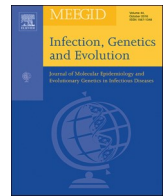




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Research Paper

Virome of crab-eating (*Cerdocyon thous*) and pampas foxes (*Lycalopex gymnocercus*) from southern Brazil and Uruguay

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ABSTRACT

Crab-eating (*Cerdocyon thous*) and Pampas foxes (*Lycalopex gymnocercus*) are wild canids distributed in South America. Domestic dogs (*Canis lupus familiaris*) and wild canids may share viral pathogens, including rabies virus (RABV), canine distemper virus (CDV), and canine parvovirus 2 (CPV-2). To characterize the virome of these wild canid species, the present work evaluated the spleen and mesenteric lymph node virome of 17 crab-eating and five Pampas foxes using high-throughput sequencing (HTS). Organ samples were pooled and sequenced using an Illumina MiSeq platform. Additional PCR analyses were performed to identify the frequencies and host origin for each virus detected by HTS. Sequences more closely related to the *Paramyxoviridae*, *Parvoviridae* and *Anelloviridae* families were detected, as well as circular Rep-encoding single-stranded (CRESS) DNA viruses. CDV was found only in crab-eating foxes, whereas CPV-2 was found in both canid species; both viruses were closely related to sequences reported in domestic dogs from southern Brazil. Moreover, the present work reported the detection of canine bocavirus (CBoV) strains that were genetically divergent from CBoV-1 and 2 lineages. Finally, we also characterized CRESS DNA viruses and anelloviruses with marked diversity. The results of this study contribute to the body of knowledge regarding wild canid viruses that can potentially be shared with domestic canids or other species.

1. Introduction

Crab-eating (*Cerdocyon thous*) and Pampas foxes (*Lycalopex gymnocercus*) are *Canidae* members with distribution ranges that overlap extensively in South America. These wild canids are widely found in the farms of southern Brazil, on the border regions of Uruguay and Argentina. The vulnerability of these animals is in part due to the destruction of their natural environment, either by deforestation for extending the agricultural borders, or spreading of urban communities on the natural environment, as well as the habitat fragmentation caused by the roads. These foxes seem to be tolerant to human disturbance and are frequently seen in rural areas and close to urban regions. These wild

canids have nocturnal scavenger habits and live in close proximity with domestic animals, which may be notable, as domestic host species can play a role in the transmission of infectious agents to wild animals (Alves et al., 2018a; Antunes et al., 2018; Ferreyra et al., 2009; Hübner et al., 2010).

It is known that domestic dogs (*Canis lupus familiaris*) and wild canids may share viral pathogens, including the rabies virus (RABV) (Antunes et al., 2018; Rocha et al., 2017), canine distemper virus (CDV) (Conceição-Neto et al., 2017; Ferreyra et al., 2009; Hübner et al., 2010; Megid et al., 2009), canine parvovirus 2 (CPV-2) (de Almeida Curi et al., 2010; Hübner et al., 2010), canine coronavirus (Alfano et al., 2019), canine adenoviruses 1 and 2 (Dowgier et al., 2018), and canine

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astrovirus (Alves et al., 2018a). However, the knowledge about sanitary conditions of these animals are still scarce.

The enhanced availability and application of high-throughput sequencing (HTS) technologies has facilitated the detection of known and unknown viruses (Goodwin et al., 2016; Paim et al., 2019). Thus, the knowledge of the virus genetic diversity present in different host species can be improved. HTS sequencing has been applied in the knowledge of the virome of domestic dogs (Moreno et al., 2017; Weber et al., 2018a, 2018b), but reports of its application in wild canids remain scarce. Therefore, the present study aimed to evaluate and characterize the spleen and mesenteric lymph node virome of crab-eating and Pampas foxes from southern Brazil and Uruguay using HTS. The results outline the viral agents that compound the microbiota of these wild dogs, helping to elucidate the viral population present in these wild canid species.

2. Materials and methods

2.1. Study design and sample sources

Five Pampas foxes (*Lycalopex gymnocercus*) and seventeen crab-eating foxes (*Cerdocyon thous*) run over by cars were collected by the Veterinary Pathology sector of Universidade Federal do Rio Grande do Sul and Plataforma de Salud Animal of Instituto Nacional de Investigación Agropecuaria Tacuarembó using an active search on highways between November 2017 and September 2019. Fig. 1 shows the location on the foxes sampled in the present study. The mesenteric lymph nodes and spleen of the 22 animals were collected, macerated and diluted to 20% (w/v) in phosphate-buffered saline (PBS) (pH 7.2), centrifuged at low speed ($1800 \times g$ for 30 min), filtered through a 0.45- μm filter for removal of small debris and stored at -80°C for subsequent analysis. These organs were selected since they concentrate antigens in order to presentation for immune system, which could

increase the chance of detecting some viral agents.

The authorization for the collection of the samples from Brazil was registered in SISBio/ICMBio under number 67053. Samples obtained of dead animals do not require authorization for molecular analysis in Uruguay.

2.2. Viral metagenomics and HTS

The 22 wild canid spleens and mesenteric lymph nodes (17 crab-eating foxes and five Pampas foxes) were assembled into one pool containing 500 μL of each sample. A total of 11 mL was passed through a 0.22- μm filter and subsequently centrifuged on a 25% sucrose cushion at 150,000g for 3 h at 4°C in a Sorvall AH629 rotor. The pellet containing the viral particles was incubated for 1.5 h with DNase and RNase enzymes (Thermo Fisher Scientific, Waltham, MA, USA) (Thurber et al., 2009). Subsequently, the total RNA and DNA were isolated using TRIzol™ LS reagent (Thermo Fisher Scientific) and a standard phenol-chloroform protocol (Sambrook and Russel, 2001), respectively. The viral DNA was enriched using the Genoplex® Complete Whole Genome Amplification (WGA) kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's recommendations. Furthermore, the viral RNA was reverse-transcribed using the TransPlex® Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich) following the manufacturer's recommendations. The DNA products from these enrichment protocols were pooled in equimolar amounts and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ (Thermo Fisher Scientific), respectively. The DNA libraries were further prepared with 1 ng of purified DNA using the Nextera XT DNA sample preparation kit and sequenced using a MiSeq Reagent kit v2 300 (2×150 paired-end) at the MiSeq platform (Illumina®).



Fig. 1. Spatial distribution of the crab-eating (*Cerdocyon thous*) and Pampas foxes (*Lycalopex gymnocercus*) sampled. The quantity of each wild canid species are shown on the map.

Table 1

Summary of sequences that matched with the animal viruses present in the pooled spleen and mesenteric lymph nodes of *Cerdocyon thous* and *Lycalopex gymnocercus* sample.

Best blast hit (BLASTX, E -value $< 1 \times 10^{-3}$)	No. of hits	No. of reads ^a	Contigs lengths	Amino acid identity range ^a
Canine bocavirus	3	22	267–343	93.5–100%
Carnivore protoparvovirus 1	2	12	435–530	99.3–100%
Canine morbillivirus	3	10	109–251	99.8–100%
Anelloviridae	171	1678	886–2415	42.3–64.8%
CRESS DNA virus	7	87	327–852	34.1–55.9%

^a Analysis performed in BLASTX tool, from GenBank database.

2.3. Bioinformatic analysis

The quality of the generated sequences was evaluated using FastQC. Furthermore, the sequences with bases possessing a Phred quality score < 20 were trimmed with the aid of Geneious software (version 9.0.5). Subsequently, the paired-end sequence reads were *de novo* assembled into contigs with SPAdes Assembler version 3.11.1 (Bankevich et al., 2012). All assemblies were confirmed by mapping reads to contigs produced by the SPAdes Assembler using Geneious software. Thereafter, the assembled contigs were examined for similarities with known sequences through BLASTX software using Blast2GO (Gotz et al., 2008). Sequences with E -values $\leq 10^{-3}$ were classified as likely to have originated from eukaryotic viruses, bacteria, bacteriophages, or unknown sources, a conclusion reached based on the taxonomic origin of the sequence with the best E -value. Gene and protein comparisons were performed with BLASTN and BLASTP programs (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Sequences representative of viruses belonging to circular Rep-encoding single-stranded (CRESS) DNA viruses and the families Anelloviridae, Parvoviridae and Paramyxoviridae were obtained from GenBank and aligned with the sequences identified in the present study with MAFFT software (Katoh and Standley, 2013). Phylogenetic trees were constructed using MEGA6 (Tamura et al., 2013).

2.4. PCR and RT-PCR

The 22 animals were screened individually for CPV-2 (Buonavoglia et al., 2001), CDV (Fischer et al., 2013), canine circovirus (CaCV) (Li et al., 2013), and RABV (Soares et al., 2002). Positive samples in the applied CDV-RT-nested PCR were submitted to an additional assay to amplify a fragment of CDV-hemagglutinin (An et al., 2008; Riley and Wilkes, 2015) for genotyping.

CBoV were searched applying two independent PCR protocols in the 22 individual wild canid samples, where the first one was designed against one of the contigs obtained in the HTS in order to amplify a 154-bp fragment from the nonstructural protein of CBoV-1 (Supplementary Table). The second PCR protocol using primers CBoV-QFX1-f1 and CBoV-QFX1-r2 amplifies a 311-bp fragment of the VP2 gene of CBoV-1 (Kapoor et al., 2012).

The sequences related to anelloviruses detected through HTS higher than 400 amino acids in ORF1 were investigated individually to define the host origin (*Lycalopex gymnocercus* or *Cerdocyon thous*). The primer pairs (Supplementary Table 1) were selected using Primer3 software (Untergasser et al., 2007). The PCRs for anelloviruses were conducted using [1 ×] PCR buffer, 1 mM MgCl₂, 0.5 mM dNTP mix, 0.2 mM each specific primer pair, and 1 unit of GoTaq® DNA Polymerase (Promega, Madison, WI, USA). Reactions were performed in a Veriti 60-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 95 °C followed by 35 cycles of 45 s at 95 °C, 45 s at 50 °C and 45 s at 72 °C with a final extension at 72 °C for 7 min.

2.5. Sanger sequencing

All positive samples in PCR and RT-PCR were submitted for Sanger sequencing to confirm the specificity of the tests. The PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA). Both DNA strands were sequenced with an ABI PRISM 3100 Genetic Analyzer utilizing a BigDye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Furthermore, overlapping fragments were aligned and assembled using Geneious software.

3. Results

3.1. HTS overview

One DNA library consisting of 22 pooled wild canid spleens and mesenteric lymph nodes (17 crab-eating foxes and five Pampas foxes) was generated and sequenced using paired-end 2×150 base runs on the Illumina MiSeq platform, which generated a total of 138,850 reads. The 13,250 assembled sequence contigs produced with SPAdes Assembler version 3.11.1 (Bankevich et al., 2012) were compared with the viral reference database and the GenBank nonredundant protein database through a BLASTX search conducted with an E -value cut-off of 10^{-5} in Blast2GO (Gotz et al., 2008). The exogenous eukaryotic virus-related sequences comprised 1.38% (1809/138,850) of the reads and 1.37% (182/13,250) of the contigs.

Furthermore, eukaryotic exogenous virus-related sequences with single-stranded DNA (ssDNA) genomes belonging to two viral families (Anelloviridae and Parvoviridae) and CRESS DNA viruses were observed, as well as with single-stranded RNA (ssRNA) genomes belonging to the Paramyxoviridae family (Table 1). Moreover, the majority of the viral sequences studied shared a high degree of identity with known animal viruses (CPV-2, CDV and CBoV), while others (anellovirus-like and CRESS DNA virus) exhibited a high degree of divergence to genomes already recorded in GenBank. Information regarding the sequences obtained is described in the following sections. All 22 samples analyzed using CaCV (Li et al., 2013) and RABV-specific PCR (Soares et al., 2002) were negatives.

3.2. Canine distemper virus (CDV)

Three contigs closely related to CDV were obtained in the pooled wild canid organs sequenced by HTS (Table 1). Applying the RT-nested PCR protocol to amplify the CDV nucleocapsid (Fischer et al., 2013), 18.18% (4/22) of the samples were positive, and all the positive samples were from *Cerdocyon thous* from Brazil. The amplification products from the positive samples were Sanger sequenced and shared 99.29–100% nucleotide identity between them. In the nucleotide BLAST search, the samples detected in this study presented 99.6 to 100% identity with CDV strains detected in domestic dogs in Brazil (GenBank accession numbers MH382872 and KU725677). The partial N gene sequences obtained in the present study presented the same single nucleotide polymorphisms (SNP) that characterize South America I lineage described previously (Fischer et al., 2013). These sequences were deposited in GenBank under accession numbers MT002482-MT002485.

Positive samples were submitted to an additional PCR assay to amplify a fragment of CDV-hemagglutinin (An et al., 2008; Riley and Wilkes, 2015) followed by Sanger sequencing for genotyping (Budaszewski et al., 2014). The sequences presented 99.06 to 99.87% nucleotide identity. In the nucleotide BLAST search, the samples detected in this study presented 99.3 to 99.6% identity with CDV strains detected in domestic dogs in Brazil (GenBank accession numbers KC879049 and KC879050). The partial CDV-hemagglutinin phylogenetic reconstruction (Fig. 2) presented 14 well-supported branches corresponding to the 14 genotypes analyzed. All the sequences

generated in the present study grouped into the Europe/South America-1 genotype cluster supported by a 72% bootstrap value. The sequences grouped in the same node of samples detected in domestic dogs from

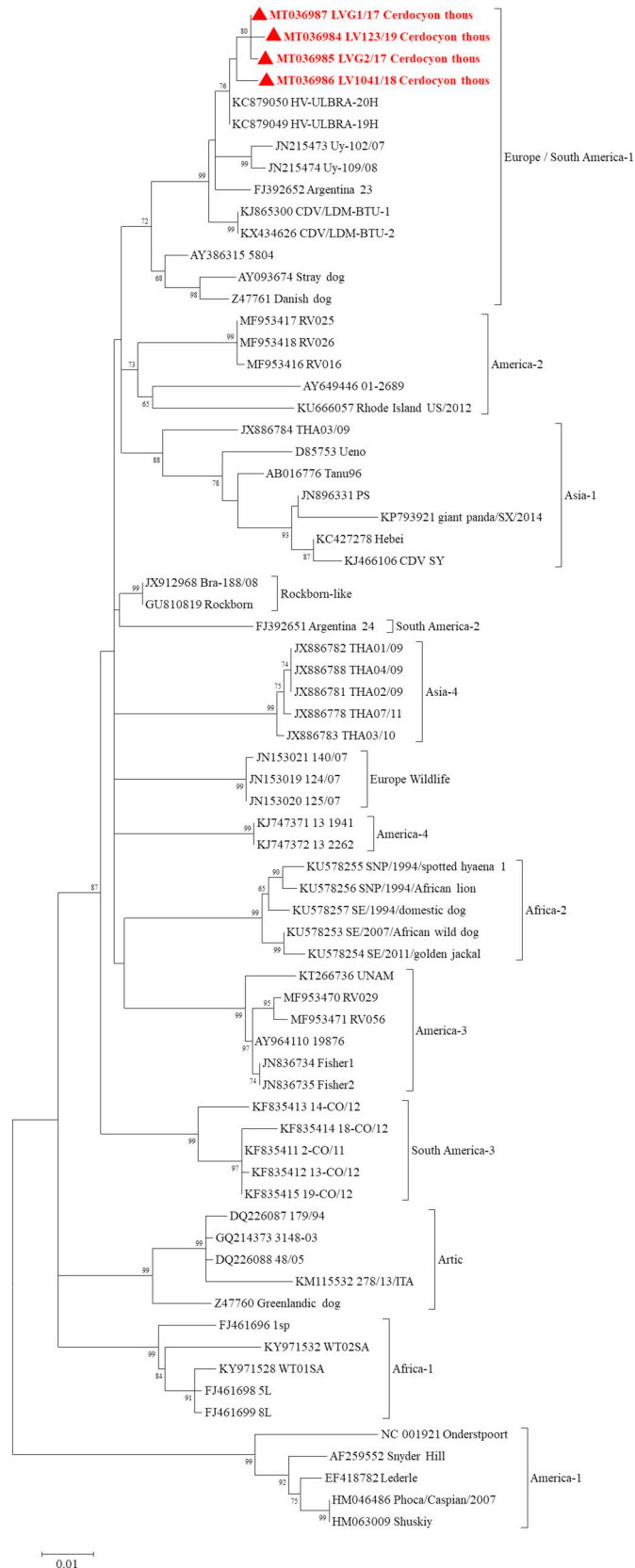


Fig. 2. Nucleotide phylogenetic reconstruction of the partial hemagglutinin of canine morbillivirus species members highlighting the sequences detected in the present study. Sequences were analyzed through maximum-likelihood method applied with the GTR + G + I model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been depicted adjacent to the branches. Bootstrap values for each node have been demonstrated if they were > 50%. The sequence detected in the present study has been highlighted with ●. GenBank accession numbers are described in the phylogenetic tree.

southern Brazil (GenBank accession numbers [KC879049](#) and [KC879050](#)).

3.3. Canine parvovirus 2 (CPV-2)

In the 22 pooled wild dog organs submitted for HTS, the presence of two contigs closely related to CPV-2 was observed (Table 1). The application of the specific CPV PCR protocol (Buonavoglia et al., 2001) revealed 13.64% (3/22) to be positive (one *Lycalopex gymnocercus* and two *Cerdocoyon thous* from Brazil).

All the CPV-2-positive PCR products were sequenced for CPV-2 type definition (CPV-2a, 2b, or 2c). Furthermore, samples were assigned to one CPV-2 type based on the presence of a deduced asparagine (2a), aspartic acid (2b), or glutamic acid (2c) at amino acid position 426 (Buonavoglia et al., 2000). The three tested samples shared 99.03 to 99.23% nucleotide identity. In the nucleotide BLAST search, the samples detected in this study presented 99.6 to 100% identity with CPV-2 strains detected in domestic dogs in southern Brazil (GenBank accession numbers [MK344466](#), [MK344470](#), and [JF796206](#)). One sample detected in *Cerdocoyon thous* was classified as CPV-2b, while the two other samples detected in the present study (one sample from a *Cerdocoyon thous* and one sample from a *Lycalopex gymnocercus*) were classified as CPV-2c. The CPV-2 sequences generated in the present study were deposited in the GenBank database under accession number [MN997122-MN997124](#).

3.4. Canine bocavirus (CBoV)

Three contigs more closely related to CBoV were obtained from the pooled wild dog organs submitted for HTS (Table 1). One of the 22 (4.55%) samples was positive in both assays and was from a *Lycalopex gymnocercus* sampled in Brazil. The two amplification products were submitted for Sanger sequencing. The sequence obtained using the primers designed in the present study (Supplementary Table) enabled us to identify the same sequence obtained in HTS.

In a nucleotide BLAST search, the VP2 fragment (Kapoor et al., 2012) named LV564/18 presented 96.9% identity with CBoV isolate Dis-021 reported in a domestic dog presenting respiratory disease in the United States (GenBank accession number [JN648137](#)).

To present the genetic relation between LV564/18 and other *Bocaparvovirus* members, a partial VP2 gene phylogenetic tree applying maximum-likelihood inference, GTR + G + I statistical method, and 1000 bootstrap was constructed in MEGA6 (Tamura et al., 2013) (Fig. 3). The sequence LV564/18 obtained from the *Lycalopex gymnocercus* sample clustered in the same node of other genetically different CBoVs (Dis-021 and HRB-C7) reported in domestic dogs from the United States and China, respectively (GenBank accession numbers [JN648137](#) and [KT192702](#), respectively), supported by a 99% bootstrap value. This genetically divergent CBoV cluster evolved from the same common ancestor that originated the CBoV-1 and CBoV-2 sequences, as supported by an 82% bootstrap value.

3.5. Anelloviruses

A total of 171 contigs with a closer association with *Anelloviridae* members were also observed (Table 1). The contigs ranged between

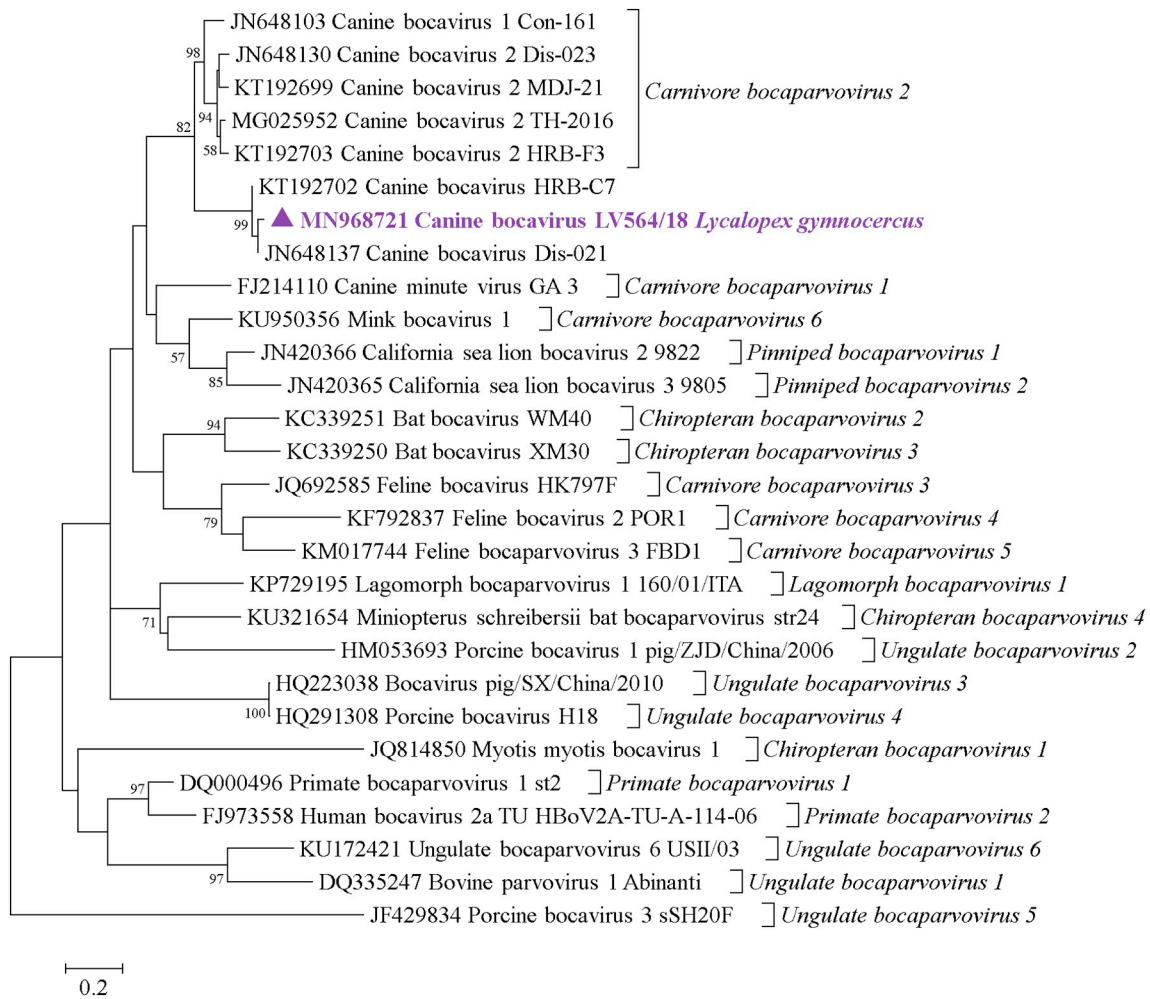


Fig. 3. Nucleotide phylogenetic reconstruction of the partial VP2 of subfamily *Parvovirinae* members highlighting the bocaparvovirus detected in the present study. Sequences were analyzed through maximum likelihood method applied with the GTR + G + I model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been depicted adjacent to the branches. Bootstrap values for each node have been demonstrated if they were > 50%. The sequence detected in the present study has been highlighted with a ▲. GenBank accession numbers are described in the phylogenetic tree.

2415 and 886 nt in length. It was possible to obtain two complete genomes (Fig. 4A and B) and five additional sequences displaying the complete ORF1 gene (Fig. 4C).

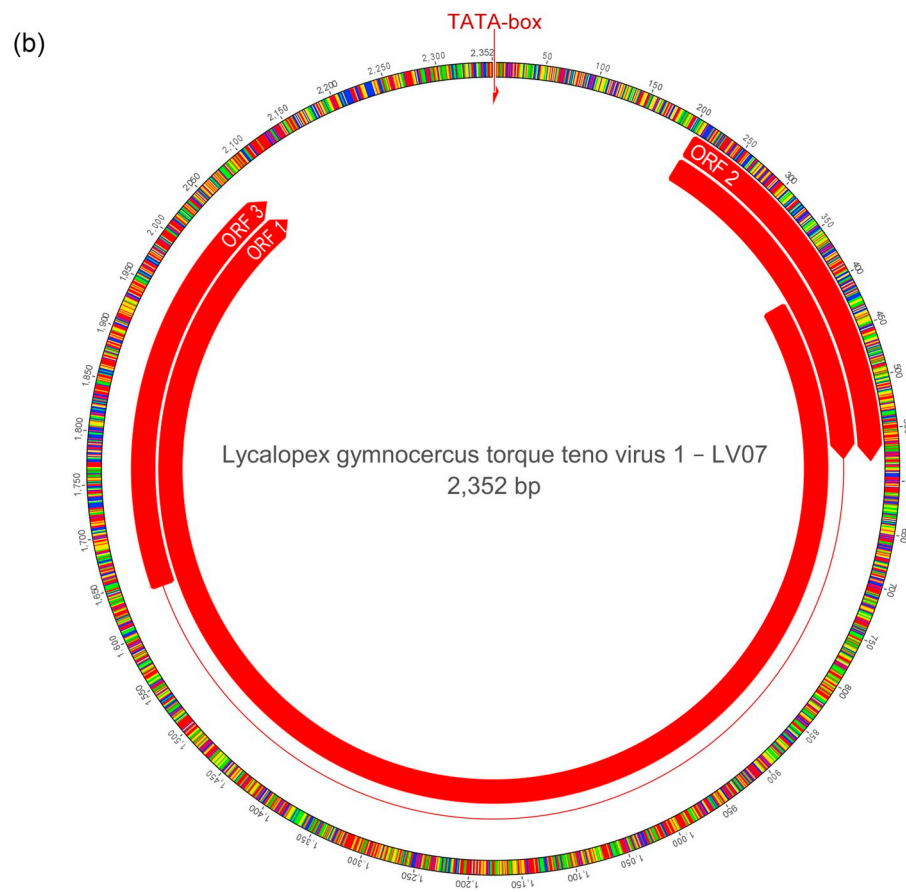
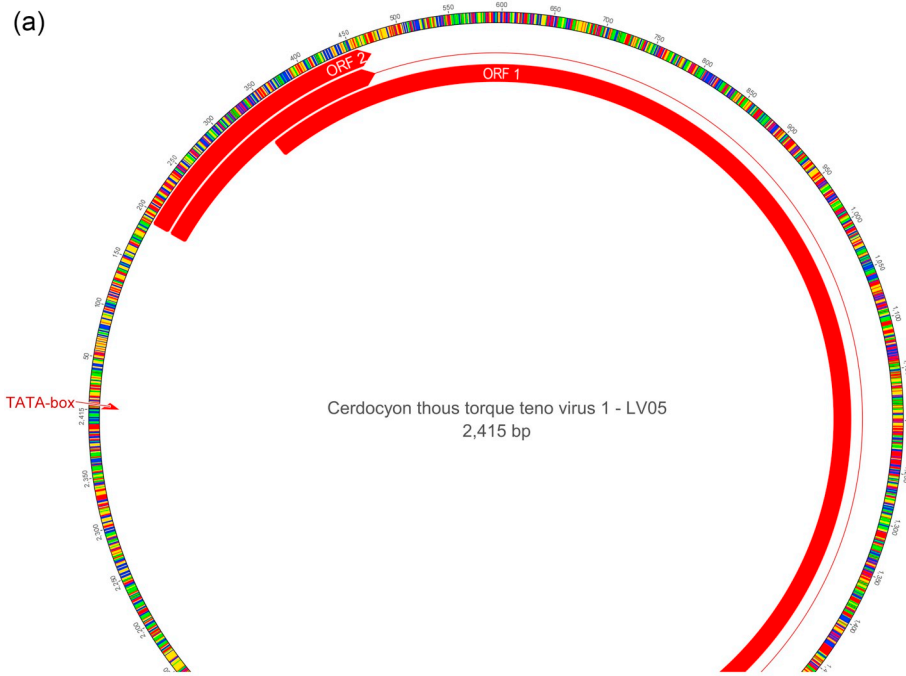
To identify the host of origin (pampas or crab-eating fox), we applied specific PCR protocols designed using the sequences identified in HTS (Supplementary Table). No sequence was identified in either *Cerdocyon thous* or *Lycalopex gymnocercus*. The sequences were named the putative *Anelloviridae* family species *Cerdocyon thous* torque teno virus 1 (CtTTV-1), CtTTV-2, CtTTV-3, CtTTV-4, *Lycalopex gymnocercus* torque teno virus 1 (LgTTV-1), and LgTTV-2, where two different sequences displaying 81.21% nucleotide identity (Table 2) were classified as LgTTV-1 putative species. According to ICTV, nucleotide divergences in the ORF1 gene higher than 35% and 56% denote the same anellovirus species and genus, respectively (ICTV, <http://www.ictvonline.org/virusTaxonomy.asp>). Apparently, six putative new *Anelloviridae* species (CtTTV-1, CtTTV-2, CtTTV-3, CtTTV-4, LgTTV-1 and LgTTV-2) belonging to five putative new genera were observed (Fig. 4C). CtTTV-1 and CtTTV-2 can be classified in the same genus, since they present 63.15% nucleotide identity in the ORF1 gene. The relation of the sequences reported in the present study and other members of the family *Anelloviridae* is reported in Table 3.

Two whole genome *Anelloviridae* sequences were obtained by HTS: CtTTV-1 LV05 and LgTTV-1 LV07. CtTTV-1 LV05 (Fig. 4A) displayed a typical *Anelloviridae* organization comprised of a circular single-

stranded DNA genome containing 2415 nucleotides (nt) and a 51.9% C + G content. The sequence presents an untranslated intergenic region comprising 556 nucleotides, putative ORF1 (nt 336 to 2048) and ORF2 genes (nt 190 to 468), and an ORF3 gene divided in two intervals (nt 190 to 465 and 1678 to 2034).

LgTTV-1 LV07 (Fig. 4B) also displayed a typical *Anelloviridae* organization comprised of a circular single-stranded DNA genome containing 2352 nucleotides (nt) and a 44.4% C + G content. The sequence presents an untranslated intergenic region comprising 460 nucleotides, putative ORF1 (nt 395 to 2095) and ORF2 genes (nt 204 to 581), and an ORF3 gene divided in two intervals (nt 204 to 578 and 1638 to 2090).

To analyze the sequences detected in the present study and representative sequences within the *Anelloviridae* family, a complete amino acid ORF1 phylogenetic reconstruction was performed (Fig. 4C). CtTTV-1 LV05, CtTTV-2 LV15 and CtTTV-3 LV08 were closely related and apparently emerged from the same common ancestor that originated Torque teno canis virus (genus *Thetatorquevirus*) that was reported in domestic dogs (Lan et al., 2011). CtTTV-4 LV23 emerged from the same common ancestor that originated unclassified anelloviruses detected in masked palm civet (*Paguma larvata*) (Nishizawa et al., 2018). LgTTV-1 LV06 and LV07 apparently evolved from the same common ancestor of CtTTV-4 LV23 and the unclassified anelloviruses reported in the masked palm civet. Finally, LgTTV-2 LV13 apparently presents the same common ancestor of *Etorquevirus* and



(caption on next page)

Fig. 4. Genetic characterization of *Anelloviridae* family members detected in the present study. (A) Genomic organization of LV05 detected in *Cerdocoyon thous* in the present study. (B) Genomic organization of LV07 detected in *Lycalopex gymnocercus* in the present study. (C) Amino acid phylogenetic tree of complete ORF1 gene highlighting the sequences obtained in the present study. The sequences were analyzed through the maximum-likelihood method with JTT + G + I model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been presented adjacent to the branches. Bootstrap values for each node have been shown if they were > 50%. The sequences detected in the present study has been highlighted with ● for sequences detected in *Cerdocoyon thous*, and with ▲ for sequences reported in *Lycalopex gymnocercus*. The sequences detected in the present study were deposited in GenBank database: CtTTV-1 LV05 (MT010524), CtTTV-2 LV15 (MT010525), CtTTV-3 LV08 (MT010526), CtTTV-4 LV23 (MT010527), LgTTV-1 LV06 (MT010528), LgTTV-1 LV07 (MT010529), and LgTTV-2 LV13 (MT010530).

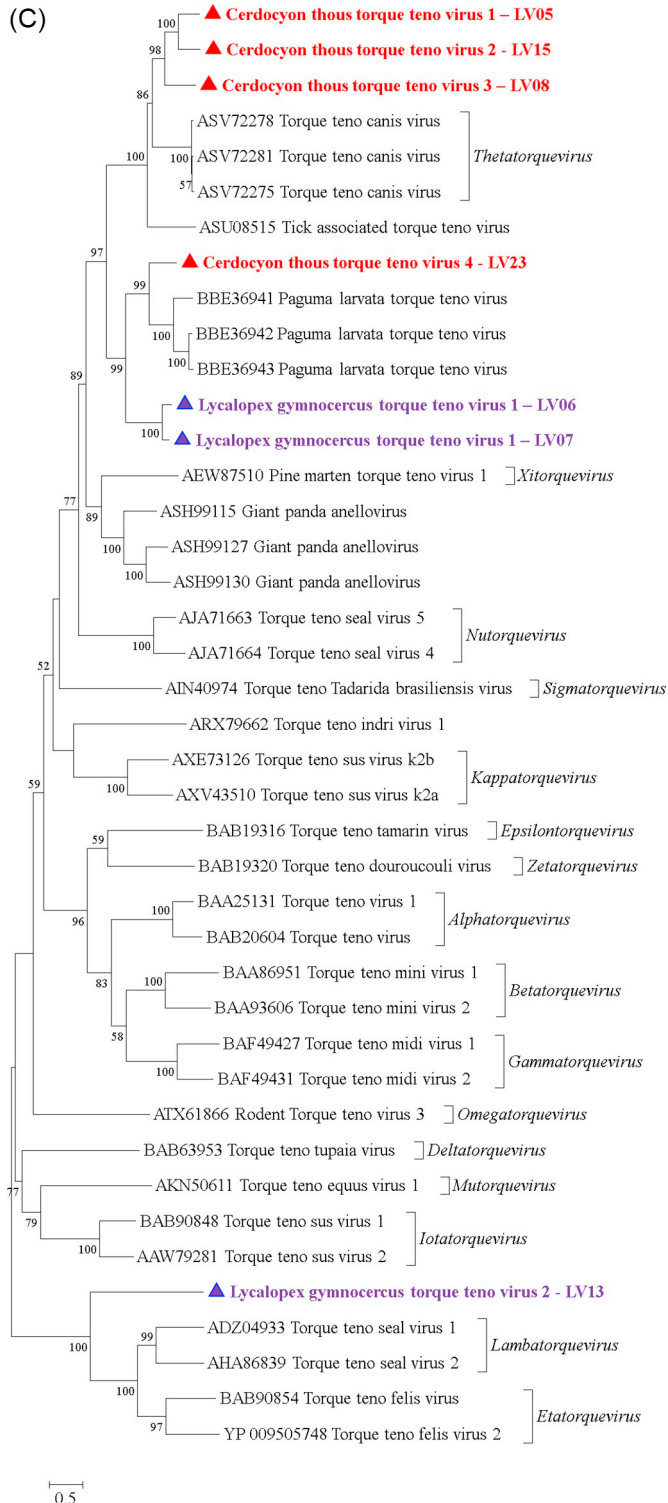


Fig. 4. (continued)

Lambdatorquevirus genera members reported in domestic cats (Okamoto et al., 2002) and seals (Ng et al., 2011), respectively.

3.6. Circular rep-encoding single-stranded (CRESS) DNA viruses

Seven contigs closely related to unclassified CRESS DNA viruses were detected by HTS in the present work (Table 1). One of the seven sequences presents the complete sequence of the putative replicase-associated (Rep) protein, which is widely used to classify CRESS DNA viruses (Zhao et al., 2019). This sequence was screened using PCR (Supplementary Table) in the 22 wild canid samples and was detected in one *Cerdocoyon thous* from Brazil and putatively named *Cerdocoyon thous*-associated circular ssDNA virus - LV03.

A complete Rep amino acid phylogenetic analysis was performed using *Cerdocoyon thous*-associated circular ssDNA virus - LV03 and other representative CRESS DNA viruses (Fig. 5). The phylogenetic reconstruction presented six-well separated clusters corresponding to classified families *Circoviridae*, *Genomoviridae*, *Geminiviridae*, the proposed potential family *Kirkoviridae*, and two independent clades containing sequences not classified in any CRESS DNA viral family supported by bootstrap values ranging between 82 and 100%. The *Cerdocoyon thous*-associated circular ssDNA virus LV03 grouped into one of the unclassified CRESS DNA viral families supported by an 87% bootstrap value and was more closely related to sequences reported in dragonflies, lakes and marine water (GenPept accession numbers ALE29688, YP_009237550, AGA18247, and AGA18478).

4. Discussion

The virome present in spleen and mesenteric lymph node samples obtained from 17 crab-eating foxes (*Cerdocoyon thous*) and five Pampas foxes (*Lycalopex gymnocercus*) has been described using HTS and metagenomic analysis (Fig. 1). Previous works described the virome in samples obtained in domestic dogs (*Canis lupus familiaris*) (Li et al., 2011; Moreno et al., 2017; Weber et al., 2018a, 2018b), but only a limited number of works analyzed wild canids as wolves (*Canis lupus signatus*) (Conceição-Neto et al., 2017) and red foxes (*Vulpes vulpes*) (Lojkić et al., 2016). Our study, which employed nonspecific amplification, revealed the presence of commonly reported dog viruses and previously unknown viral agents (Table 1). The outstanding presence of *Parvoviridae* and *Anelloviridae* members is consistent with the findings reported in domestic dogs (Weber et al., 2018a, 2018b). We also observed the presence of highly prevalent viral agents in domestic dogs from Brazil, such as CPV-2 and CDV (Alves et al., 2018b), that are genetically closely related to those reported in dogs of the same Brazilian region where the wild canids were sampled.

RABV was not detected in the analyzed samples using either HTS (Table 1) or RABV-specific PCR (Soares et al., 2002). In Brazil, RABV was controlled in domestic dogs by intense public vaccination campaigns (Freire de Carvalho et al., 2018). However, the circulation of RABV in wildlife has become a major concern for public health (Antunes et al., 2018; Campos et al., 2019; Rocha et al., 2017), where biting of humans by wild animals has been reported. Moreover, crab-eating foxes were identified as reservoirs of RABV variants (Campos et al., 2019; Rocha et al., 2017). It is important to highlight that the central nervous system is the optimal sample to detect RABV, instead of

Table 2

Summary of *Anelloviridae* family-related sequences reported in the present study, reporting host of origin, ORF1 size, and nucleotide identity of ORF1 gene between them.

Sequence name	Host	ORF1 size (aa)	Nucleotide identity							
			LV05	LV06	LV07	LV08	LV13	LV15	LV23	
LV05	<i>Cerdocyon thous</i>	570								
LV06	<i>Lycalopex gymnocercus</i>	566	42.08							
LV07	<i>Lycalopex gymnocercus</i>	566	41.71	81.21						
LV08	<i>Cerdocyon thous</i>	581	57.23	43.93	42.57					
LV13	<i>Lycalopex gymnocercus</i>	471	29.36	31.53	32.10	29.54				
LV15	<i>Cerdocyon thous</i>	583	63.32	41.26	40.72	53.53	28.40			
LV23	<i>Cerdocyon thous</i>	524	43.14	51.50	51.97	42.61	29.88	42.59		

the samples used in the present study, and this factor may have contributed to the non-detection of RABV in the samples analyzed.

The CDV species, renamed canine morbillivirus, was detected in four of the 17 crab-eating foxes sampled from Brazil in the present study by applying an RT-nested PCR protocol against the CDV nucleocapsid (Fischer et al., 2013). Two of these samples had a partial sequence within the CDV-hemagglutinin (An et al., 2008; Riley and Wilkes, 2015) sequenced and were genotyped as South America I/Europe (Fig. 2), which is the most prevalent CDV genotype in southern Brazil and Uruguay (Budaszewski et al., 2014; Fischer et al., 2016; Sarute et al., 2014) and is also reported in Argentina (Panzer et al., 2012). Other CDV genotypes as South America 2, 3 and 4 are frequently reported in other South American countries as Argentina, Ecuador and Colombia (Espinal et al., 2014; Panzer et al., 2014; Panzer et al., 2012; Sarute et al., 2014). The CDV sequences detected in crab-eating foxes of the present study were assigned as closely related to CDV infecting domestic dogs from southern Brazil in both nucleocapsid and hemagglutinin sequencing. CDV infection causes high mortality in domestic and wild dogs (Beineke et al., 2015), and it is a common cause of wildlife population declines (Cleaveland et al., 2000; Roelke-Parker et al., 1996; Viana et al., 2015). Additionally, CDV was determined to be the cause of death of crab-eating foxes in Brazil (Megid et al., 2009) and Argentina (Ferreira et al., 2009). Our data reinforce that crab-eating foxes may be wild reservoirs of CDV and suggest a possible spillover between crab-eating foxes and domestic dogs.

CPV-2 was reclassified as carnivore protoparvovirus 1 (genus *Protoparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae*) by the International Committee of Taxonomy of Viruses (ICTV) (Cotmore et al., 2014). In the present study, CPV-2 genome segments were found in one Pampas fox and two crab-eating foxes from Brazil. These sequences were subtyped as CPV-2b and -2c. CPV-2b and CPV-2c strains are more frequently found in domestic dogs from Brazil, where CPV-2c substituted CPV-2b as the most prevalent CPV-2 subtype (Pinto et al., 2012). Additionally, the CPV-2 fox sequences were closely related to those reported in domestic dogs of the same Brazilian region. Wild canids are also likely to act as reservoirs of CPV-2 infection for domestic canine populations (Truyen et al., 1998; Van Arkel et al., 2019), and CPV-2-positive serology was previously reported in Pampas and crab-eating foxes from southern Brazil (de Almeida Curi et al., 2010; Hübner et al., 2010). CPV-2 is the most frequent canine pathogen worldwide (Alves et al., 2018b; Decaro et al., 2011; Decaro and Buonavoglia, 2012) and an important cause of severe diarrhea in puppies (Decaro and Buonavoglia, 2012). Moreover, CPV-2 is highly stable in the environment and can persist in domestic dog populations due to its indirect faecal-oral transmission and circulation in susceptible dogs (Van Arkel et al., 2019). Our data reinforce that Pampas and crab-eating foxes may be wild reservoirs of CPV-2. However, more studies are required to understand the impact of CPV-2 on these two wildlife species.

CBoV was detected in one Pampas fox from Brazil in the present study (Fig. 3). This sequence was closely related to bocaparvoviruses reported in domestic dogs presenting respiratory disease (Kapoor et al.,

2012). This CBoV is genetically different from CBoV-1 and CBoV-2, which were reclassified as carnivore bocaparvovirus 2 (CBPV-2) in the genus *Bocaparvovirus* of the subfamily *Parvovirinae* and family *Parvoviridae* (Cotmore et al., 2014). Apparently, the CBoV sequence from the present study is from a new species within the genus *Bocaparvovirus*. This putative new genus was only reported in domestic dogs from the United States (Kapoor et al., 2012) and China (Guo et al., 2016). To the best of our knowledge, this report is the first to describe these genetically divergent CBoVs in South America and in canids other than domestic dogs.

The *Anelloviridae* members represent nonenveloped ssDNA viruses comprised of more than 65 species grouped in 16 genera (ICTV, <http://www.ictvonline.org/virusTaxonomy.asp>), where some anelloviruses remain unassigned (Nishizawa et al., 2018). The anellovirus-related sequences were the most abundant viral sequences detected by HTS in the present study (Table 1). The predominance of anelloviruses in the mesenteric lymph nodes and spleen of both wild canid species is similar to what was reported in the serum of domestic dogs (Weber et al., 2018a, 2018b). Moreover, we described two complete genomes (Fig. 4A and B) and five additional sequences that displayed the complete ORF1 gene, comprising five putative new genera within the *Anelloviridae* family (Fig. 4C). The present study expands the host range of this viral family and suggests that novel anelloviruses of marked diversity can be found in wild canids. It is important to emphasize that anellovirus pathogenicity in canids has not been examined in depth (Lan et al., 2011; Sun et al., 2017; Weber et al., 2018a, 2018b).

We also detected sequences related to Rep and capsid (Cap) proteins of different circular ssDNA viruses (Table 1). One of these sequences presented the complete Rep protein that is used for the classification of the so-called CRESS-DNA viruses (Fig. 5). This sequence was genetically distinct from the other reported genomes and apparently was not classified in any assigned viral family. CRESS DNA viruses were previously described as novel circovirus-like viruses, since these viruses present Rep and Cap genes in agreement with *Circoviridae* members (Rosario et al., 2012b). Recently, with the recognition of its diversity and low degree of genome similarity with members of the family *Circoviridae*, the term CRESS-DNA viruses was proposed (Rosario et al., 2012a). These viruses have been detected in samples from a number of widely different sources, including samples from humans and other mammals, fishes, and insects, as well as from the environment (López-Bueno et al., 2016; Rosario et al., 2012a; Steel et al., 2016; Weber et al., 2018a, 2018b). Apparently, CRESS viruses were not reported in previous works that analyzed the virome of domestic (Moreno et al., 2017; Weber et al., 2018a, 2018b) and wild canids (Conceição-Neto et al., 2017; Lojkić et al., 2016), which may suggest that these viruses were not abundant in those animal species.

5. Conclusion

The present study categorized the virome of crab-eating and Pampas foxes. We reported viruses commonly detected in domestic dogs,

Table 3
Comparison of the ORF1 sequence of anelloviruses detected in *Cerdocyon thous* and *Lycalopex gymnocercus* obtained in the present study.

Genera	Species	Accession number	Host	ORF1 size (aa)	Difference (%) within the ORF1 amino acid sequence										
					LV05	LV06	LV07	LV08	LV13	LV15	LV23				
<i>Alphatorquevirus</i>	Torque teno virus 1	BAA25131	Human (<i>Homo sapiens</i>)	770	82.56	82.09	82.35	82.22	88.33	16.26	84.32				
	Torque teno virus 2	BAB20604	Chimpanzee (<i>Pan troglodytes</i>)	727	81.73	83.71	82.65	82.63	88.20	17.24	84.52				
<i>Betatorquevirus</i>	Torque teno mini virus 1	BAA86951	Human (<i>Homo sapiens</i>)	662	85.45	80.91	80.91	84.79	90.18	84.59	86.50				
	Torque teno mini virus 2	BAA93606	Human (<i>Homo sapiens</i>)	628	83.64	81.36	81.07	83.59	87.94	82.71	86.16				
<i>Deltatorquevirus</i>	Torque teno tupaia virus	BAB63953	Tupaia (<i>Tupaia belangeri chinensis</i>)	502	80.65	81.83	81.83	81.27	83.54	82.97	82.99				
<i>Epsilontorquevirus</i>	Torque teno tamarin virus	BAB19316	Cotton-top tamarin (<i>Saguinus oedipus</i>)	712	80.84	81.31	80.63	80.44	87.65	79.95	84.38				
	Torque teno felis virus 1	BAB90854	Cat (<i>Felis catus</i>)	436	86.95	86.58	86.74	87.70	79.61	86.92	89.26				
<i>Gammatorquevirus</i>	Torque teno felis virus 2	YP_009505748	Cat (<i>Felis catus</i>)	404	88.71	88.60	88.11	88.56	79.68	88.00	89.63				
	Torque teno midi virus 1	BAP49427	Human (<i>Homo sapiens</i>)	673	82.92	82.27	81.98	82.62	88.03	82.11	85.33				
<i>Iotatorquevirus</i>	Torque teno midi virus 2	BAP49431	Human (<i>Homo sapiens</i>)	677	82.26	83.02	82.73	83.36	89.28	83.88	85.31				
	Torque teno sus virus 1	BAB90848	Pig (<i>Sus scrofa domestica</i>)	635	80.84	81.91	82.07	81.02	83.09	81.32	82.78				
<i>Kappatorquevirus</i>	Torque teno sus virus 2	AAW79281	Pig (<i>Sus scrofa domestica</i>)	437	80.45	82.12	81.52	80.09	84.73	80.21	83.09				
	Torque teno sus virus k2a	AXV43510	Pig (<i>Sus scrofa domestica</i>)	620	78.98	81.02	80.12	79.82	86.76	79.38	80.82				
<i>Lambdatorquevirus</i>	Torque teno sus virus k2b	AXE73126	Wild boar (<i>Sus scrofa</i>)	633	79.43	81.53	79.73	81.83	85.42	80.21	80.69				
	Torque teno seal virus 1	ADZ04933	Seal (<i>Phoca vitulina richardsi</i>)	469	86.49	85.88	85.88	87.48	79.37	87.26	87.28				
<i>Miotorquevirus</i>	Torque teno seal virus 2	AHA86839	Seal (<i>Phoca vitulina richardsi</i>)	464	86.63	86.84	87.50	86.95	79.85	87.14	89.07				
	Torque teno equus virus 1	AKN50611	Horse (<i>Equus caballus</i>)	435	81.11	80.83	79.60	80.59	86.74	80.96	79.12				
<i>Nutorquevirus</i>	Torque teno seal virus 4	AJA71664	Seal (<i>Phoca vitulina richardsi</i>)	642	76.30	78.00	77.39	78.12	86.10	78.59	78.74				
	Torque teno seal virus 5	AJA71663	Seal (<i>Phoca vitulina richardsi</i>)	439	77.43	77.34	78.15	78.01	86.34	78.15	77.59				
<i>Omeigatorquevirus</i>	Rodent Torque teno virus 3	ATX61866	Hairy-tailed bolo mouse (<i>Bolomys lasiurus</i>)	585	79.87	78.77	77.80	79.04	84.86	81.69	79.64				
<i>Sigmatorquevirus</i>	Torque teno <i>Tadarida brasiliensis</i> virus	AIN40974	Bat (<i>Tadarida brasiliensis</i>)	547	77.54	78.54	77.47	77.29	84.28	78.49	77.62				
<i>Thetatorquevirus</i>	Torque teno canis virus	ASV72275	Dog (<i>Canis lupus familiaris</i>)	576	56.29	71.24	70.05	56.13	83.78	56.52	71.85				
<i>Xitorquevirus</i>	Pine marten torque teno virus 1	AEW87510	Pine marten (<i>Martes martes</i>)	568	74.71	74.84	73.52	73.06	85.97	76.11	76.27				
<i>Zetatorquevirus</i>	Torque teno douroucouli virus	BAB19320	Douroucouli (<i>Aotus trivirgatus</i>)	720	82.50	82.93	81.84	81.56	86.67	82.00	86.24				
Unassigned	Giant panda anellovirus	ASH19115	Giant panda (<i>Ailuropoda melanoleuca</i>)	559	71.61	69.30	68.12	69.19	84.79	71.57	71.61				
	<i>Paguma larvata</i> torque teno virus	BBE36941	Masked palm civet (<i>Paguma larvata</i>)	582	70.59	64.91	66.23	69.34	86.83	71.06	52.51				
Unassigned	Tick-associated torque teno virus	ASU08515	Tick (<i>Dermacentor variabilis</i>)	578	59.35	73.66	72.32	58.42	83.53	58.38	74.03				
	Torque teno indri virus	ARX79662	Lemur (<i>Indri indri</i>)	520	79.26	75.97	77.31	80.23	85.44	79.24	79.23				

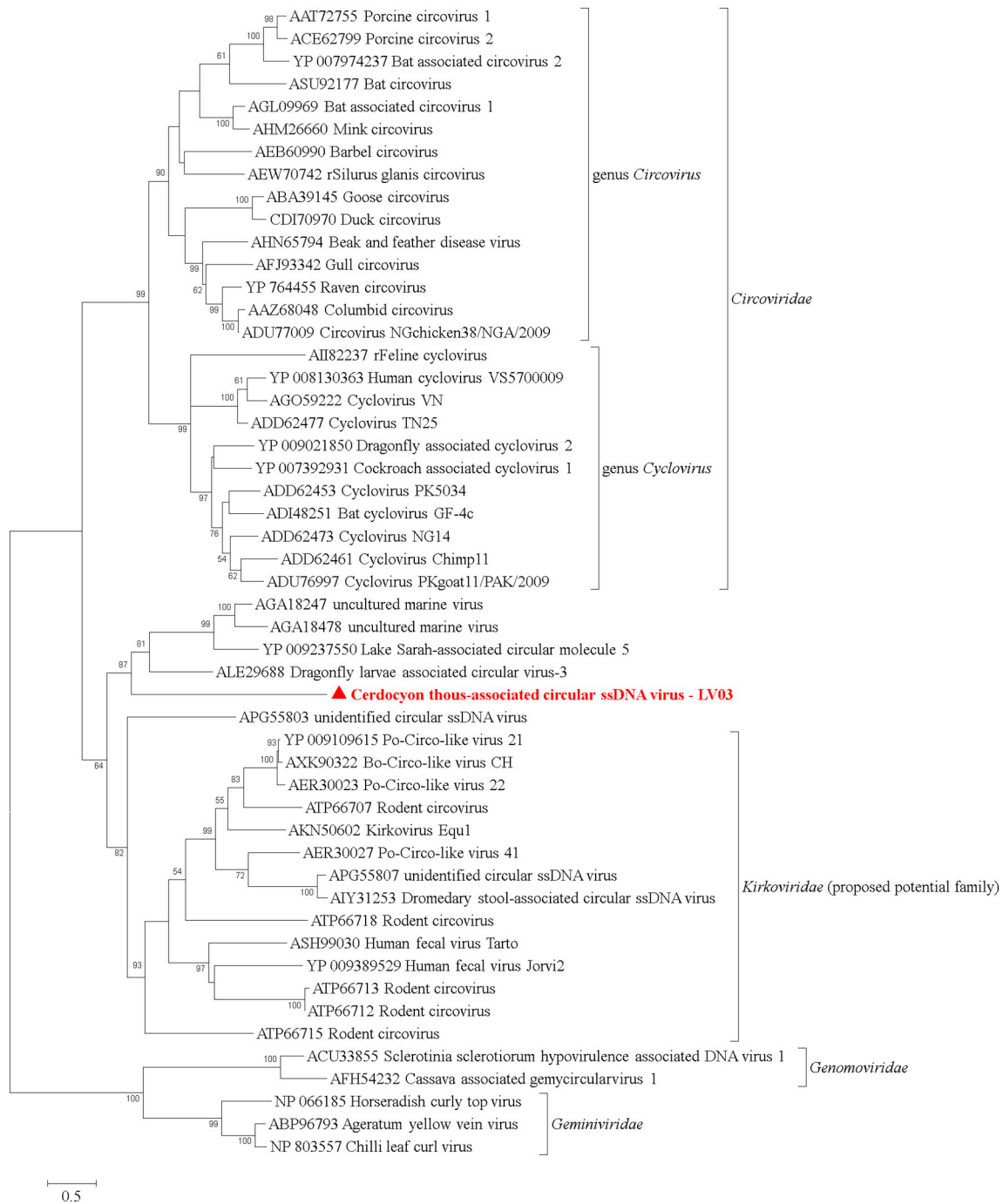


Fig. 5. Amino acid phylogenetic reconstruction of complete putative replicase-associated (Rep) protein of CRESS DNA virus members highlighting the sequence detected in the present study. Sequences were analyzed through maximum-likelihood method applied with the JTT + G + I model. All analyses were conducted with 1000 bootstrap replicates, and the percentage of replicate trees in which the sequences clustered together have been depicted adjacent to the branches. Bootstrap values for each node have been demonstrated if they were > 50%. The sequence detected in the present study has been highlighted with ●. The sequence generated in the present study was deposited in GenBank database under accession number MT013549.

including the pathogenic and prevalent ones CDV and CPV-2, that may impact wildlife. Moreover, the reported CDV and CPV-2 sequences were closely related to genomes reported in domestic dogs from southern Brazil. The present study also expands the host range of CBoV, CRESS DNA viruses and anelloviruses of marked diversity. The results of this study contribute to the body of knowledge regarding the wild canid virome and viral agents that are potentially transmitted through domestic and wild canids.

Declaration of Competing Interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2020.104421>.

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