

Whole-genome sequencing of parvoviruses from wild and domestic animals in Brazil provides new insights into parvovirus distribution and diversity

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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.

47 1. Introduction

48 Parvoviruses are small, linear and non-enveloped viruses with single-stranded DNA (ssDNA)
49 genomes ~5-6 kilobases (kb) in length [1]. All parvoviruses possess two major genes, a
50 non-structural (NS) gene encoding the viral replicase, and a capsid (VP) gene encoding the
51 structural proteins of the virion [2]. The *Parvoviridae* family is divided into two subfamilies. All
52 parvoviruses that infect vertebrates falling into one subfamily (*Parvovirinae*), which currently
53 contains 41 viral species, classified into eight genera [1].

54 Parvoviruses cause disease in humans and domestic animals. For example, parvovirus B19, a
55 species in the genus *Tetraparvovirus*, causes 'erythema infectiosum' in children and polyarthropathy
56 syndrome in adults [2], while canine parvovirus, a member of the genus *Protoparvovirus*, can cause
57 haemorrhagic enteritis in dogs, with lethality around 80% of cases [3].

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

66 2. Materials and Methods

67 2.1. Samples

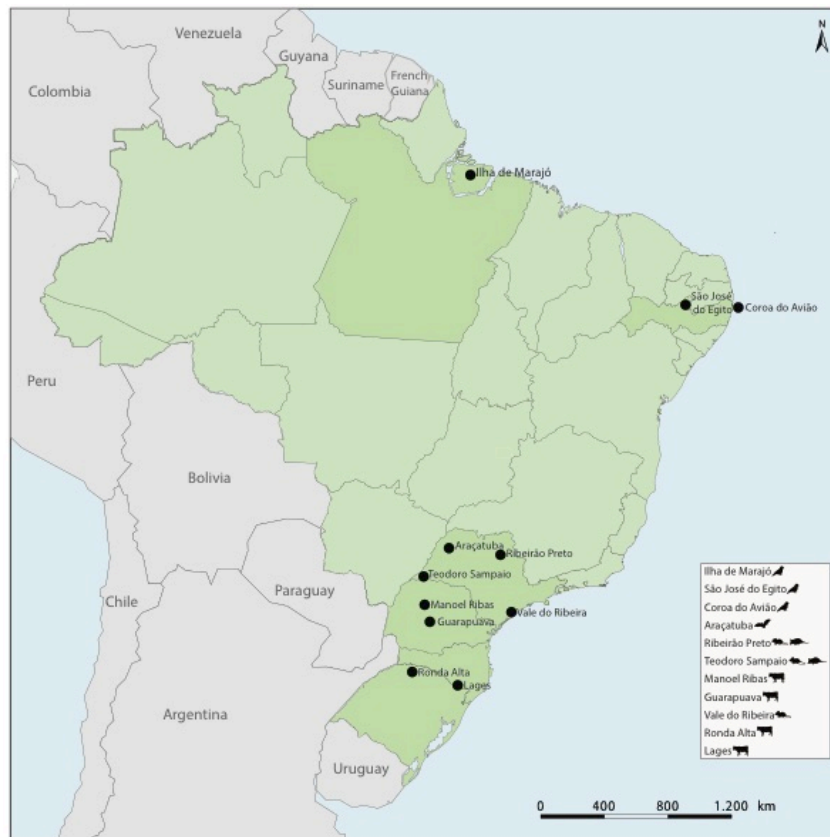
68 A total of 1073 specimens obtained from 21 different animal species were collected from 2007
69 to 2016 from rural areas of Pará, Pernambuco, São Paulo, Paraná, Santa Catarina and the Rio
70 Grande do Sul states in Brazil. Individual specimens were distributed in 60 pools based on the
71 species,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

76 2.2. Preparation of pools, viral genome sequencing and assembly

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

84 The DNAs were prepared for high-throughput sequencing using the RAPID module with the
85 TruSeq Universal adapter (Illumina, USA) protocols and standard multiplex adaptors. A
86 paired-end, 150-base-read protocol in RAPID module was used for sequencing on an Illumina
87 HiSeq 2500 instrument as recommended by the manufacturer. Sequencing was performed in Life
88 Sciences Core Facility from University of Campinas, Brazil. A total of 7,059,398 to 94,508,748
89 paired-end reads per pool were generated with 64.85% to 91.45% of bases \geq Q30 with a base call
90 accuracy of 99.9% (Supplementary Table 1). The sequencing reads were assembled using the de
91 novo approach in the metaViC pipeline (<https://github.com/sejmodha/MetaViC>) [12].

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93
94 **Figure 1.** Geographic locations of collected samples in Brazil.

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96 **2.3. Genome characterization**

97 Genome size, coding potential and molecular protein weight were assessed with Geneious
98 9.1.2 (Biomatters, New Zealand). The annotations of protein domains were performed using the
99 Conserved Domain Database [13]. The nucleotide sequences determined in this study have been
100 deposited in GenBank under the accession numbers listed in **Table 1**.

101
102 **2.4. Phylogenetic analysis**

103 Maximum likelihood (ML) phylogenetic trees were reconstructed using alignments of NS and
104 VP proteins identified in the present study with representative members of *Parvovirinae* subfamily
105 [1]. Multiple sequence alignment (MSA) was carried out using RevTrans 2.0 [14] with manual
106 adjustment. The alignments of core of NS and VP proteins ML trees were inferred by IQ-TREE
107 version 1.4.3 software based on LG+F+G4 protein substitution model to core of NS protein with 145
108 amino acids, and LG+F+I+G4 protein substitution model to core of VP protein with 245 amino acids,
109 both with 1,000 replicates [15,16]. Statistical support for individual nodes was estimated via
110 bootstrap replicates. Phylogenetic trees were visualized using Figtree 1.4.2. Nucleotide divergence
111 calculations were performed using the Sequence Demarcation Tool (SDT) version 1.2 in muscle
112 mode [17].

113 **3. Results**

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

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

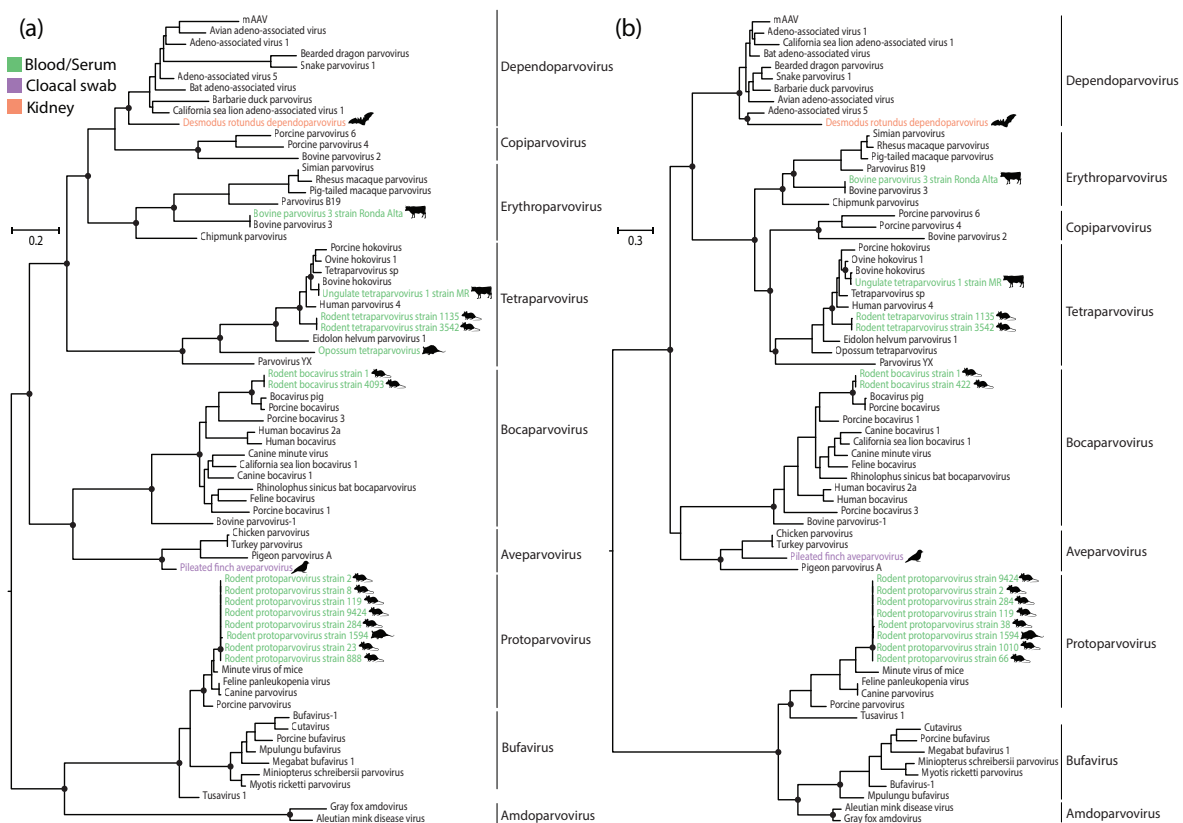
Genus	Viral Species	Strain	Genome	Size (nt)	Host Species	Sample	N	Location	Date	GenBank
<i>Tetraparvovirus</i>	Rodent tetraparvovirus	1135	Complete	5494	<i>Necromys lasiurus</i>	Blood	59	Ribeirão Preto, SP	2008	MG745669
<i>Tetraparvovirus</i>	Rodent tetraparvovirus	3542	Complete	5494	<i>Necromys lasiurus</i>	Blood	52	Ribeirão Preto, SP	2009	MG745670
<i>Tetraparvovirus</i>	Opossum tetraparvovirus	4113	Complete	5420	<i>Didelphis albiventris</i>	Serum	14	Teodoro Sampaio, SP	2009	MG745671
<i>Aveparvovirus</i>	Pileated finch aveparvovirus	29	Complete	5368	<i>Coryphospingus pileatus</i>	Cloacal Swab	4	São José do Egito, PE	2010	MG745672
<i>Bocaparvovirus</i>	Rodent bocaparvovirus	1	Complete	5227	<i>Necromys lasiurus</i>	Blood	58	Ribeirão Preto, SP	2008	MG745673
<i>Protoparvovirus</i>	Rodent protoparvovirus	9424	Complete	5219	<i>Necromys lasiurus</i>	Blood	58	Ribeirão Preto, SP	2008	MG745674
<i>Protoparvovirus</i>	Rodent protoparvovirus	284	Complete	5196	<i>Akodon montensis</i>	Blood	41	Ribeirão Preto, SP	2009	MG745675
<i>Protoparvovirus</i>	Rodent protoparvovirus	119	Complete	4998	<i>Calomys tener</i>	Blood	38	Ribeirão Preto, SP	2008	MG745676
<i>Dependoparvovirus</i>	Desmodus rotundus dependoparvovirus	246	Complete	4894	<i>Desmodus rotundus</i>	Kidney	8	Araçatuba, SP	2010	MG745677
<i>Protoparvovirus</i>	Rodent protoparvovirus	2	Complete	4898	<i>Necromys lasiurus</i>	Blood	59	Ribeirão Preto, SP	2008	MG745678
<i>Tetraparvovirus</i>	Ungulate tetraparvovirus	MR	Complete	5368	<i>Bos taurus</i>	Blood	15	Manoel Ribas, PR	2016	MG745679
<i>Erythroparvovirus</i>	Bovine parvovirus 3	Ronda Alta	Complete	5220	<i>Bos taurus</i>	Blood	6	Ronda Alta, RS	2016	MG745680
<i>Protoparvovirus</i>	Rodent protoparvovirus	1594	Partial	2255	<i>Didelphis albiventris</i>	Blood	32	Ribeirão Preto, SP	2012-2013	MG745681
<i>Bocaparvovirus</i>	Rodent bocaparvovirus	4093	Partial	2844	<i>Necromys lasiurus</i>	Blood	52	Ribeirão Preto, SP	2009	MG745682
<i>Protoparvovirus</i>	Rodent protoparvovirus	8	Partial	1679	<i>Calomys tener</i>	Blood	34	Ribeirão Preto, SP	2009, 2012-2013	MG745683
<i>Protoparvovirus</i>	Rodent protoparvovirus	888	Partial	1606	<i>Oligoryzomys nigripes</i>	Blood	20	Ribeirão Preto, SP	2012-2013	MG745684
<i>Protoparvovirus</i>	Rodent protoparvovirus	23	Partial	1566	<i>Akodon montensis</i>	Blood	55	Ribeirão Preto, SP	2008	MG745685
<i>Bocaparvovirus</i>	Rodent bocaparvovirus	422	Partial	1362	<i>Necromys lasiurus</i>	Blood	52	Ribeirão Preto, SP	2009	MG745686

<i>Protoparvovirus</i>	Rodent	1010	Partial	1283	<i>Oligoryzomys</i>	Blood	20	Ribeirão	2012-2	MG745687
protoparvovirus					<i>nigripes</i>			Preto, SP	013	
<i>Protoparvovirus</i>	Rodent	66	Partial	1099	<i>Akodon</i>	Blood	55	Ribeirão	2008	MG745688
protoparvovirus					<i>montensis</i>			Preto, SP		
<i>Protoparvovirus</i>	Rodent	38	Partial	1067	<i>Calomys tener</i>	Blood	34	Ribeirão	2009,2	MG745689
protoparvovirus								Preto, SP	012-20	
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122 Legend: “N” number of samples per pool, SP (São Paulo State), PR (Paraná State), PE (Pernambuco State), RS (Rio
123 Grande do Sul State).

124
125 To investigate the phylogenetic relationships of the novel parvoviruses to those described
126 previously, we inferred ML phylogenetic trees from alignments of 71 NS proteins and 71 VP
127 peptide sequences. Phylogenies revealed eight distinct clades corresponding to recognized genera,
128 each having high bootstrap support (values >75%). The sequences recovered in this study grouped
129 into six distinct genera (Figure 2). In most cases, the newly identified sequences grouped robustly
130 within the established diversity of their respective genera. Only the *Dependoparvovirus*-like sequence
131 identified in our study grouped in a basal position with respect to previously characterized taxa in
132 both NS and VP trees.

133



134

135 **Figure 2.** Maximum likelihood phylogenies showing the evolutionary relationships of newly
136 identified parvoviruses. (a) Phylogenetic tree of NS proteins. (b) Phylogenetic tree of VP proteins.
137 Phylogenies are midpoint rooted for clarity of presentation. The scale bar indicates evolutionary
138 distance in substitutions per amino acid site. Black lines indicate genera within the *Parvovirinae*
139 subfamily. Black circles indicate nodes with maximum likelihood bootstrap support levels >75%,
140 based on 1,000 bootstrap replicates. Taxa names of parvoviruses identified in our study are colored
141 according to sample type, as shown in the key. Silhouettes indicate host species groups.

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144 According to the species demarcation criteria of the International Committee on Taxonomy of
145 Viruses (ICTV), parvoviruses in the same species should share >85% amino acid sequence identity
146 across the entire NS polypeptide sequence [1]. On this basis, the 21 genomes described in this study
147 represent six novel species of parvoviruses, and two that have been described previously (bovine
148 parvovirus 3 and ungulate tetraparvovirus 1) (**Supplementary Figures 1 and 2**).

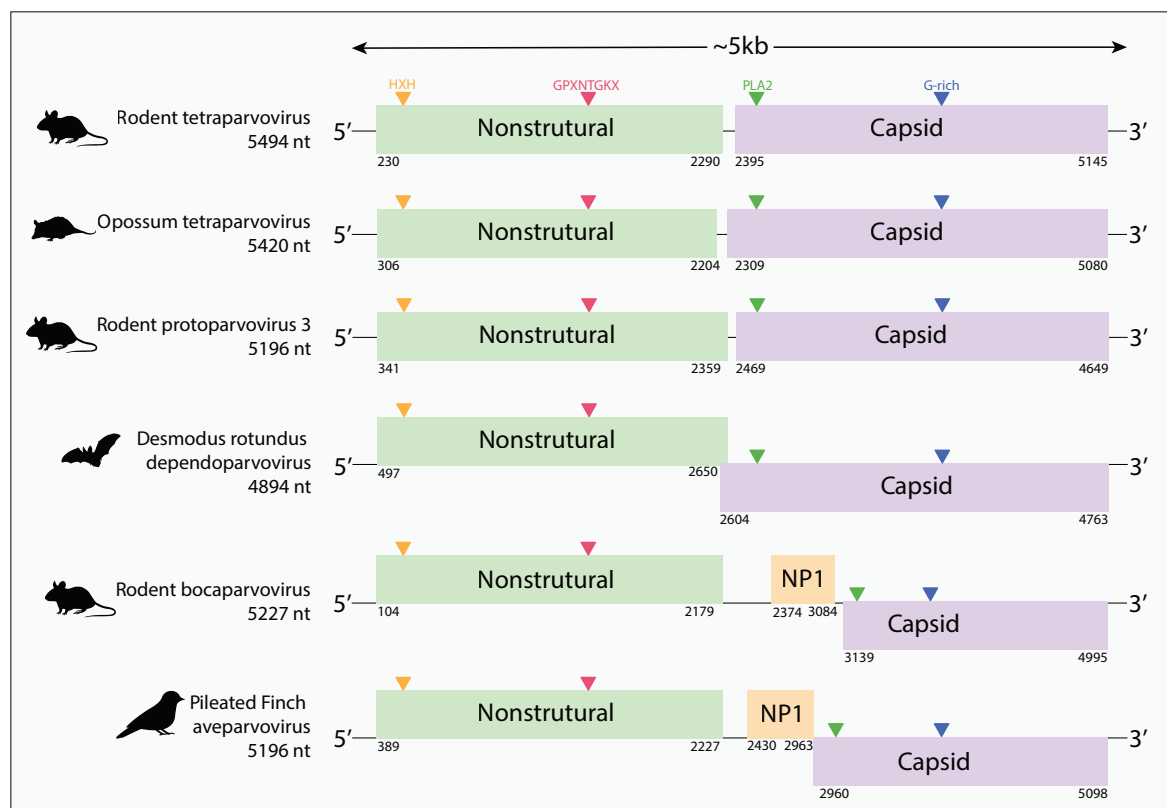
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149 We identified a novel species of protoparvovirus in sigmondontine rodents. This virus, which
150 was detected in samples from several distinct animal and species (**Table 1**), is quite similar to
151 minute virus of mice (MVM), but is sufficiently distinct based on ICTV criteria to be considered a
152 distinct species. We also identified novel tetraparvoviruses in the opossum and hairy-tailed bolo
153 mouse, and a novel dependoparvovirus in tissue samples derived from common vampire bats
154 (*Desmodus rotundus*). We identified a novel bocaparvovirus species - rodent bocaparvovirus - in two
155 distinct sample pools obtained from hairy-tailed bolo mice, and a novel aveparvovirus in grey
156 pileated finch in São José do Egito, Pernambuco State, Brazil. We also identified strains of two
157 parvoviruses that previously detected in cattle, bovine parvovirus 3 and ungulate tetraparvovirus 1,
158 identified in cattle serum of Ronda Alta in the Rio Grande do Sul State and Manoel Ribas in Paraná
159 State, respectively, both located in South of Brazil.

159

160 All the viruses identified in our study have typical parvovirus genome structures encoding NS
161 and VP proteins. The deduced NS protein sequences from these viruses contains the "HxH" and
162 "GPASTGKS" motifs, which play a critical role in viral DNA replication [20]. Most of the capsid
163 proteins also possess the PLA₂ motif involved in particle release [21], and the glycine-rich (G-rich)
164 region required for cellular entry [22]. Interestingly, we observed that one species, *Desmodus*
165 *rotundus* dependoparvovirus encodes NS and VP as overlapping ORFs, with a shared region of 47
166 nucleotides (**Figure 3**).

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Figure 3. Genome structures of newly identified parvoviruses. The length of the determined nucleotide sequences of the viral sequences are shown in the left. Boxes indicate the open reading frames (ORFs), and the number represent their respective position of ORFs.

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Notably, the rodent bocaparvoviruses and pileated finch 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].

180 4. Discussion

181 Brazil has a great diversity and abundance of wildlife, and is considered a hotspot for the
182 potential emergence of novel zoonotic viruses [23]. However, parvovirus studies in Brazil have
183 focused predominantly on canine parvovirus and human parvovirus B19 [2,24]. In this study, we
184 used a HTS approach to investigate parvovirus infections among wild mammals and birds
185 apparently without symptoms or disease from Brazil. We identified 21 parvovirus sequences,
186 representing six novel and two previously described parvovirus species. We report the first
187 examples of parvoviruses in samples derived from *Sigmondontinae* rodents, opossums and New
188 World bats. Interestingly, all of the viruses detected here were sequenced from serum or blood
189 samples suggesting that viremia may have been a factor in their identification.

190 We detected strains of ungulate tetraparvovirus – a virus in the genus *Tetraparvovirus* - in cattle
191 from the South of Brazil. Ungulate tetraparvovirus 2 (formerly known as porcine hokovirus) has
192 previously been identified in swine in Brazil [29]. However, ungulate tetraparvovirus 1 (formerly
193 known as bovine hokovirus) has not previously been reported outside Asia. This virus, which was
194 originally identified in bovine spleen samples obtained from food markets in Hong Kong, has also
195 been identified in domestic yaks (*Bos grunniens*) in northwestern China [18,19]. The identification of
196 this virus in an entirely distinct population (Brazilian cattle) not only establishes that it occurs
197 outside Asia, but also suggests it may be present in cattle populations throughout the world. In
198 addition, we identified novel species of tetraparvovirus in samples obtained from rodents, and
199 from an opossum. Interestingly, the opossum sequence grouped basal relative to the largest
200 *Tetraparvovirus* clade, which contains isolates from diverse eutherian mammals. Further sampling
201 may reveal if this basal position reflects the broad co-divergence of tetraparvoviruses and mammals
202 dating back to the common ancestor of marsupials and eutherians. Such ancient origins of the
203 *Tetraparvovirus* genus are consistent with evidence from endogenous viral element (EVE) sequences
204 that parvoviruses have been infecting mammals for millions of years [30,31].

205 Recently, studies have reported numerous novel dependoparvoviruses in samples derived
206 from Asian bats [32,33]. Here, we provide the first report of a dependoparvovirus in a New World
207 bat - the vampire bat (*Desmodus rotundus*). In trees based on Rep, this virus groups basally within
208 the *Dependoparvovirus* genus, consistent with these viruses potentially having an ancestral origin in
209 bats, as has been proposed previously [32].

210 Currently, only one species is recognised in the genus *Aveparvovirus*. This virus (*Galliform*
211 *aveparvovirus 1*) infects chickens and turkeys and is widespread in poultry farms in the United
212 States and Europe [34,35]. We identified a novel *Aveparvovirus* species in samples derived from
213 pileated finch (*Coryphospingus pileatus*), an indigenous (and non-migratory) South American bird,
214 suggesting that viruses belonging to the *Aveparvovirus* genus may circulate widely among avian
215 species, including wild as well as domestic birds.

216 We detected bovine parvovirus 3 (genus *Erythroparvovirus*) in Brazilian cattle. Since this virus -
217 to the best of our knowledge – has only been described as a contaminant of commercial bovine
218 serum [36], our study is the first to report detection of bovine parvovirus 3 in cattle populations.

219 We also identified a novel protoparvovirus species infecting sigmodontine rodents in Brazil.
220 Sigmodontine rodent protoparvovirus was identified in several species of rodents (all belong to
221 subfamily) that we captured in the Ribeirão Preto region of São Paulo State. These viruses are

222 closely related to *Minute virus of mice* (MVM), a common pathogen of laboratory mice [37], but
223 following official taxonomic criteria, they are sufficiently divergent from MVM (>85% in NS and
224 >73% aa in VP) to be considered a distinct species within the *Protoparvovirus* genus.

225 Bocaparvoviruses are associated with pathogenic conditions in human, bovine and canine
226 hosts [2,38]. Rodent bocaparvoviruses have recently been reported [39], but relatively little is
227 known about their broader distribution. We identified novel rodent bocaparvoviruses in
228 sigmodontine rodents that are closely related to bocaparvoviruses recently reported in brown rats
229 (*Rattus rattus*) in China [39] (data not shown). Together, these findings suggest a broad distribution
230 for rodent bocaparvoviruses.

231 Parvoviruses that infect domestic and wild carnivores (including amdoviruses and
232 protoparvoviruses) have been studied fairly extensively in the field. These studies have shown that
233 groups of closely related parvoviruses circulate widely among species in the order Carnivora, with
234 the barriers to transmission between species within the order apparently being relatively low
235 [40-42]. The findings of our study suggests that this pattern might be reflected more broadly in
236 parvovirus ecology, with many parvovirus genera containing sublineages that circulate within
237 particular taxonomic groups of hosts (and are largely restricted to this host group). For example, the
238 phylogenetic relationships shown in Figure 1 indicate that closely related protoparvoviruses
239 circulate widely among rodents, and that closely related tetraparvoviruses circulate widely in
240 ungulates. With further sampling of parvovirus diversity it should quickly become apparent
241 whether these inferences are accurate.

242

243 5. Conclusions

244 In this study, we used a sequencing-based approach to characterize parvovirus infections in
245 wild and domestic animals in Brazil. Our findings extend the known biogeographic range of
246 previously characterized parvovirus species, and the known host range of three parvovirus genera
247 (*Dependovirus*, *Aveparvovirus*, and *Tetraparvovirus*). More broadly, our findings indicate that many
248 parvovirus genera contain well-defined sub-lineages that circulate widely throughout the world
249 within particular taxonomic groups of hosts.

250 **Supplementary Materials:** The following are available online at www.mdpi.com/link, Table S1: Samples
251 information, host, sources, sample type, location, date, reads, and %Bases \geq Q30. Figure S1: Heatmap of
252 pairwise amino acid identities of the NS protein of parvoviruses identified in this study and representative
253 members of *Parvovirinae* subfamily based on ICTV criteria. The viruses described in this study are highlighted
254 with bold, Figure S1: Heatmap of pairwise amino acid identities of the VP protein of parvoviruses identified in
255 this study and representative members of *Parvovirinae* subfamily based on ICTV criteria. The viruses described
256 in this study are highlighted with bold.

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269 Wesley Dennis and Robert James Gifford analyzed the data; Sejal Modha, Márcio Roberto Teixeira Nunes and
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275 **Conflicts of Interest:** The authors declare no conflict of interest.

276

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