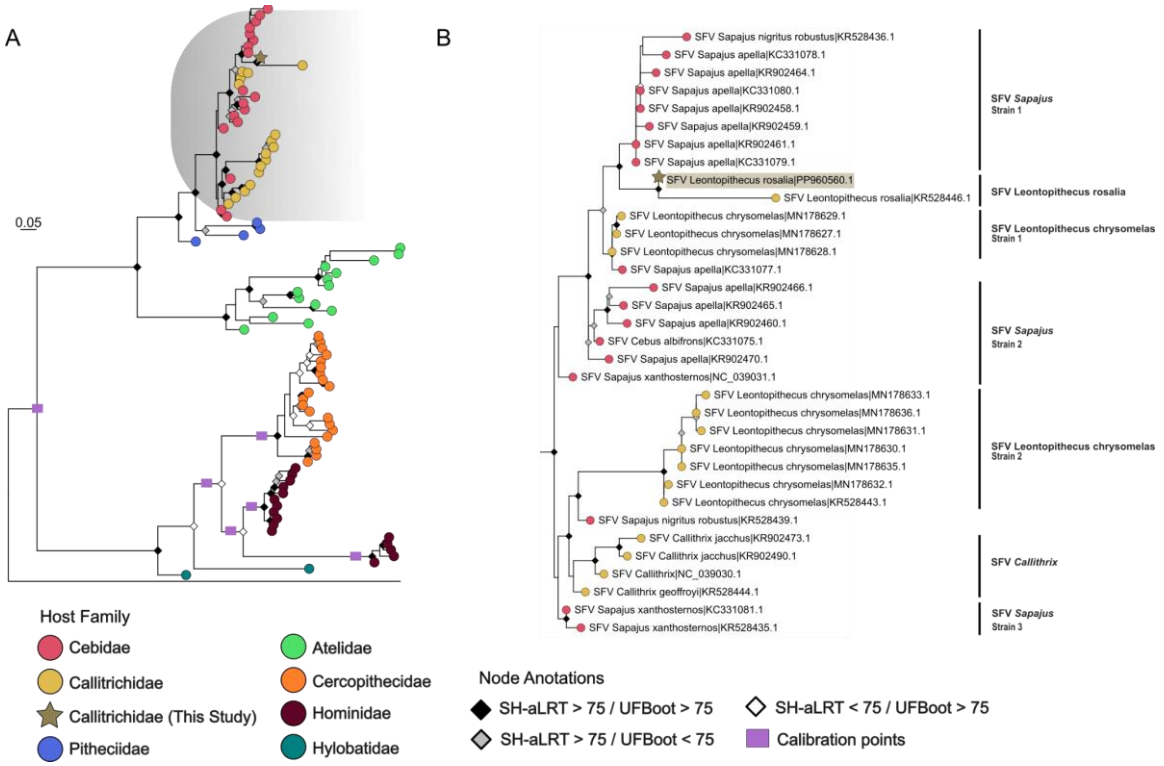

Figure 4. Phylogenetic tree inferred using maximum likelihood analysis with a fragment of SFV viral polymerase (213-bp). The new sequence generated in the current study is marked with a golden star. The host species within the Callitrichidae and the Cebidae families are listed. The node labels are colored according to the host family used in the dataset. The node labels colored in black represent support for SH-aLRT and bootstrap equal to or greater than 75%. When only SH-aLRT is superior to the cutoff, the label is represented in gray, while only UFBoot node labels are represented by white squares. When both parameters are lower than 75%, no label is depicted. Purple rectangles represent the calibration points used to further explore the timescale of the phylogeny.

While our phylogeny inferred the SFV infecting Old World primates (OWP) as a monophyletic clade within each of the host family that they infect, we do not observe the same happening in the SFV infecting AP. For instance, the SFV infecting the family Pitheciidae did not form a monophyletic clade in our analysis. Also, we can observe the presence of two lineages of SFV circulating both in the *Sapajus* and *Leontopithecus* genus. Although the SFV families are mixtured in the AP, we observe monophyletic clades in each SFV lineage. The SFV infecting *Leontopithecus* genus always forms a sister clade of SFV from Cebidae or Callitrichidae families.

Considering this unique pattern, a time scale phylogenetic tree was obtained by the RelTime-ML method. The SFV found in OWP served as calibration points using its host divergence dates to calibrate internal nodes of the viral tree. The *L. rosalia* SFV sequence obtained in this work has an origin calculated of 79.6 thousands of years ago (Tya), indicating a recent circulation among *L. rosalia*. The SFVlro shares recents ancestors with *Sapajus* SFV (673.7 Tya) and *L. chrysomelas* SFV 1 (1.09 Mya), belonging to an older monophyletic lineage of Cebidae SFV (3.61 Mya). A similar pattern is found for the *L. chrysomelas* SFV 2, who shares an ancestor with *Sapajus nigritus* SFV (2.36 Mya) and is a sister clade to a lineage of *Callithrix* SFV (2.88 Mya), outgrouped by a *Sapajus xanthosternos* SFV clade (3.49 Mya). Interestingly, a pattern of host switching between Callitrichidae and Cebidae is observed, as

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the monophyletic lineage of all Callitrichidae SFV and Cebidae SFV (4.032 Mya) is not exclusive to either host family.

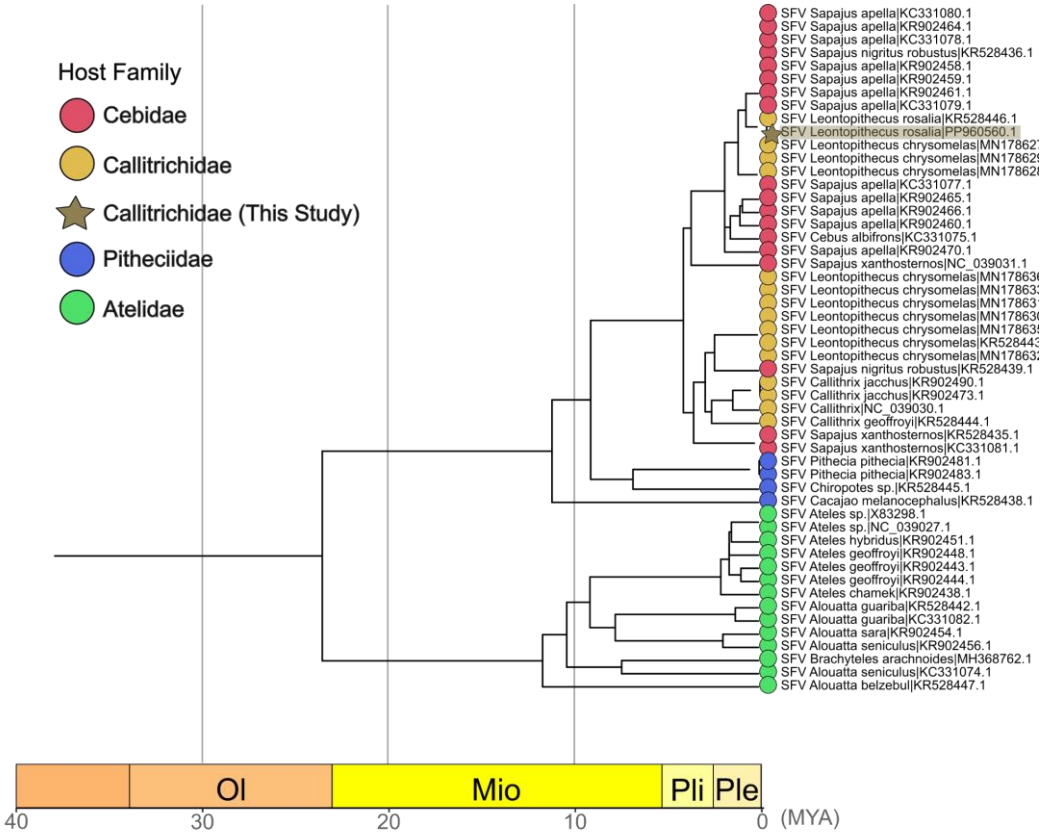

Figure 5. Timescale phylogenetic tree generated by RelTime-ML. Estimated host divergence dates were used to calibrate internal nodes of the viral tree. The node labels are colored according to the host family used in the dataset. The sequence generated in the current study is marked with a golden star. The x-axis summarizes geological time scale of the timetree: Oligocene (Ol), Miocene (Mio), Plioceno (Pli) and Pleistocene (Ple).

4. Discussion

Despite APs being a highly diverse group with a wide geographic distribution in South and Central Americas [51], data about the prevalence of viral agents, including SFV, remain scarce, especially in free-living populations. SFV are widely disseminated retroviral agents that coevolve with their primate hosts [7] and their diagnosis and monitoring can serve as a biomarker of zoonotic transmission of the virus between different callitrichid species. This is the first study to show the prevalence of SFV in free-living populations of *L. chrysomelas* and *L. rosalia*. The SFV prevalence in both species are in agreement with those reported in previous studies, between 20 and 50% in free-living AP [8,11,31]. We did observe a significant difference in the SFV proviral load ($p = 0.005$) between *L. chrysomelas* (average SFV proviral load of 3.03 log per 10^6 cells) and *L. rosalia* (3.27 log). Host immunogenetic diversity can act as a factor that impacts the viral burden in different individuals, with some managing to maintain low levels of proviral DNA and others failing to prevent viral replication and integration [52]. The immune response to SFV is not yet well understood, however it is thought that IFN-γ production can play a role in SFV replication [53]. In this sense, each individual could have different immune responses to SFV infections that lead to different proviral loads. Falcone *et al*. argue that the distribution of infected cells in the oral mucosa might not be homogeneous [54]. Thus, the mode of oral swabs collection may also impact the results of the proviral load detected in qPCR. In fact, Falcone *et al*. found, through *in situ* hybridization, that actively infected cells are distributed in small sparse foci [54].

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When comparing the prevalence of SFV infecting free-living *L. chrysomelas* in this study with that of recently captured *L. chrysomelas* [11], the prevalence was similar, either in the comparison of individuals with up to six months of captivity ($p = 0.72$) or those that have been in captivity for more than six months ($p = 0.13$). In agreement to what has been found previously for OWP and AP, we did not find any significant difference between the SFV prevalence in males and females of both species, showing that sex is not a factor that impacts SFV transmission [11,31,55].

Hood et al. [52] showed that the SFV prevalence in animals tends to grow with advancing age in *Macaca fascicularis*. As SFV promotes a chronic infection, implying that there is greater exposure to SFV as age advances [20,56], we sought to compare the SFV prevalence in juveniles, subadults and adults. The prevalence in juveniles was 29%, 22% in subadults and 42% in adults, and therefore there were no significant differences in SFV prevalence between the age groups.

In general, there was a high variability in SFV prevalence among the collection points sampled at AMLD (0 - 100%). The *L. rosalia* population is distributed in 13 forest fragments, in an area of approximately 4,500 km² of lowland Atlantic coastal rainforest. Each fragment has limited or no forest connection with other fragments. These fragments are called management units (MUs). In 2019, AMLD had detected 24 social groups [57]. The main factor responsible for habitat fragmentation is the presence of physical structures, such as roads, cars or traffic. This factor is known as the "barrier effect" and can cause restrictions on individual movement, ultimately resulting in losses in population size and persistence [58]. The dispersion of *L. rosalia* occurs more frequently from small and nearby fragments than from large, isolated forests. That being so, a fragmented landscape may lead to low dispersal rates [59]. The population dynamics and viability is highly affected by dispersion [60]. As some fragments inhabited by *L. rosalia* are closer to roads than others, that may have impacted the number of collections [58].

We attempted to correlate the geospatial arrangement of *L. rosalia* with SFV prevalence, assuming that nearby groups would have similar prevalence, but due to the small sample size of each group, a robust statistical analysis was not possible to be conducted. With the provided data, we also observed the prevalence in each group within each of the AMLD animal collection points. Some points were better represented than others, as was the case at the Afetiva collection point (Table 3). On the other hand, at other collection points, sampling probably did not represent the local population, such as at Sítio Quelinho and Andorinha points, with samples from a single group of two and four animals, respectively.

For phylogenetic analysis, a conventional PCR of the *pol* region was carried out and 8% (4 out of 45) of the positive samples for SFV were amplified. SUch limited PCR amplification success may be indicative of a great genetic variability of SFV strains circulating among this group of animals. The pol PCR was developed with the few available sequences of SFV infecting the primate families Cebidae and Callitrichidae, which included SFV from *Sapajus*, *Callithrix*, and *Leontopithecus* [11]. In this sense, the primers used may not comprise the diversity of SFV present in these families of primates, being a major limitation for the study of molecular characterization by conventional methods. As new strains of SFV are sequenced, especially through massive sequencing techniques, an improvement in molecular techniques for detecting this virus is warranted.

The only SFV sequence of *L. rosalia* obtained formed a monophyletic clade with a previous SFVlro sequence from a captive *L. rosalia* specimen from the Rio de Janeiro Primate Center obtained in the study by Muniz and collaborators [20]. However, there was no grouping with any clade of the SFVlchrysom lineages, which would be expected by the co-divergence hypothesis. The identification of two circulating strains in *L. chrysomelas* was observed in [11], in which viral strains SFVlcm-1 and SFVlcm-2 were identified. In agreement with what was found in Muniz *et al.* [8], we also observe two circulating SFV strains in the *Sapajus* genus. One hypothesis is that the co-speciation process between SFV and AP might still be in progress. On the other hand, the SFVs infecting the *Leontopithecus* genus do not group in the same clade, and one alternative explanation would be that ongoing zoonotic transmission events occurred that resulted in multiple SFV strains coevolving with their primate hosts in *Leontopithecus*. That could explain the fact that we observe monophyletic clades of SFV within

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the primates families Cebidae and Callitrichidae, but the SFV infecting the same families do not cluster together.

The same phenomenon may take place in *L. rosalia*, and it is possible that we have sequenced only one of the SFV strains infecting that species. A more recent cross species SFV transmission event from *Sapajus* to *L. rosalia* may not be discarded, but only could have occurred in captivity, since the *Sapajus apella* is not autochthonous of the state of Rio de Janeiro [61]. This could explain the grouping between the sequences of SFVlro and SFV from *S. apella* seen herein, since some *L. rosalia* specimens have been reintroduced into the wild or are descendants of reintroduced animals. Thus, the animals may have had direct or indirect contact with *Sapajus* during their time in captivity, although it is not common for animals of different species to be housed in the same enclosure. Those hypotheses could be better investigated through the use of molecular dating techniques. However, since the sequence generated is short (213 bp) compared to the *pol* gene (approximately 3,440 bp), testing such hypotheses is not currently permitted. The same event of SFVlro clustering with SFVsxa was observed in captive *L. rosalia* in [20], and the hypothesis of two independent cross-species SFV transmission events is debated, with a possible ancient host-switching. The sequencing of larger regions of *pol* and/or other viral genes from the wide range of SFVs infecting AP will allow more robust phylogenetic analyses, bringing a better resolution and understanding onto the evolution of SFV in AP.

The SFV infecting Pitheciidae form a monophyletic clade with the previous Cebidae family classification [8] and, with the recently division in the Cebidae family in Cebidae, Callitrichidae and Aotidae, multiple strains are noted infecting the three families, similar to what occurs with papillomavirus [62]. Although the SFV infecting the Pitheciidae family do not form a monophyletic clade in our analysis, they grouped together in other studies by our group [8,20]. The SFV of the Atelidae family is the only one within the AP to exhibit a unique clade. We observe monophyletic clades with multiple SFV strains, except for *Sapajus xanthosternos*. Several strains do not form single monophyletic clades, but rather multiple strains circulating between *Sapajus* and *Leontopithecus* in a virus complex for which we do not have representative viruses sequenced. Besides the short fragment used for phylogenetic and timescale analysis, the dating matches the dates of separation of the of the Old World primates and New World primates, with the split between catarrhines and platyrrhines occurring between 41.1 and 36.7 My [21], with the crown Platyrrhini diverging in 26.5-22.7 Mya [21]. The most recent common ancestor (MRCA) of Pitheciidae diversified approximately in 18.08 Mya [63], the MRCA of Atelidae radiated at 15.29 My [63] and the MRCA of Cebidae diverged in 20.86 My. The diversification between *Leontopithecus*–*Callimico* and *Callithrix* is estimated to have occurred at 10–11 Mya [64].

5. Conclusions

In conclusion, we describe for the first time the prevalence of SFV in free-living populations of *L. rosalia* and *L. chrysomelas*. We did not observe any significant difference in SFV prevalence according to infected species or associated with sex. We also did not observe any significant difference in SFV proviral loads associated with the sex or age of the animals. However, the SFV proviral load was higher in *L. rosalia* than in *L. chrysomelas*. It was not possible to establish relationships between the geographic distribution of animals at AMLD and SFV prevalence. We identified that the SFV strain circulating in *L. rosalia* does not cluster with any of the SFV strains circulating in *L. chrysomelas* but rather with SFVs that infect the *Sapajus* genus, which may be the result of one or more zoonotic transmission events, and further molecular and phylogenetic analyses are necessary to elucidate this issue. With the split of the Cebidae family into three families, the evolutionary history of SFV proved to be more complex in four of five AP families. The molecular dating of SFV infecting AP was also determined herein.

Supplementary Materials: The following supporting information can be downloaded at: https://github.com/deagirardi/Supplementary_data_SFV_L.rosalia_L.chrysomelas, Table S1: Geographic

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Coordinates of *Leontopithecus rosalia*, Table S2: Alignment dataset, Table S3: Anotation Dataset, Sanger sequencing output, qPCR output and report.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee on the Use of Animals (CEUA) of UFRJ (reference number 037/14). All procedures were conducted in full compliance with Federal permits issued by the Brazilian Ministry of the Environment (SISBIO 75941-4) and samples were collected following the national guidelines and provisions of IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Brazil; permanent license number 11375–1).

Data Availability Statement: The sequence generated in the present work was submitted to Genbank under the accession number PP960560.

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