**Abstract:** Parvoviruses (family Parvoviridae) are small, single-stranded DNA viruses. Many parvoviral pathogens of medical, veterinary and ecological importance have been identified. In this study, we used high-throughput sequencing (HTS) to investigate the diversity of parvoviruses infecting wild and domestic animals in Brazil. We identified 21 parvovirus sequences (including twelve nearly complete genomes and nine partial genomes) in samples derived from rodents, bats, opossums, birds and cattle in Pernambuco, São Paulo, Paraná and Rio Grande do Sul states. These sequences were investigated using phylogenetic and distance-based approaches and were thereby classified into eight parvovirus species (six of which have not been described previously), representing six distinct genera in the subfamily Parvovirinae. Our findings extend the known biogeographic range of previously characterized parvovirus species and the known host range of three parvovirus genera (Dependovirus, Aveparvovirus and Tetraparvovirus). Moreover, our investigation provides a window into the ecological dynamics of parvovirus infections in vertebrates, revealing that many parvovirus genera contain well-defined sub-lineages that circulate widely throughout the world within particular taxonomic groups of hosts.

**Keywords:** parvovirus; Parvoviridae; ssDNA viruses; zoonotic viruses
1. Introduction

Parvoviruses are small, linear and non-enveloped viruses with single-stranded DNA (ssDNA) genomes ~5–6 kilobases (kb) in length [1]. All paroviruses possess at least two major genes, a non-structural (NS) gene encoding the viral replicase and a capsid (VP) gene encoding the structural proteins of the virion [2]. The Paroviridae family is divided into two subfamilies. All paroviruses that infect vertebrates fall into one subfamily (Parovirinae), which currently contains 41 viral species, classified into eight genera [1].

Paroviruses cause disease in humans and domestic animals. For example, parovirus B19—a species in the genus Erythroparvovirus—causes "erythema infectiosum" in children and polyarthropathy syndrome in adults [2], while canine parovirus—a member of the genus Protoparvovirus—can cause haemorrhagic enteritis in dogs, with lethality in around 80% of cases [3].

In recent years, high throughput sequencing (HTS) approaches have been instrumental in the discovery of many novel parovirus species [4–7]. Consequently, the known diversity of parovirus species has expanded greatly and recent studies have suggested that the parovirus host range may encompass the entire animal kingdom [8]. To understand the natural biology of vertebrate paroviruses—that is, their dynamics in natural hosts, propensity to cause disease and zoonotic potential—it is important to document their distribution and diversity across a wide range of vertebrate species and populations. In this study, we used an HTS approach to investigate parovirus infections among wild mammals and birds in Brazil.

2. Materials and Methods

2.1. Samples

A total of 1073 specimens obtained from 21 different animal species were collected between 2007 and 2016 from rural areas of Pará, Pernambuco, São Paulo, Paraná, Santa Catarina and the Rio Grande do Sul states in Brazil. Individual specimens were distributed in 60 pools based on the species, sample type (i.e., tissue, blood, sera and cloacal swab), date and place of collection (Table S1). The species of wild animals were identified using morphological characteristics keys as previously described [9–11]. The geographical distribution of the pools is shown in Figure 1.

![Figure 1. Geographic locations of collected samples in Brazil.](image-url)
2.2. Preparation of Pools, Viral Genome Sequencing and Assembly

Tissue samples were individually homogenized with Hank's balanced salt solution using the TissueLyser system (Qiagen, Germantown, MD, USA). Then, the homogenized tissue, sera and cloacal swabs were centrifuged for 5 min at 10,000 g and the pools were prepared as previously described [12]. The viral genomes were extracted with a QIAamp viral RNA mini kit (Qiagen, USA) and stored at −80 °C. Subsequently, the nucleic acid was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, NM, USA) and the purity and integrity of nucleic acid of samples were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

The ssDNAs were converted to dsDNA and sequenced in high-throughput sequencing using the RAPID module with the TruSeq RNA Universal kit (Illumina, San Diego, CA, USA) protocol and standard multiplex adaptors. A paired-end, 150-base-read protocol in the RAPID module was used for sequencing on an Illumina HiSeq 2500 instrument, as recommended by the manufacturer. Sequencing was performed at the Life Sciences Core Facility of the University of Campinas, Brazil. A total of 7,059,398 to 94,508,748 paired-end reads per pool were generated with 64.85% to 91.45% of bases ≥ Q30 with a base call accuracy of 99.9% (Table S1). The sequencing reads were assembled using the de novo approach in the metaViC pipeline (https://github.com/sejmodha/MetaViC) [12]. The parvovirus contigs longer than length of 200 nucleotides and supercontigs were merged and classified using DIAMOND against NCBI RefSeq protein database [13,14].

2.3. Genome Characterization

Genome size, coding potential and molecular protein weight were assessed with Geneious 9.1.2 (Biomatters, Auckland, New Zealand). The annotations of protein domains were performed using the Conserved Domain Database [15]. The nucleotide sequences determined in this study have been deposited in GenBank under the accession numbers listed in Table 1.

Table 1. Sequences information, sources, sample, location, location, date and environment of viruses identified in wild animals from Brazil.

| Genus                  | Viral Species               | Strain                  | Genome            | Size (nt) | Host Species   | Sample | Samples Per Pool | Location         | Date   | GenBank            |
|------------------------|----------------------------|-------------------------|-------------------|-----------|----------------|--------|------------------|------------------|--------|--------------------|
| Tetraparvovirus         | Rodent tetraparvovirus     | 1135                    | Nearly complete   | 5494      | Necromys lasiurus | Blood  | 59               | Ribeirão Preto, SP | 2008   | MG745669           |
| Tetraparvovirus         | Rodent tetraparvovirus     | 3542                    | Nearly complete   | 5494      | Necromys lasiurus | Blood  | 52               | Ribeirão Preto, SP | 2009   | MG745670           |
| Tetraparvovirus         | Didelphimorph tetraparvovirus | 4113               | Nearly complete   | 5420      | Didelphis alleminia | Serum  | 14               | Teodoro Sampaio, SP | 2009   | MG745671           |
| Aveparvovirus           | Ratamurine aveparvovirus   | 29                      | Nearly complete   | 5368      | Lophochoerus pipiens | Swab   | 4                | São José do Egito, PE | 2010   | MG745672           |
| Bocaparvovirus          | Rodent bocaparvovirus      | 1                       | Nearly complete   | 5227      | Necromys lasiurus | Blood  | 58               | Ribeirão Preto, SP | 2008   | MG745673           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 9424                    | Nearly complete   | 5219      | Necromys lasiurus | Blood  | 58               | Ribeirão Preto, SP | 2008   | MG745674           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 284                     | Nearly complete   | 5196      | Ateles montensis | Blood  | 41               | Ribeirão Preto, SP | 2009   | MG745675           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 119                     | Nearly complete   | 4998      | Calomys tener   | Blood  | 38               | Ribeirão Preto, SP | 2008   | MG745676           |
| Dependoparvovirus       | Chimonapteran dependoparvovirus | 246              | Nearly complete   | 4894      | Dendropsophus reticulatus | Kidney  | 8                | Araçatuba, SP | 2010   | MG745677           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 2                       | Nearly complete   | 4998      | Necromys lasiurus | Blood  | 59               | Ribeirão Preto, SP | 2008   | MG745678           |
| Tetraparvovirus         | Rodent tetraparvovirus     | MR                      | Nearly complete   | 5368      | Boa taurus      | Blood  | 15               | Maracaju, RR | 2016   | MG745679           |
| Erythroparvovirus       | Ungulate erythroparvovirus | 5                       | Nearly complete   | 5220      | Boa taurus      | Blood  | 6                | Rondônia, RS | 2016   | MG745680           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 1594                    | Partial           | 2255      | Didelphis alleminia | Blood  | 32               | Ribeirão Preto, SP | 2012–2013 | MG745681          |
| Bocaparvovirus          | Rodent bocaparvovirus      | 4093                    | Partial           | 2844      | Necromys lasiurus | Blood  | 52               | Ribeirão Preto, SP | 2009   | MG745682           |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 8                       | Partial           | 1679      | Calomys tener   | Blood  | 34               | Ribeirão Preto, SP | 2009–2013 | MG745683          |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 888                     | Partial           | 1606      | Ateles montensis | Blood  | 20               | Ribeirão Preto, SP | 2012–2013 | MG745684          |
| Prototetraparvovirus    | Rodent prototetraparvirus  | 23                      | Partial           | 1566      | Ateles montensis | Blood  | 55               | Ribeirão Preto, SP | 2008   | MG745685           |
Table 1. Cont.

| Genus          | Viral Species          | Strain | Genome Size (nt) | Host Species          | Sample | Samples Per Pool | Location | Date       | GenBank       |
|----------------|-----------------------|--------|------------------|-----------------------|--------|------------------|----------|------------|---------------|
| Bocaparvovirus| Rodent bocaparvovirus | 422    | Partial          | Necromys lasiurus     | Blood  | 52               | Ribeirão Preto, SP | 2009       | MG745686     |
| Protoparvovirus| Rodent protoparvovirus| 1010   | Partial          | Oligoryzomys nigripes | Blood  | 20               | Ribeirão Preto, SP | 2012-2013  | MG745687     |
| Protoparvovirus| Rodent protoparvovirus| 66     | Partial          | Akodon montensis     | Blood  | 55               | Ribeirão Preto, SP | 2008       | MG745688     |
| Protoparvovirus| Rodent protoparvovirus| 38     | Partial          | Calomys tener        | Blood  | 34               | Ribeirão Preto, SP | 2009,2012   | MG745689     |

Legend: SP (São Paulo State), PR (Paraná State), PE (Pernambuco State), RS (Rio Grande do Sul State).

2.4. Phylogenetic Analysis

Maximum likelihood (ML) phylogenetic trees were reconstructed using alignments of non-structural (NS) proteins and viral proteins (VPs), identified in the present study with representative members of the Parovirinae subfamily [1]. Multiple sequence alignment (MSA) was carried out using RevTrans 2.0 [16] with manual adjustment. The alignments of the core of the NS and VP protein ML trees were inferred using IQ-TREE version 1.4.3 software based on an LG+F+G4 protein substitution model to the core of an NS protein with 145 amino acids and an LG+F+I+G4 protein substitution model to the core of a VP protein with 245 amino acids, both with 1000 replicates [17,18]. Statistical support for individual nodes was estimated via bootstrap replicates. Phylogenetic trees were visualized using Figtree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Nucleotide divergence calculations were performed using the Sequence Demarcation Tool (SDT) version 1.2 in muscle mode [19].

3. Results

Using HTS, we identified 21 parvovirus sequences in samples derived from rodents, bats, opossums, birds and cattle in Pernambuco, São Paulo, Paraná and Rio Grande do Sul states in Brazil (Figure 1). These sequences comprised twelve nearly complete genomes and nine partial genomes (Table 1) and included the first examples of parvoviruses identified in opossums, New World bats and sigmodontine rodents. Parvovirus sequences recovered in our study were classified on the basis of (i) phylogeny and (ii) pairwise distance.

To investigate the phylogenetic relationships between the novel parvoviruses and those described previously, we inferred ML phylogenetic trees from alignments of 71 NS proteins and 71 VP peptide sequences. Phylogenies revealed eight distinct clades corresponding to recognized genera, each having high bootstrap support (values > 75%). The sequences recovered in this study were grouped into six distinct genera (Figure 2). In most cases, the newly identified sequences grouped robustly within the established diversity of their respective genera. Only the Dependoparvovirus-like sequence identified in our study was grouped in a basal position with respect to previously characterized taxa in both NS and VP trees.

According to the species demarcation criteria of the International Committee on Taxonomy of Viruses (ICTV), parvoviruses in the same species should share >85% amino acid sequence identity across the entire NS polypeptide sequence [1]. On this basis, the 21 genomes described in this study represent six novel species of parvoviruses and two that have been described previously—Ungulate erythroparvovirus 1 and ungulate tetraparvovirus 1 (Figures S1 and S2).

We identified a novel species of protoparvovirus in sigmodontine rodents. This virus, which was detected in samples from several distinct animals and species (Table 1), is quite similar to the minute virus of mice (MVM) but is sufficiently distinct based on ICTV criteria to be considered a distinct species. We also identified novel tetraparvoviruses in the opossum and hairy-tailed bolo mouse and a novel dependoparvovirus in tissue samples derived from common vampire bats (Desmodus rotundus). We identified a novel bocaparvovirus species—rodent bocaparvovirus—in two distinct sample pools obtained from hairy-tailed bolo mice and a novel aveparvovirus in the grey piliated finch in Sào José do Egito, Pernambuco State, Brazil. We also identified strains of two parvoviruses that were previously detected in cattle—Ungulate erythroparvovirus 1 and Ungulate tetraparvovirus 1—identified in cattle
serum of Ronda Alta in the Rio Grande do Sul State and Manoel Ribas in Paraná State, both located in South of Brazil.

All the viruses identified in our study have typical parvovirus genome structures encoding NS and VP proteins. The deduced NS protein sequences from these viruses contain the “HxH” domain, which is similar to “HIH,” a metal binding domain previously described in the endonuclease domain [20,21]. This domain is a catalytic unit of the endonuclease, which was described to cleave one of the strands of dsDNA in viral cycle replication [2]. Also, we identified helicase motifs including Walker motifs [22], which are involved in viral DNA synthesis (Figure S3) [2,21]. Most of the capsid proteins also possess a glycine-rich (G-rich) region required for cellular entry [23] and the PLA2 motif involved in the viral release from the endosome and entry into the nucleus [24]. However, we did not identify a PLA2 motif in passeriform aveparvovirus or rodent bocaparvovirus. Interestingly, we observed that one species—chiropteran dependoparvovirus 2—encodes NS and VP as overlapping open reading frames (ORFs), with a shared region of 47 nucleotides (Figure 3).
Figure 3. Genome structures of nearly complete coding sequences of newly identified parvoviruses. The length of the determined nucleotide sequences of the viral sequences are shown on the left. Boxes indicate the open reading frames (ORFs) and the number represents the respective position of their ORFs.

Notably, the rodent bocaparvoviruses and passeriform aveparvovirus contain a putative additional ORF (NP1). This gene is located in the middle of the viral genome and overlaps with the C-terminus region of the NS ORF but in a different reading frame (Figure 3). In the case of the rodent bocaparvoviruses, this ORF may correspond to the NP1 protein, which has been reported to play a role in efficient replication for human and canine bocaparvoviruses [25–27] and in immune evasion for porcine bocaparvoviruses [28].

4. Discussion

Brazil has a great diversity and abundance of wildlife and is considered a hotspot for the potential emergence of novel zoonotic viruses [29]. However, parvovirus studies in Brazil have focused predominantly on canine parvovirus and human parvovirus B19 [2,30]. In this study, we used an HTS approach to investigate parvovirus infections among wild mammals and birds from Brazil that were apparently without symptoms or disease. We identified 21 parvovirus sequences, representing six novel—and two previously described—parvovirus species. We report the first examples of parvoviruses in samples derived from Sigmodontinae rodents, opossums and New World bats. Interestingly, almost all the viruses detected here were sequenced from serum or blood samples suggesting that viremia may have been a factor in their identification.

We detected strains of ungulate tetraparvovirus—a virus in the genus Tetraparvovirus—in cattle from the South of Brazil. Ungulate tetraparvovirus 2—formerly known as porcine hokovirus—has previously been identified in swine in Brazil [31]. However, ungulate tetraparvovirus 1—formerly known as bovine hokovirus—has not previously been reported outside Asia. This virus, which was originally identified in bovine spleen samples obtained from food markets in Hong Kong, has also been identified in domestic yaks (Bos grunniens) in northwestern China [32,33]. The identification of this virus in an entirely distinct population (Brazilian cattle) not only establishes that it occurs outside Asia but also
suggests it may be present in cattle populations throughout the world. In addition, we identified novel species of tetraparvovirus in samples obtained from rodents and from an opossum. Interestingly, the opossum sequence grouped basal relative to the largest Tetraparvovirus clade, which contains isolates from diverse eutherian mammals. Further sampling may reveal whether this basal position reflects the broad co-divergence of tetraparvoviruses and mammals dating back to the common ancestor of marsupials and eutherians. Such ancient origins of the Tetraparvovirus genus are consistent with evidence from endogenous viral element (EVE) sequences that paroviruses have been infecting mammals for millions of years [34,35].

Recently, studies have reported numerous novel dependoparvoviruses in samples derived from Asian bats [36,37]. Here, we provide the first report of a dependoparvovirus in a New World bat—the vampire bat (Desmodus rotundus). In trees based on Rep, this virus groups basally within the Dependoparvovirus genus, consistent with these viruses potentially having an ancestral origin in bats, as has been proposed previously [36].

Currently, only one species is recognised in the genus Aveparvovirus. This virus (Galliform aveparvovirus 1) infects chickens and turkeys and is widespread in poultry farms in the United States and Europe [38,39]. We identified a novel Aveparvovirus species in samples derived from pileated finch (Coryphospingus pileatus), an indigenous (and non-migratory) South American bird, suggesting that viruses belonging to the Aveparvovirus genus may circulate widely among avian species, including wild as well as domestic birds.

We detected Ungulate erythroparvovirus 1 (genus Erythroparvovirus) in Brazilian cattle. Since this virus—to the best of our knowledge—has only been described as a contaminant of commercial bovine serum [40], our study is the first to report detection of Ungulate erythroparvovirus 1 in cattle populations.

We also identified a novel protoparvovirus species infecting sigmodontine rodents in Brazil. Sigmodontine rodent protoparvovirus was identified in several species of rodents (all belong to the subfamily) that we captured in the Ribeirão Preto region of São Paulo State. These viruses are closely related to the Minute virus of mice (MVM), a common pathogen of laboratory mice [41] but, following official taxonomic criteria, they are sufficiently divergent from MVM (>85% in NS and >73% aa in VP) to be considered a distinct species within the Protoparvovirus genus.

Bocaparvoviruses are associated with pathogenic conditions in human, bovine and canine hosts [2,42]. Rodent bocaparvoviruses have recently been reported [43] but relatively little is known about their broader distribution. We identified novel rodent bocaparvoviruses in sigmodontine rodents that are closely related to bocaparvoviruses recently reported in brown rats (Rattus rattus) in China [43] (data not shown). Together, these findings suggest a broad distribution for rodent bocaparvoviruses.

Paroviruses that infect domestic and wild carnivores (including amdoviruses and protoparvoviruses) have been studied fairly extensively in the field. These studies have shown that paroviruses circulate widely among species in the order Carnivora, with the barriers to transmission between species within the order apparently being relatively low [44–46]. The findings of our study suggest that this pattern might be reflected more broadly in parovirus ecology, with many parovirus genera containing sublineages that circulate within particular taxonomic groups of hosts (and are largely restricted to this host group). For example, the phylogenetic relationships shown in Figure 1 indicate that closely related protoparvoviruses circulate widely among rodents and that closely related tetraparvoviruses circulate widely in ungulates. With further sampling of parovirus diversity, it should quickly become apparent whether these inferences are accurate.

5. Conclusions

In this study, we used a sequencing-based approach to characterize parovirus infections in wild and domestic animals in Brazil. Our findings extend the known biogeographic range of previously characterized parovirus species and the known host range of three parovirus genera (Dependovirus, Aveparvovirus and Tetraparvovirus). More broadly, our findings indicate that many
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Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/10/4/143/s1, Table S1: Samples information, host, sources, sample type, location, date, reads and %Bases ≥ Q30. Figure S1: Heatmap of pairwise amino acid identities of the NS protein of parvoviruses identified in this study and representative members of the Parovirinae subfamily based on ICTV criteria. The viruses described in this study are highlighted in bold. Figure S2: Heatmap of pairwise amino acid identities of the VP protein of parvoviruses identified in this study and representative members of the Parovirinae subfamily based on ICTV criteria. The viruses described in this study are highlighted in bold. Figure S3: Alignment of helicase enzymatic motif showing walker’s motifs.

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Author Contributions: William Marcel Souza and Robert James Gifford conceived and designed the experiments; William Marcel Souza, Tristan Dennis, Marcello Jorge Fumagalli, Marilia Farignoli Romeiro and Luiz Carlos Vieira performed the experiments; William Marcel Souza, Tristan Dennis and Robert James Gifford analyzed the data; Sejal Modha, Márcio Roberto Teixeira Nunes and Luiz Tadeu Moraes Figueiredo contributed reagents/materials/analysis tools; Gilberto Sabino-Santos Jr, Felipe Gonçalves Motta Maia, Gustavo Olszanski Acrani, Adriano de Oliveira Torres Carrasco, Luzia Helena Queiroz, Jansen Araujo, Tatiana Lopes Ometto, Edison Luiz Durigon collected samples and performed fieldwork; William Marciel Souza, Tristan Dennis, Luiz Tadeu Moraes Figueiredo and Robert James Gifford wrote the paper.

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