Identification and Evolutionary Characterization of Papillomavirus Sequences in New World Monkeys (Genera *Sapajus* and *Alouatta*) from Argentina

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Abstract

Objective: In this study, we investigated the occurrence of papillomavirus (PV) infection in non-human primates (NHP, Platyrrhine) of northeastern Argentina by using broad-spectrum PCR primers at the L1 gene. In addition, we conducted a phylogenetic and coalescence analysis of viral sequences to explore their evolutionary history and evaluate the co-speciation hypothesis in the context of primate evolution.

Methods: We obtained samples of 57 individuals from wild and captive populations of *Alouatta caraya*, *Sapajus nigritus* and *Sapajus cay*. We assessed PV infection by PCR amplification with the CUT primer system and sequencing of 337 bp (112 amino acids) of the L1 protein. The viral sequences were analyzed by phylogenetic and Bayesian coalescence methods to estimate the age of the most common recent ancestor (tMCRA) with BEAST, v1.4.8 software. We evaluated viral/host tree congruence with TreeMap v3.0.

Results: We identified two novel putative PV sequences of the genus Gamma- PV in *Sapajus* sp and *Alouatta caraya* (SPV1 and AcPV1, respectively). The tMRCA of SPV1 was estimated at 11,941,682 years before present (ybp) and that of AcPV1 at 46,638,071 ybp, both predating the coalescence times of their hosts: 6.4 million years (MYA) and 6.8 MYA, respectively. Based on the comparison of primate and viral phylogenies, we could not reject the null hypothesis that the PV tree is no more congruent with the host tree than a random tree would be ($P>0.05$). Thus, a model of virus-host coevolution was rejected.

Conclusion: This study presents the first report of PV infection in Platyrrhine species from Argentina, expands the range of described hosts for these viruses, and proposes new scenarios for their origin and dispersal.

Introduction

Papillomaviruses (PVs) are a highly diverse group of viruses (*Papillomaviridae* family) that were initially described in mammals, but have since been found in birds, turtles, snakes and fishes, and probably infect all vertebrates [1–3]. PVs have a circular double-stranded DNA genome with a size close to 8 kb, and their taxonomy is based on the DNA nucleotide identity of the L1 gene and complete genome sequencing [4–5]. Thus far, a total of 429 viral types have been identified. They include 211 types identified from animals (86 species) and 218 exclusively from humans (HPV), which are the most intensively studied hosts [2, 4, 6]. Because there is a larger body of research focused on the study of PVs of clinical significance for humans (grouped on the *Alpha*, *Beta*, *Gamma*, *Mu*, and *Nu*-PVs genera), the described host range is not probably a true reflection of the biology of these viruses [7–9].

PVs have evolved in close relationship with their hosts, leading to the hypothesis of co-speciation [10–12]. Yet, multiple incongruences between their evolutionary history and those of their hosts have previously been demonstrated [13–16], suggesting that a strict host-pathogen coevolution has not

shaped the PV phylogeny [17–19]. For this reason, additional evolutionary forces such as cross-species infection, recombination, and gene duplication are likely to have influenced PV evolution [17–23].

PVs infect animals in an anatomically site-specific fashion (epithelia and mucosa), with genital infections being associated with cervical dysplasia and carcinomas in humans, macaques, and baboons [1, 24, 25, 26]. Moreover, at least 30 PVs types/putative types have been detected in the genital mucosa of non-human primate (NHP) species, such as *Macaca fascicularis* (MfPV1-11 and MmPV2-7) *Macaca mulatta* (MmPV1-7), *Macaca fuscata* (MfuPV1-2); *Pan paniscus* (PpPV1), *Pan troglodytes* (PtPV1), *Colobus guereza* (CgPV1-2) and *Papio hamadryas anubis* (PhPV1) [2, 6, 8, 26, 27, 28, 29, 30, 31]. However, information about PV infection in neotropical NHP species is still scarce, with only four PVs identified in *Saimiri sciureus* (SscPV1-3), and *Alouatta guariba* (AgPV1) [30, 32, 33] and two putative types in *Ateles geoffroyi* (isolate SMAA1) and *Callicebus cupreus* (PV006) [7, 34].

In Argentina, there are five species of NHPs. These include *Alouatta caraya* (black and gold howler monkeys), *Alouatta guariba clamitans* (brown howler monkeys), *Aotus azarae* (owl monkeys), *Sapajus nigritus* (black capuchins), and *Sapajus cay* (Brown-capped capuchins) [35, 36]. They inhabit the remaining forest in Argentina, and their populations are facing severe threats due to habitat alteration for agriculture (soy and rice crops, deforestation, etc.), and illegal hunting [36]. Moreover, the expansion of human populations and human activities within primate habitats has resulted in a high potential for pathogen exchange [37].

This research investigated the occurrence of PV infection in the New World monkey species *Alouatta caraya*, *Sapajus nigritus*, and *Sapajus cay* from northeastern Argentina. We conducted a phylogenetic and coalescence analysis of viral and host sequences in order to describe their evolutionary history and evaluate the co-speciation hypothesis in the context of primate evolution. The results of this study expand our knowledge about viral infections in those species, and contribute to the understanding of PV evolution in primate species more broadly.

Material And Methods

Biological samples and bioethics

For this study, we collected biological samples (feces, genital and oral swabs) from 57 NHP individuals (19 from captive and 38 from wild populations) from Argentina. These included the following species: *Alouatta caraya* (Family *Atelidae*), *Sapajus nigritus*, *S. cay* and unidentified monkeys from the genus *Sapajus* (Family *Cebidae*). The latter belong to a captive population living in the Ecological Park El Puma in Misiones Province (see 1.2.2. Sampling sites and methods).

This study complied with the Code of Best Practices for Field Primatology (International Primatological Society, 2014) [38]. It was conducted with the approval of authorities from Corrientes and Chaco, the Administración de Parques Nacionales in Argentina (Number: NEA350), and the Ministerio de Ecología y Recursos Renovables de la Provincia de Misiones (Number: DISP 121 EXP 9910-00076/12). The animal

capture and identification techniques were designed to be less invasive to preserve the welfare of the animals and relieve potential stress.

Sampling sites and methods

Wild populations

Sample sites were located at the Biological Station of Corrientes, Argentine Museum of Natural Sciences (EBCo-MACN) and State Park San Cayetano Corrientes [27º30' S, 58º41' W]; Paraje Santa Rita, Chaco [26º 01’ 32.46” S, 59º 58’ 33.49” W] and the Iguazú National Park, Misiones [25°40'S, 54°30'W].

Fifteen fecal samples were collected at the Iguazú National Park at Misiones during 2012-13. The park is part of the Upper Paraná Atlantic Forest in the southwestern edge of the South American Atlantic Forest complex. Researchers were trained for a period of three months at the site in order to be able to individually recognize every member of the troop by their physical features (facial color pattern, body size, and shape of tufts). The sampling method consisted in following *S. nigritus* troops and waiting until an identified monkey defecated. Immediately after defecation, approximately 5 g of feces per individual were taken from the forest floor and then transferred into a sterile 50-ml polypropylene tube. Samples were kept cool (2 – 8°C) until reaching the lab and conserved at -20°C until DNA extraction.

A total of 31 sample swabs from genital (n=23) and oral (n=8) cavity of *A. caraya* were obtained through capture and anesthesia in EBCo-MACN at the Corrientes and State Park San Cayetano from Corrientes and Paraje Santa Rita, Chaco. The research group has conducted long term studies with these troops over the past 15 years [36, 39, 40, 41]. Individuals were immobilized with methomidine hydrochloride combined with ketamine hydrochloride, administered via a dart driven by compressed air. A trained veterinarian collected the samples by swabbing the oral or genital area and then transferring them into a 15 ml sterile tube with Buffer Phosphate Saline (PBS 1X). During this procedure, the body temperature of the NHPs was maintained by covering the animals with blankets and placing warm water bottles next to their bodies. After sampling, each animal was transferred to the exact site of capture and observed until it fully recovered. The samples were maintained at a cold temperature until they were taken to the lab, whereupon they were frozen at -20ºC until subjected to DNA extraction.

Captive populations

Eighteen archival samples of DNA extracted from oral and genital samples were available at the Laboratory (LaBiMAp-FCEQyN-UNaM) as a part of previously unpublished studies from our group conducted at the Ecological Reserve “El Puma” (Candelaria, Misiones) [27°27’36.5” S, 55°48’00.5” W]. This site houses rescued animals kept as pets and receives confiscated animals from illegal trafficking in Misiones Province and nearby areas. The Institution is open to the public and agrees with local regulations. Briefly, animals are in outdoor cages of wire mesh with soil floor and roof. These cages have crossbars, ropes, platforms, dry tree trunks, and some refuge to provide environmental and behavioural

enrichment to the animals. Different species occupy different cages. The primates are grouped according to their physical characteristics (see below), age, size of the resident troop (approximately 15 individuals per cage). They are feed with a balanced and varied diet containing seasonal fruits, vegetables and seeds, and red and white meats. Water is freely available. The well being of the animals is monitoring daily by the trained personal from the park.

The sampling was conducted as follows: the animals were captured with a net and received an injection with anaesthesia within it (0,1 - 0,3 ml de Zelazol o Ketamine, 25 mg/kg). Once asleep, a trained veterinarian collected the samples by swabbing the oral or genital area and then transferring them into a 15 ml sterile tube with Buffer Phosphate Saline (PBS 1X). These samples were kept cold on an ice bucket and later transferred to the Lab for DNA extraction. After sampling, each animal received a vitamin complement and was observed until it fully recovered and returned to the home colony.

At the time of sampling, the species assignment was based on the phenotypic diagnosis of the individual as follows: Sapajus nigritus (black capuchin): very dark brown or grey, even blackish, pelage; with no (or very vague) dorsal stripe, the face is white and contrasts with the colour of the body. Sapajus cay (yellow-bearded capuchin): yellow to white head with black crown and sideburns, which contrast with the light-coloured body; the presence of a prominent dark dorsal stripe; limbs mainly dark to blackish; upper arms not lighter than the body; the underside is yellowish or reddish, often overlaid with black, as described in [41].

Following these criteria, most samples at this site were recorded as belonging to S. nigritus and S. cay. However, not all species were unambiguously identified by the zookeepers and/or veterinarians and, therefore, were denoted as Sapajus sp. Details about the species and study sites are shown in Fig. 1.

Sample processing and PV identification

DNA from feces was extracted using the PowerFecal® DNA Isolation Kit (12830-50 MO BIO Laboratories, Inc., Carlsbad, United States) according to the manufacturer's instructions. DNA from genital and oral swabs was extracted using ADN PuriPrep-S kit (K1205-250, Inbio HighWay®, Buenos Aires, Argentina). We quantified DNA concentration with a Qubit™ fluorometer (Thermo Fisher Scientific; Waltham, United States).

The general strategy for PV DNA identification involved the use of an improved version of the original CUT primer system with a "hanging droplet PCR" amplification strategy, as previously described [42]. Briefly, the primary 20 μl-reaction mixture was placed in a 0.2 ml tube and covered with one-drop of mineral oil. After the addition of a 5 μl sample, the mixture had a final volume of 25 μl containing 0.4 μM of CUT1Fw, 0.4 μM of CUT1EFw (5-$TR\mathbb{C}iGAY\mathbb{C}i\forall TAGA\ \top\ TG$ - 3), 0.266 μM of CUT1BRv, 0.266 μM of CUT1CRv (5-$TCiCACATRTCi\mathbb{C}RTCYTG$ - 3) and 0.266 μM of CUT1DRv (5 -$TCiS\mathbb{C}ATRTCi\mathbb{C}RTCYTG$ - 3), 200 μM of each dNTP, 3.5 mM $MgCl_2$, 1.5x PCR buffer with $(NH_4)_2SO_4$ and 1 U Taq DNA polymerase (Thermo Fisher Scientific). Before closing the tube, a 25-μl droplet

containing the same reaction mixture (but with 400 μM of each dNTP and 2.5 U Taq DNA polymerase) was placed in the center of the inside of the reaction tube cap.

Using a thermocycler programmed for block temperature without heated lid, the mixture was heated for 2 min at 94°C, followed by 20 cycles of a step down protocol (4 cycles: 30 s at 94°C, 30 s at 52°C and 40 s at 72°C; 4 cycles: 30 s at 94°C, 30 s at 51°C and 40 s at 72°C; 4 cycles: 30 s at 94°C, 30 s at 50°C and 40 s at 72°C; 4 cycles: 30 s at 94°C, 30 s at 49°C and 40 s at 72°C; and 4 cycles: 30 s at 94°C, 30 s at 48°C and 40 s at 72°C). After the first round of amplification, we incorporated the "hanging droplet" into the reaction mixture (final volume 50 μl) by centrifugation 1 min (11,000 × g) and a second round of 40 cycles (30 s at 94°C, 2 s at 60°C followed by a ramp of 0.2°C/s to 50°C, 50°C for 10 s and 40 s at 72°C) was performed.

Amplicons derived from the modified CUT primer systems (~370 bp) were purified with a spin column (Nucleospin II, Thermo Fisher Scientific) and eluted in 30 μl of elution buffer. All purified amplicons were cloned using a pGEM®-T Easy Cloning kit (Promega). Clones containing target inserts were identified by PCR amplification using M13 Fw/Rv primers. We sequenced at least three recombinant clones from each sample. All samples were subjected to Sanger sequencing using commercial facilities (Macrogen, Inc., Seoul, South Korea). The resulting sequences were compared to available PV-sequences in the GenBank database by using the Blast algorithm "Somewhat similar sequences (blastn)." A new putative PV type was defined when the L1 fragment sequence showed less than 90% sequence identity to any of the previously known PV types [4]. The resulting sequences have been deposited in GenBank [43] under accession numbers: MT450752 (AcPV1) and MT450753 (SPV1).

Cytochrome B amplification of non-human primate DNA

In order to confirm the NHP origin of the PV-positive samples, we characterized the mitochondrial DNA (mtDNA) cytochrome b (CYTB) sequence in each sample. Briefly, we conducted PCR amplifications using the following conditions: 75 μl of solution containing 10-50 ng of template DNA, 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer (FW: 5'-CCATCCAACATCTCAGCATGATGAAA-3'; RV: 5'-CCCCTCAGAATGATATTTGTCCTCA-3') and 2 U of Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific). We included negative (no template) controls in all amplifications. Ten μl of PCR products were run in 2% agarose gels and visualized through SYBR™ Safe DNA Gel Stain (S33102, Invitrogen™, Thermo Fisher Scientific). A single band of 359 bp was indicative of a positive amplification of L1 gene. Positive amplicons were purified with the ADN PuriPrep-GP kit (K1206-100 Inbio HighWay) and then sequenced using the original primers through sequencing services by Sanger methodology (Macrogen, Inc). The two sequences belonging to the positive PV samples were deposited in Genbank [43] under accession numbers: MT451931 (*Alouatta caraya*) and MT451932 (*Sapajus sp*).

Phylogenetic Analysis

A PV phylogeny was constructed using published reference sequences of primate PVs for the genera *Alpha*, *Beta*, and *Gamma* (n=266) available at Papillomavirus Episteme [6] and GenBank [43] and those obtained in this study (n=2). Details of the dataset are provided in Supplementary Table 1. The dataset was aligned using MUSCLE v3.8.31 [44], and the best-fit model of nucleotide substitution was selected with the Bayesian Information Criterion using the FindModel procedure [45]. A phylogenetic tree was constructed with the Maximum Likelihood methodology using IQTree 1.6.8 for Linux [46], which automatically sets the substitution model according to the results of ModelFinder (in this case GTR + Γ + I). The branch support was evaluated by ultrafast bootstrapping with 1,000 pseudo-replicates [47]. The tree was visualized and prepared for publication using FigTree V1.4.4 [48].

Molecular dating of Gamma-PVs

To estimate the t_{MRCA} of novel isolates within the *Gamma*-PV genus, we used a dataset of 126 sequences that were 337 nucleotides in length (the L1 CUT PCR fragment). Details about accession numbers, PV types, and host species are provided in S1 Table 1. An initial phylogenetic reconstruction was carried out with Maximum Likelihood Methodology and ultrafast bootstrapping as branch support, both implemented in IQTree v 1.6.8 [46]. This tool allows the construction of a phylogenetic tree and the selection of the best-fit nucleotide substitution model [45]. The molecular dating was then obtained by using the Bayesian Markov Chain Monte Carlo (MCMC) method in BEAST v1.10.4 program [49]. The priors were as follows: General Time Reversible substitution model [50] with gamma-distribution and invariant sites (GTR+G+I); a relaxed (uncorrelated lognormal) molecular clock; a Bayesian skyline plot (BSP) demographic growth [49]; and a substitution rate of 1.84×10^{-8} s/s/y (substitutions per site per year) [12].

The MCMC was run for 5×10^{6} generations, sampling every 5,000th generation in order to achieve an Effective Sample Size (ESS) > 200. We analyzed all BEAST run logs with the TRACER program version 1.7 [51] after discarding 2% of the run-length as burn-in. We constructed a maximum clade credibility tree (MCCT) with the TreeAnnotator tool after discarding 2% of the sampling [49]. We further visualized the MCCT summarizing the posterior information of topologies and the median branch lengths from the trees sampled with FigTree V1.4.4 [48].

Cophylogeny Analysis

To evaluate the hypothesis that *Gamma*-PV genus evolved in association with their host, we used TreeMap v3 [52]. This program evaluates the significance of any congruence between viral-host trees through randomization, using a Markov model to reconstruct random associate trees. The null hypothesis was that the parasite tree is no more congruent with the host tree than a random tree would be [53]. A practical limit to the size of tanglegrams that are manageable by older versions of TreeMap is less than 50 samples, as large numbers may have unreasonable calculation times or memory requirements [53]. Hence, we reduced our data set to a single representative PV sequence per monophyletic lineage occurring in a single host species (n=30). A mirror primate host phylogeny was constructed by using

complete mitochondrial genomes of *Homo*, *Pan*, *Gorilla*, *Macaca*, *Alouatta*, *Ateles*, and *Cebus* using sequences available in GenBank [43]. Details about the accession numbers and host species are shown in S2 Table 2.

A phylogenetic tree was constructed using the Maximum Likelihood method in the PhyML platform [54]. Both phylogenies were visualized as a tanglegram and subjected to reconciliation analysis through cophylogeny mapping (25 random maps and 25 generations) [55]. The maximum number of codivergence events (CEs=28) was later used to run the statistical test with 50 randomized phylogenies. The latter gave us a p-value and 95% confidence interval, with the null hypothesis being rejected if $p<0.05$.

Results

NHP population characteristics

We analyzed 64 samples from 57 individuals from the species *S. nigritus*, *S. cay*, and *A. caraya*. Of them, 47.7% were female and 52.3% were male. The characteristics of the NHP study sample are shown in Table 1.

Table 1
Characteristics of the Study Samples

		A. caraya *n=24*	*S. nigitus* *n=16*	*S. cay* *n=10*	*Sapajus sp.* n= 7	Total n=57
Sex (*)	Female	6 (54.5)	7 (43.8)	6 (60.0)	2 (28.6)	21 (47.7)
	Male	5 (45.5)	9 (56.2)	4 (40.0)	5 (71.4)	23 (52.3)
Status	Wild	23 (95.8)	15 (93.7)	0 (0.0)	0 (0.0)	38 (66.7)
	Captive	1 (4.2)	1 (6.3)	10 (100.0)	7 (100.0)	19 (33.3)
Sample type (**)	Oral	8 (25.8)	1 (6.3)	3 (30.0)	5 (71.4)	17 (26.6)
	Genital	23 (74.2)	0 (0.0)	7 (70.0)	2 (28.6)	32 (50.0)
	Fecal	0 (0.0)	15 (93.7)	0 (0.0)	0 (0.0)	15 (23.4)
Location (***)		[1] [2] [4]	[3] [4]	[3]	[3]	

(*) The sum of samples exceeds the number of individuals in some columns because more than one sample per individual was analyzed (oral, genital or feces).

(**) Some columns do not add to total because some information was not available.

(***) Location points, as described in Fig. 1.

PV detection typing and phylogenetic analysis

Of the 64 samples analyzed, 62 were negative and two were positive for PV. One of the positive samples was detected in an oral swab sample of a wild female *A. caraya* from Corrientes, while the other was found in an oral swab of a captive female *Sapajus sp.* from Misiones. We provisionally named these sequences AcPV1 (isolate CUT-Pr145) and SPV1 (isolate CUT-Pr035), respectively. The translation to protein confirmed the amplification of L1 gene for both sequences. Pairwise comparison by nucleotide Blast algorithm indicated that AcPV1 shared 71.9% nucleotide identity with HPV126 (species Gamma-11) and 71.6% nucleotide identity with non-human putative PV type MfAA11 (Gamma-4) identified from *Macaca fascicularis*. By contrast, putative lineage SPV1 shared 98.1% nucleotide identity with HPV-msk022 (Gamma-unclassified) and 70.2% nucleotide identity with putative non-human PV type MfAA13 (Gamma– 4) identified from *Macaca fascicularis*. Thus, Blast analysis indicated that these new sequences met the criteria for defining novel types and variants within the genus *Gamapapillomavirus*, respectively. No sequences from non-primate hosts were retrieved in the Blast search.

Phylogenetic Analysis

In Fig. 2, we show the phylogenetic relationships between the new putative PV sequences AcPV1 and SPV1 and other known primate PV types (*Alpha*, *Beta*-, and *Gamma*-PV). The tree topology was consistent in revealing three supported monophyletic clusters for genera Alpha- Omicron-, Beta-, and Gamma-PV. Phylogenetic analysis confirmed that both PV sequences were members of the *Gamma*-PV genus.

In Fig. 3, we show the phylogenetic relationships and molecular dating of the novel putative PV types AcPV1 and SPV1 in relation to other known *Gamma*-PV types. In our dataset, all *Gamma*-PVs coalesced to a tMCRA of 51,367,435 years (HPD 95% = 33,282,867 - 72,480,552 years), with the emergence of SPV1 during the last 11,941,682 years (HPD 95% = 6,751,416 – 18,042,367 years) and AcPV during the last 46,638,071 years (HPD 95% = 26,810,762 – 61,367,537 years). However, since a number of clusters were not strongly supported across the tree, these dates were viewed with caution.

Cophylogeny analysis

The obtained tanglegram is shown in S3 Fig. 1. The reconciliation analysis through cophylogeny mapping indicated that we could not reject the null hypothesis that the PV tree was no more congruent with the host tree than a random tree would be, with a P value of 0.340 (0.212, 0.484).

Discussion

This study explored the occurrence of PV infections in NHP in northern Argentina and the possible scenarios for the evolution and dispersal of these viruses in the primate lineage. We identified two putative PV types. One was found in the oral mucosa of a wild *Alouatta caraya* female from Corrientes Province (called AcPV1), and the other was identified in a captive *Sapajus sp.* female (SPV1) from Misiones Province. The genetic and phylogenetic characterization of these PVs sequences allowed us to

assign them to the *Gamma*-PV genus. Before this study, PV infections were reported for a number of other Platyrrhine species, including *Saimiri sciureus*, *Alouatta guariba*, *Ateles geoffroyi Callicebus cupreus* and *Callithrix penicillata* [7, 30, 32, 33, 34, 56]. Thus, our findings expand the range of described hosts for these viruses.

The diversity of cutaneous *Gamma*-PVs is known to be high. Several hundred partial PCR sequences and more than 100 complete reference genomes have been described in the last 10 years (6, 22, 42, 57, 58]. The evolutionary basis for this genetic diversity is unknown. However, it has been suggested that UV light-induced damage may contribute to a higher mutation rate of the sun-exposed PV in the skin of the *Beta* and *Gamma* genera [57].

Interestingly, the *Gamma*-PV genus was initially reported as belonging to the group of cutaneous PV because of being predominantly isolated in the cutaneous epithelium of the human skin [22, 42, 57, 59]. However, certain studies have shown that the oral cavity contains a broad spectrum of *Gamma*-PVs, which enlarges their proposed tropism [9, 58, 60]. In a recent publication, Chen et al. (2019) studied PV infection in different body parts of macaques, and found frequencies of 55.6% in genital swabs, 35.9% in oral swabs, and 29.9% in perianal swabs. Moreover, they found a significant difference in the distribution of PV genera at different body sites, with *Alpha*-PV infections being more frequent in genital samples (86.2%) and *Gamma*-PV infections more frequent in the oral cavity (90.3%) [9]. Therefore, our identification of AcPV1 and SPV1 in samples of desquamated cells of the oral mucosa is consistent with these recent discoveries.

On the other hand, cross body site infection was also reported as being common among macaques [9]. Thus, an alternative explanation for our finding is that primate skin-to-mouth contact could be responsible for the transmission of skin PV types to the oral cavity. In the case of NHP, grooming is a widespread activity that involves looking for parasites in the fur of peers and eating them [61, 62]. In this context, oral mucosa could represent a satellite niche produced by grooming. This is not a minor issue since tissue tropism has been indicated as one of the main determinants for the evolution of PV genera [18, 20, 30]. Therefore, future studies addressing the tropism of *Gamma*-PVs will be crucial for understanding virus niche adaptation.

Moreover, the *Gamma*-PV tree topology indicates the absence of a monophyletic pattern for viruses that infect the same species, with humans being the most striking example. Consequently, our statistical test rejected the host/hosted coevolution model. This finding is consistent with previous reports for *Gamma*-PV [34] and other PV-genera such as *Alpha.* In the later viruses from *Papions* (PCPV), *Rhesus* (RhPV-1) and *Colobus* (CCPV) are closely related to the human oncogenic types of the species *Alpha*-9, hence, do not possess a basal position in the phylogeny [13–15]. Increasing evidence supports the view that the first step in PV evolution was niche adaptation to tissue tropism. The explanation may explain the conflicts between the pathogen and host phylogenies [15, 17, 30].

Regarding the evolutionary history of PVs, the molecular dating of the *Gamma*-PV genus was estimated at 51 million years ago (MYA). Previous reports based on complete PV genomes generated dates ranging

from 33 MYA to 45 MYA [9, 30, 56] and 50 to 60 MYA [21, 63]. This extent of variation can be attributed to the diversity (the number of species) and length (base pairs) of sequences involved in these different datasets. Nevertheless, all studies agree that the ancestral virus dates back to the Eocene (56 - 34 MYA). Current views on primate taxonomy agree that extant primate genera originated from a common ancestor during the Cretaceous/Paleocene boundary roughly 80 – 90 MYA, with the expansion of the major extant lineages Strepsirrhini, Tarsiiformes, and Simiiformes occurring during the Eocene [64]. The importance of these data for our study concerns the Simiiformes Group, which comprises Platyrrhini (New World monkeys) and Catarrhini (Old World monkeys and humans) and has an estimated tMRCA of 43 (36 – 50) MYA [64]. Therefore, molecular dating of the *Gamma*-PV types produces estimates that fall within the time frame of the evolution of the Primate Order.

Yet, the emergence of SPV1 during the last 11.9 MYA and of AcaPV at 46.6 MYA is not consistent with the evolutionary history of these primate *host species*. The biogeographic history of capuchins suggests a late Miocene geographic isolation of the gracile (*Cebus*) and robust (*Sapajus*) forms at 6.7 MYA [65]. The divergence time between *Alouatta* species has also been estimated at 6.6 – 6.8 MYA [66]. Therefore, it is clear that the evolution of the PVs predates the speciation of their respective hosts.

Finally, it is important to mention the potential role of cross-species transmission in our findings. For example, it is known that bovine *Delta*-PV infection causes tumors in horses, cape mountain zebras, giraffes, sable antelopes and buffaloes [67–70]. Unfortunately, there is relatively little information about humans as sources of PVs. Recent studies have revealed that a zookeeper temporarily tested positive for a chimpanzee PV [7], while a cat was infected with human HPV9 (possible by a cat owner, who was not tested) [71]. These examples raise the possibility of viral transfer between human and non-human species. In our study, the *Sapajus* sp. sample came from a captive animal, although we were unable to include the zookeepers' samples in this study.

The discovery of novel PVs, mainly in hosts in whom PV infection had not been previously reported, is significant, as it increases our knowledge about PV evolution and diversification. However, one of the study limitations is the use of a small DNA fragment for taxonomic, phylogenetic and molecular dating inferences. Unfortunately, we were unsuccessful in retrieving larger genes from our samples to expand this analysis. For this reason, other approaches such as enrichment of circular DNA by rolling circle amplification, and/or next generation sequencing may be needed for the characterization of these novel viruses in the future [72].

Conclusions

This study presents the first report of PV infection of Platyrrhine species from Argentina, expands the range of described hosts for these viruses and proposes new scenarios for their origin and emergence. Due to the importance of the description of new animal PV types, we believe that additional experimental analyses should be performed to characterize the putatively new PVs herein described.

Declarations

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Author contributions

Sanchez-Fernandez, C: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition. Bolatti EM: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Review & Editing. Culasso A.C.A.: Methodology, Formal analysis, Visualization, Writing - Review & Editing. Chouhy D: Methodology, Formal analysis, Writing - Review & Editing. Kowalewski MM: Conceptualization, Resources, Supervision, Project administration, Writing - Review & Editing. Stella EJ: Investigation, Writing - Review & Editing. Schurr TG: Formal analysis, Resources, Writing - Review & Editing. Rinas MA; Liotta DJ and Campos RH: Resources; Writing - Review & Editing. Giri AA.: Resources; Project administration; Funding acquisition; Writing - Review & Editing. Badano Ines: Conceptualization, Methodology, Formal analysis, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declarations of interest

The authors declare no conflicts of interest.

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Figures

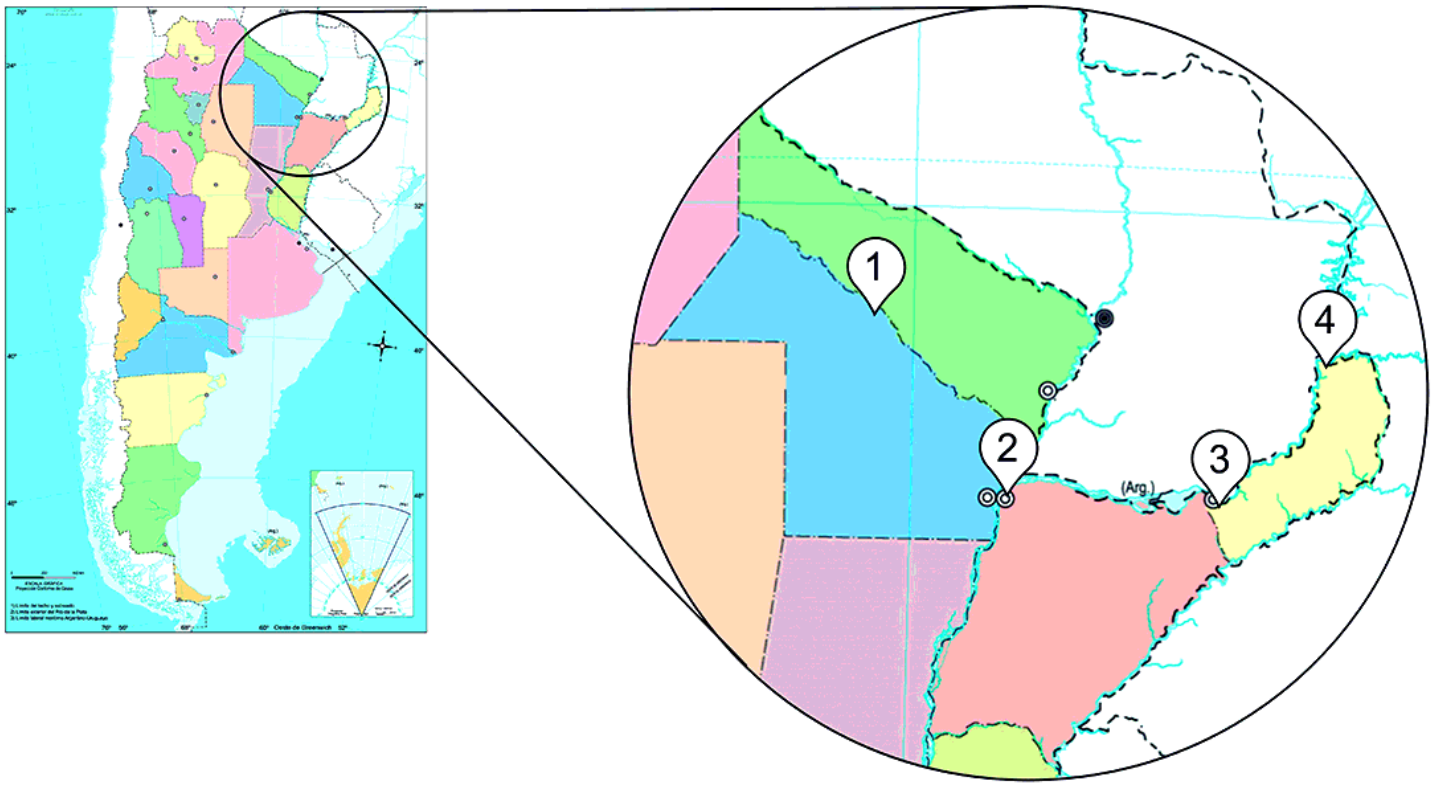

Figure 1

Study sites in Misiones, Corrientes and Chaco provinces, Northeastern Argentina. Legend: Locations: 1. Paraje Santa Rita, Chaco [26º 01’ 32.46” S, 59º 58’ 33.49” W]: A. caraya; 2. Biological Station of Corrientes, Argentine Museum of Natural Sciences and State Park San Cayetano, Corrientes [27º30' S, 58º41' W]: A. caraya; 3. Ecological Reserve “El Puma”, Misiones [27°27'S, 55°48'W]: A. caraya, Sapajus sp., S. nigritus and S. caraya; 4. Iguazú National Park, Misiones [25°40'S, 54°30'W]: S. nigritus.

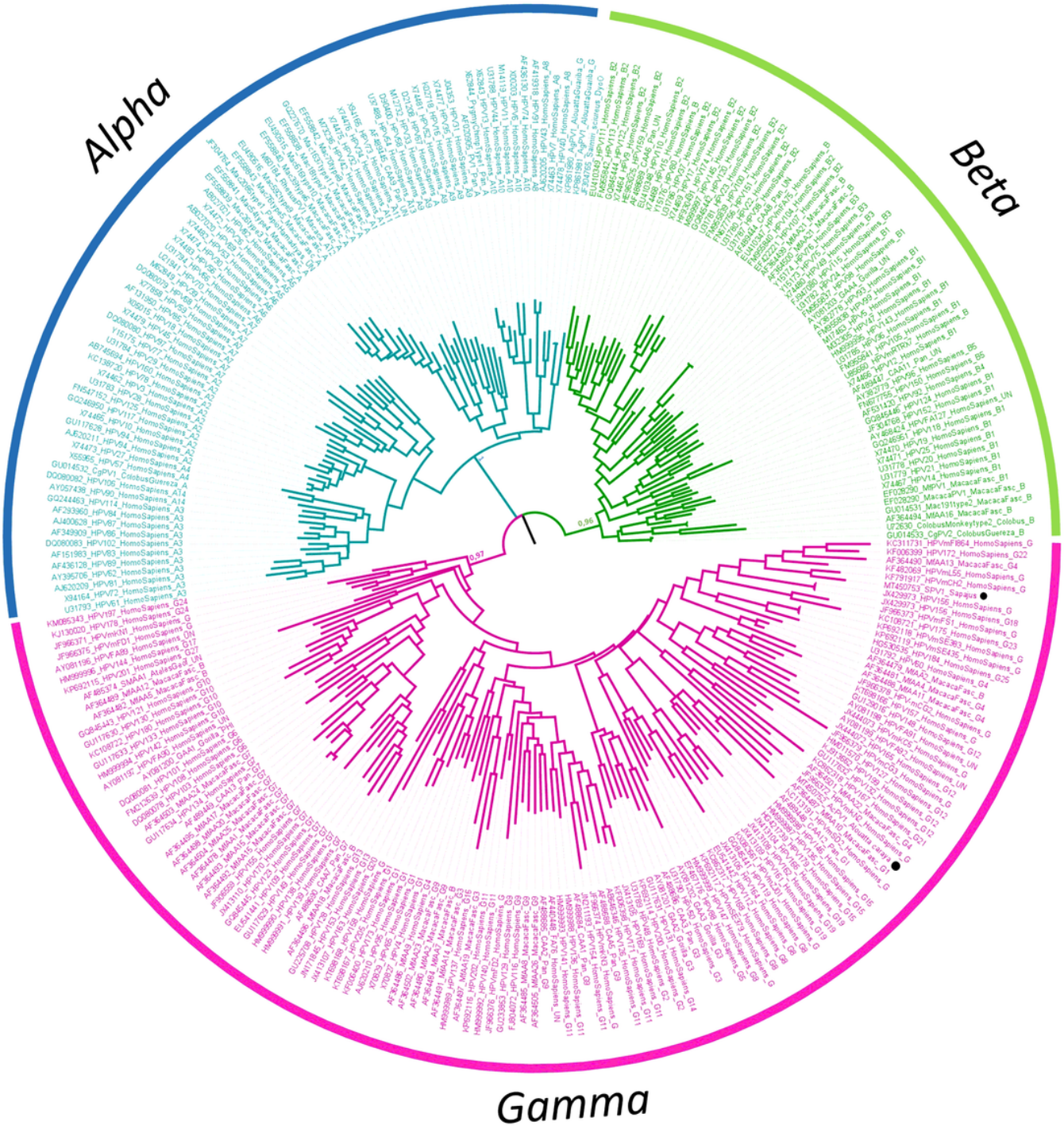

Figure 2

Phylogenetic classification of novel putative PV types AcPV1 and SPV1 within Papillomaviridae Family. Legend: The evolutionary history of Papillomaviridae Family (genera: Alpha-, Beta-, and Gamma-PV) was inferred using Maximum Likelihood method. The analysis involved 266 nucleotide sequences and 337 bp. Samples from this study belong to Gamma-PV genera, and are marked with black dots.

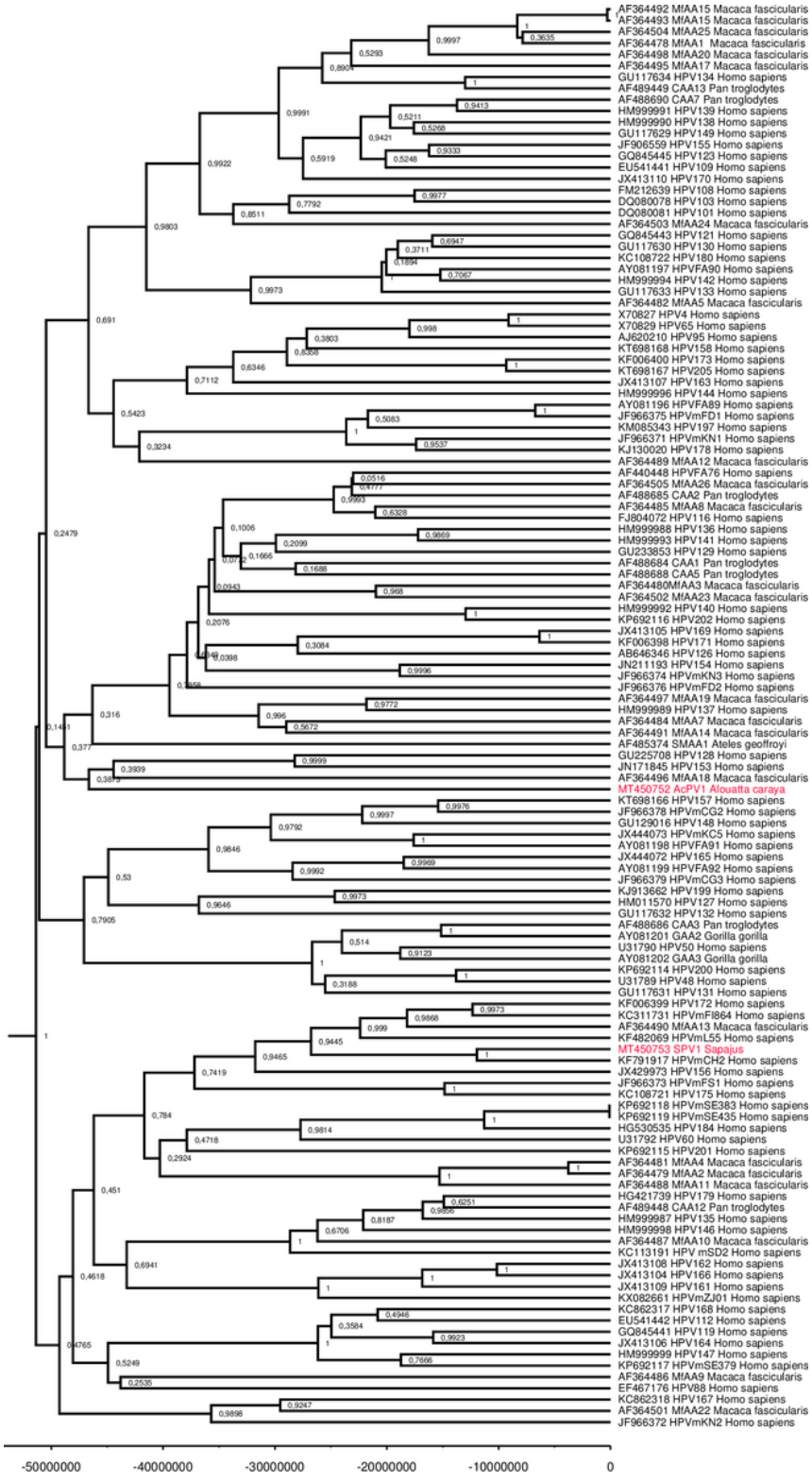

Figure 3

Phylogenetic analysis and molecular dating of primate Gamma-PVs. Legend: The evolutionary history was inferred using the Bayesian method. The maximum clade credibility tree is shown. The analysis involved 126 nucleotide sequences. The final dataset included a total of 337 positions. Timeline: the X axis indicates years ago. The posterior probability values are shown at the nodes of the tree. Novel putative PV types identified in this study are depicted in red.

Supplementary Files

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