Received: 20 January 2022 | Revised: 2 June 2022 | Accepted: 16 June 2022

DOI: 10.1111/tbed.14636

Transboundary and Emerging Diseases

WILEY

ORIGINAL ARTICLE

High genetic diversity of alphacoronaviruses in bat species (Mammalia: Chiroptera) from the Atlantic Forest in Brazil

Larissa Mayumi Bueno[1] | Laís Santos Rizotto[2] | Amanda de Oliveira Viana[3] | Laura Morais Nascimento Silva[2] | Maria Vitória dos Santos de Moraes[2] | Julia Cristina Benassi[1] | Guilherme Pereira Scagion[3] | Erick Gustavo Dorlass[3] | Bruna Larotonda Telezynski Lopes[3] | Irineu Noberto Cunha[4] | Ramiro Melinski[4] | Igor Ferreira de Alvarenga[4] | Gabriel Lins Leitão[4] | Roberta Costa Rodrigues[5] | Iolanda Maria da Silva Pereira[5] | Lilia D'ark Nunes dos Santos[5] | Fabiane Fisch[6] | Alana Drielle Rocha[6] | Dagoberto Port[7] | Gabriela Stahelin Pereira[6] | Alessandra Greatti[8] | Ana Caroline de Souza Barnabé[8] | Junko Tsukamoto[8] | Erika Hingst-Zaher[4] | Severino Mendes de Azevedo Jr.[5] | Wallace Rodrigues Telino Jr.[9] | Joaquim Olinto Branco[6] | Tatiana Ometto[3] | Jansen de Araujo[3] | Clarice Weis Arns[8] | Helena Lage Ferreira[1,2] | Edison Luiz Durigon[3]

[1]Department of Veterinary Medicine, FZEA-USP, University of Sao Paulo, Pirassununga, Brazil

[2]Graduate Program in Experimental Epidemiology Applied to Zoonoses, FMVZ-USP, University of São Paulo, São Paulo, Brazil

[3]Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil

[4]Biological Museum, Instituto Butantan, São Paulo, Brazil

[5]Department of Biology, Federal Rural University of Pernambuco, Recife, Brazil

[6]School of Sea, Science and Technology, University of Vale do Itajaí, Itajaí, Brazil

[7]Brusque Educational Foundation, Brusque, Brazil

[8]Department of Genetics, Evolution and Bioagents, Institute of Biology, University of Campinas, Campinas, Brazil

[9]Zoological Studies Laboratory, Federal University of Pernambuco Agreste, Garanhuns, Brazil

Correspondence
Helena Lage Ferreira, Department of Veterinary Medicine, FZEA-USP, University of Sao Paulo, 225 Av Duque de Caxias Norte, Pirassununga, SP, 13635-900, Brazil.
Email: hlage@usp.br

Funding information
Ministry of Science, Technology, and Innovation (MCTI-Brazil); National Council for Scientific and Technological Development, Grant/Award Numbers: 403761/2020-4, 400172/2022-4; Brazilian Post and Telegraph Company (Correios)

Abstract

Bat coronaviruses (Bat-CoVs) represent around 35% of all virus genomes described in bats. Brazil has one of the highest mammal species diversity, with 181 species of bats described so far. However, few Bat-CoV surveillance programmes were carried out in the country. Thus, our aim was to jevaluate the Bat-CoV diversity in the Atlantic Forest, the second biome with the highest number of bat species in Brazil. We analysed 456 oral and rectal swabs and 22 tissue samples from Atlantic Forest bats, detecting *Alphacoronavirus* in 44 swab samples (9.6%) targeting the RdRp gene from seven different bat species, three of which have never been described as Bat-CoV hosts. Phylogenetic

Larissa Mayumi Bueno, Laís Santos Rizotto, and Amanda de Oliveira Viana contributed equally to this work.

analysis of the amino acid (aa) sequences coding the RdRp gene grouped the sequences obtained in our study with Bat-CoV previously detected in identical or congeneric bat species, belonging to four subgenera, with high aa identity (over 90%). The RdRp gene was also detected in three tissue samples from *Diphylla ecaudata* and *Sturnira lilium*, and the partial S gene was successfully sequenced in five tissues and swab samples of *D. ecaudata*. The phylogenetic analysis based on the partial S gene obtained here grouped the sequence of *D. ecaudata* with CoV from *Desmodus rotundus* previously detected in Peru and Brazil, belonging to the Amalacovirus subgenus, with aa identity ranging from 73.6% to 88.8%. Our data reinforce the wide distribution of Coronaviruses in bats from Brazil and the novelty of three bats species as Bat-CoV hosts and the co-circulation of four *Alphacoronavirus* subgenera in Brazil.

KEYWORDS
coronavirus, RdRg gene, S gene, virus surveillance

1 | INTRODUCTION

Coronaviruses (CoV) are enveloped viruses with a positive-sense single-stranded RNA genome with the size of 26–32 kb, belonging to the *Coronaviridae* family and *Orthocoronavirinae* subfamily (Banerjee et al., 2019), which is divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The former two infect mainly humans and other mammals, while the latter two can be found mainly in birds and in two mammal species (Barbosa et al., 2019; Mihindukulasuriya et al., 2008).

Bats have been the main spotlight in several studies among mammals infected by alpha and beta CoV (Banerjee et al., 2019). Coronaviruses represent about 35% of all virus genomes described in bats (Letko et al., 2020), with *Alphacoronavirus* genus the most detected and widespread in these mammals (Wong et al., 2019).

Most studies on bat coronavirus (Bat-CoV) surveillance were carried out in Old World bats (Ruiz-Aravena et al., 2021), mainly after SARS was related to a bat species in Asia (Lau et al., 2005). Data about the diversity of viruses from the *Orthocoronavirinae* subfamily in the New World, especially Neotropical bats, remain limited. Alphacoronaviruses seem widespread worldwide (Ruiz-Aravena et al., 2021), corresponding to the 89.4% of coronavirus sequences detected in 43 different bat species from the New World (Hernández-Aguilar et al., 2021).

The CoV detection rate during surveillance studies had the highest values in Old World bats (from 1.4 to 50) (Fischer et al., 2016; Shirato et al., 2011), but still up to 30% in New World bats (Anthony et al., 2013; Moreira-Soto et al., 2015; Subudhi et al., 2017), with the high detection rate values usually related to viral surveillance by sampling bats within the same bat colony (Ge et al., 2016). Sequences from Bat-CoV detected in bats from both Old and New World have a high identity when comparing congeneric bat species (Anthony et al., 2013; Latinne et al., 2020).

Brazil has one of the highest mammal species diversity, with 181 bat species described so far (Abreu et al., 2021), and with 123 of them occurring in the Atlantic Forest (Hingst-Zaher & Brandão, 2021). The high abundance of bat species found in the Atlantic Forest and the high CoV abundance found in bats, combined with the native forest loss that has been occurring in this biome, make it a hotspot for zoonotic spillover risk (Góes et al., 2016; Jones et al., 2013; Rosa et al., 2021; Ruiz-Aravena et al., 2021).

Few Bat-CoV surveillance studies were carried out in Brazil, which accessed three different biomes (Amazonian, Atlantic Forest, and Tropical savanna/Cerrado) and different habitats (preserved wild areas, rural, peri-urban, and urban environments), and most of these studies investigated CoV in faeces/enteric content (Asano et al., 2016; Brandão et al., 2008; Corman et al., 2013; Lima et al., 2013) and tissues (mainly intestine) (Alves et al., 2022; Bittar et al., 2019; Corman et al., 2013; Góes et al., 2013, 2016), while only two studies analysed oral/rectal swab samples (Anthony et al., 2017; Barnabé et al., 2015).

In Brazil, Bat-CoV were described in 22 different bat species belonging to Phyllostomidae (11 species), Molossidae (eight species), and Vespertilionidae (three species), with diets varying from omnivorous, frugivorous, nectarivorous, insectivorous, and the hematophagous species *Desmodus rotundus*, the Common Vampire bat (Alves et al., 2022; Asano et al., 2016; Brandão et al., 2008; Hernández-Aguilar et al., 2021). Similar to other places, most Bat-CoV sequences found in Brazil belong to the *Alphacoronavirus* genus, and only three sequences of *Betacoronavirus* genus were detected in three different bat species, *D. rotundus*, *Artibeus lituratus* (the Great fruit-eating bat), and *Eumops glaucinus* (the Wagner's bonneted bat) (Brandão et al., 2008; Góes et al., 2016).

The present study aimed to evaluate the Bat-CoV diversity in the Atlantic Forest, the second biome in a number of bat species, by sampling localities at the Northeast, Southeast, and South of Brazil.

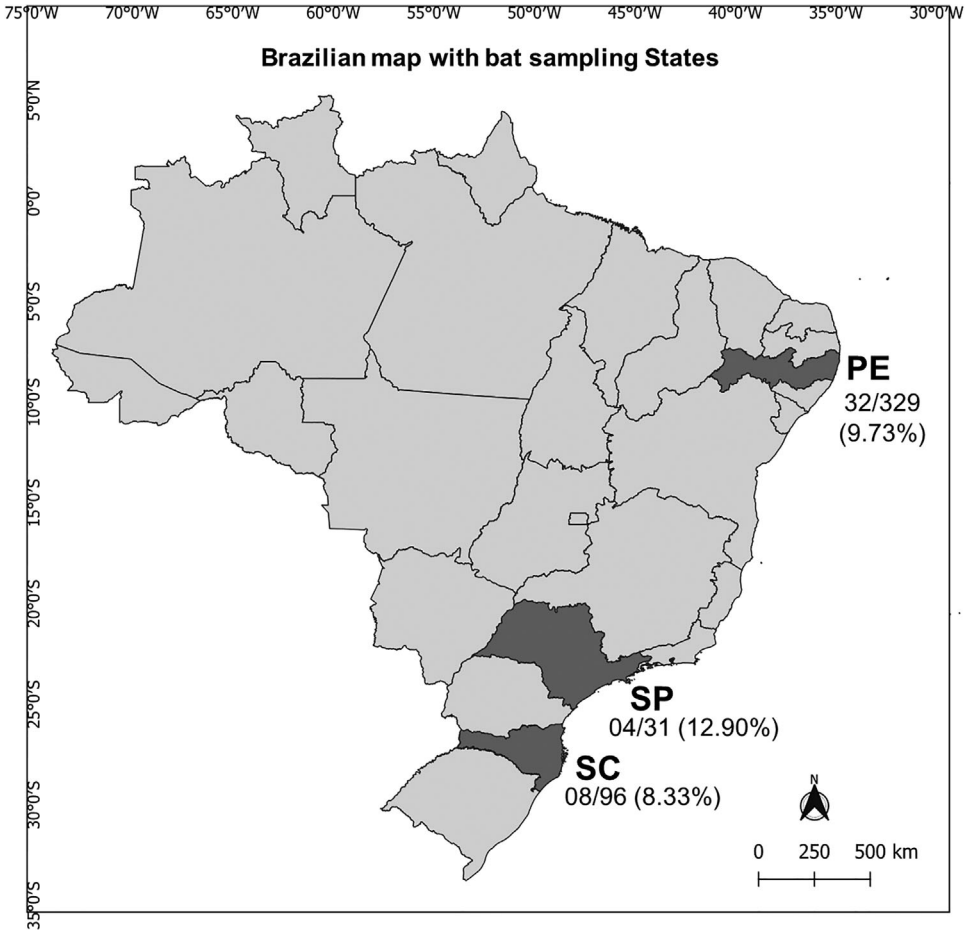

FIGURE 1 Geographic map showing the Brazilian States of bat captures and sampling (dark grey), with the number of positive samples, total samples analysed, and the detection rate for each state: Pernambuco (PE) with 32 positive samples and 329 analysed samples, and detection rate of 9.7%; São Paulo (SP) with 27 four positive samples and 31 analysed samples, and detection rate of 12.9%; Santa Catarina (SC) with eight positive samples 96 analysed samples, and detection rate of 8.3%.

The Bat-CoVs and the bat species diversity within each region were also accessed. Therefore, this study can contribute to the Bat-CoV epidemiology in Neotropical bats.

2 | METHODOLOGY

2.1 | Ethics statement and licensing

All the sample collections were authorized by the Brazilian Institute of Environment and Natural Renewable Resources (SISBIO/IBAMA: 76621-1, 12475-3, 10698-3, 76814-1, 11597-4) and by the Ethics Committee on the Use of Animals of the University of São Paulo (CEUA/FZEA-USP: 1128141120).

2.2 | Sites of capture and sampling

Bats were captured in three different sampling localities in the Atlantic Forest biome in Brazil (Figure 1) between November/2020 and June/2021, using mist nets (Figure 2). In the Northeast region, bats were captured in the city of Aliança, State of Pernambuco (PE), which has remnants of sub-deciduous and deciduous forests. In the southeast region, the captures were made in the city of São Lourenço da Serra in the State of São Paulo (SP). In the south region, bats were captured in the cities of Botuverá and Itajaí in the State of Santa Catarina (SC). In the southeast and south regions, bats were captured in Dense Ombrophilous Forest. The sampling effort for bat captures varied between the northeast, southeast, and south regions, totalizing 68,040, 29,160, and 30,195 net-h/m^2 of sample effort, consecutively.

The animals were identified to the species level according to morphological characteristics following the literature (Reis et al., 2017). For each specimen, ecological (sex, weight, age, and reproductive status) and morphometric (body measures) data and biological samples (oral and rectal swabs) were collected (Figure 2). For five specimens, we also collected 22 tissue samples (lung, kidney, liver, intestines, heart, placenta, and foetus). Swab and tissue samples were immersed in Viral Transport Medium (VTM), transported in dry ice, and stored at −80°C before use.

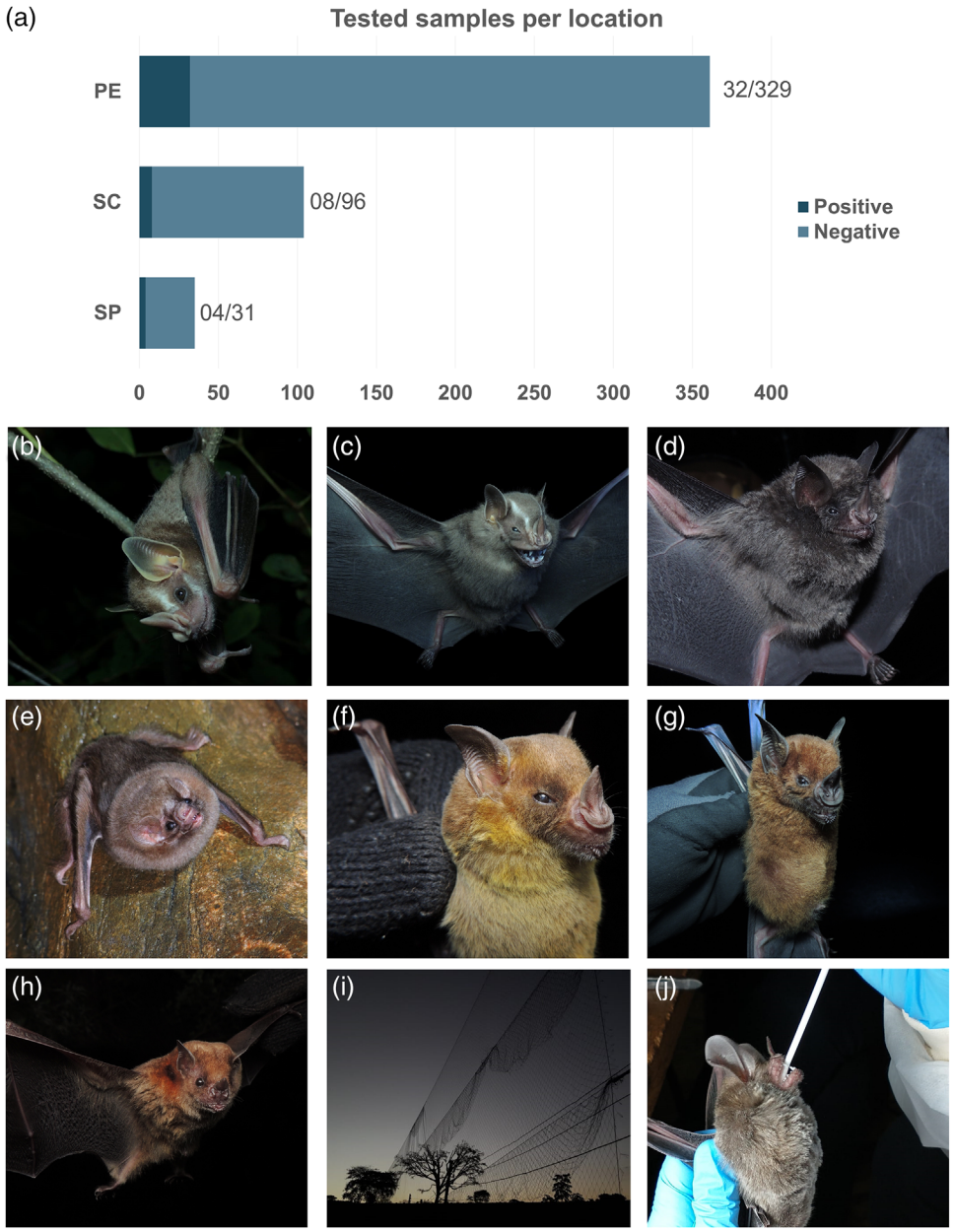

FIGURE 2 Active surveillance was carried out in Atlantic Forest for eight months from 2020 to 2021. (a) The number of positive and negative samples per location: Pernambuco (PE) with 293 negative and 32 positive samples (total: 329 samples); São Paulo (SP) with 27 negative and four positive samples (total: 31 samples); and Santa Catarina (SC) with 88 negative and eight positive samples (total: 96 samples). Bat-CoV was detected in seven bat species, including three new Bat-CoV hosts (*): (b) *Artibeus cinereus* (*), (c) *Artibeus lituratus*, (d) *Carollia perspicillata*, (e) *Diphylla ecaudata* (*), (f) *Phyllostomus discolor*, (g) *Phyllostomus hastatus* (*), and (h) *Sturnira lilium*. (i) Mist-net used for the bat capture. (j) *Trachops cirrhosus* was sampled with an oral swab. Pictures were taken by Irineu Cunha.

2.3 | Samples processing, virus detection, and sequencing

In total, 456 animals were captured and their oral and rectal swabs were pooled for molecular analysis. The captured animals were classified into 21 different bats species from Aliança city in PE (329 individuals), São Lourenço da Serra in SP (31 individuals), and Botuverá and Itajaí cities in SC (96 individuals) (Table S1), belonging to the *Phyllostomidae* (17 species) and *Vespertilionidae* (seven species) families. We also analysed 22 tissue samples (heart, kidney, liver, lungs, and intestine) from five animals (one *D. ecaudata* and four *S. lilium*), and placenta and foetus were available for one animal (*S. lilium*).

The nucleic acids from the pools of oral and rectal swabs or tissue samples from each individual were purified using the MagMAX Core Nucleic Acid Isolation Kit with the equipment KingFisher Flex 96 (Thermo Fisher Scientific). The nucleic acid was quantified by DeNovix Spectrophotometer DS-11 and stored at −80°C. The cDNA

TABLE 1 Sequences of primers designed using Primal Scheme tool targeting the entire sequence of the S-gene of bat coronavirus

	Forward (5′–3′)	Reverse (5′–3′)
BatCoV_S_1	GGTTGTGCGTGTTTATGGCATT	GTCTGCACTACGGCTGTTGTAG
BatCoV_S_2[a]	GTGCATGTTTTTAGAGTGCCTGG	GGCCAAACATGTAGCCATTGAG
BatCoV_S_2_RD[b]	GGATCGCAGTGTGCATGTTT	GCCTGCATTATCCTGCGTTG
BatCoV_S_3	ACCTGAGTGTTGAGAATGCCAC	TGGCTGTATTTGTGAGATCTGCA
BatCoV_S_4	ACCTTACGTTTGACTCTATATGCTTCT	ACTAACTGGTGTATTTGCAATCTCC
BatCoV_S_5	GCTATACAGCCTATTTCCACGGG	AAGCTGCCAATTGCTTCTGGTT
BatCoV_S_6	ATGGTTGGTGGTGCCTTTACAG	TCAAACATGGTACGAGGTGTAAGA
BatCoV_S_7	CGGCTCCTAATGGTTTCCTGTT	AGTGTAGTAAGGTAACCGTTTGCT

[a]The initially designed primers based on the MT663548 sequence could not amplify the detected virus.
[b]New primers were designed based on the obtained sequence by Sanger Sequencing.

synthesis was performed using the SuperScript III enzyme (Thermo Fisher Scientific, EUA).

We tested the presence of viruses from the *Orthocoronavirinae* subfamily using a pan-Coronavirus nested-PCR assay targeting the nsp12 genomic region of the RNA-dependent RNA polymerase gene (RdRP) (Chu et al., 2011). We also designed specific primers based on a complete sequence of a *D. rotundus* Bat-CoV (GenBank MT663548, reference sequence of the subgenus *Amalacovirus*), using the web-based primer design tool Primal Scheme (Quick et al., 2017), to amplify the S gene. In total, we designed seven sets of primers targeting the whole S gene. One pair of the initially designed primers based on the MT663548 sequence could not amplify the detected virus. Therefore, new primers were designed based on the obtained sequence by Sanger Sequencing (Table 1). All the S gene reactions were done using the Taq Platinum enzyme (Thermo Fisher Scientific) and were performed following 40 cycles of 94°C for 20 s, 55°C/56°C for 30 s, and 72°C for 60 s, with band sizes of 700 bp in a 1.5% agarose gel.

Positive samples were purified using the BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific) and sequenced by the Sanger method with the 3730 XL DNA Analyzer (Applied Biosystems) using the set of primers from the nested-PCR reactions.

2.4 | Phylogenetic and statistical analysis

The phylogenetic analyses of the virus sequences were performed based on the amino acid (aa) sequences coded by the RdRp and S genes, which were aligned using ClustalW by MEGA7 (Kumar et al., 2016). The best fit models for aa substitution were inferred using ModelTest-NG. The Maximum Likelihood analyses were also done using MEGA7, and the Bayesian Markov Chain Monte Carlo (MCMC) analyses were done using BEAST v.1.10.4 (Suchard et al., 2018). The phylogenetic trees were summarized using Tree Annotator and edited in FigTree v1.4.4. Evolutionary distances were estimated between the aa sequences obtained in this study, and the most closely related sequences available in GenBank applying the Maximum Composite Likelihood model (Tamura et al., 2004) and JTT matrix-based model with 1000 bootstrap replicates using MEGA7 (Kumar et al., 2016).

For the RdRp aa analyses, a data set was composed of 38 available GenBank sequences representing *Alpha* and *Beta* genera, and 44 sequences obtained in this study. The Maximum Likelihood phylogenetic trees were constructed with 1000 replications on the bootstrap and the Le Gascuel model and gamma distribution (LG + G). The Bayesian analyses were performed using the Whelan and Goldman model plus gamma distribution (WAG + G), under a lognormal relaxed clock and constant population size, with the MCMC chain, running for 10,000,000 steps sampling every 5000 steps.

For the S gene analyses, a data set was composed of one sequence obtained in this study and 14 sequences from *Alpha* and *Beta* genera. The Maximum Likelihood phylogenetic trees were constructed with a total of 1000 replications on the bootstrap, using the Le Gascuel model with a gamma distribution (LG + G). The Bayesian analyses were performed using the Whelan and Goldman model with a gamma distribution (WAG + G), under a lognormal relaxed clock and constant population size, with the MCMC chain, running for 10,000,000 steps sampling every 5000 steps.

A statistical analysis based on chi-squared test was performed to compare the Bat-CoV detection in young and adult bat individuals.

2.5 | GenBank numbers

The obtained sequences for the viruses were submitted to GenBank and are available under the accession numbers OM265165–OM265209 (Table S4).

2.6 | Maps and graphic design

The maps representing the sites of bat captures were designed using the software QGIS 3.2 with maps of Brazilian states provided by the Brazilian Institute of Geography and Statistics (IBGE) (IBGE, 2022), and graphics were designed using Tableau software 2020.1. The animal draws used to identify the origin of sequences in the phylogenetic trees (Figures 3–6) were obtained in Canva (www.canva.com).

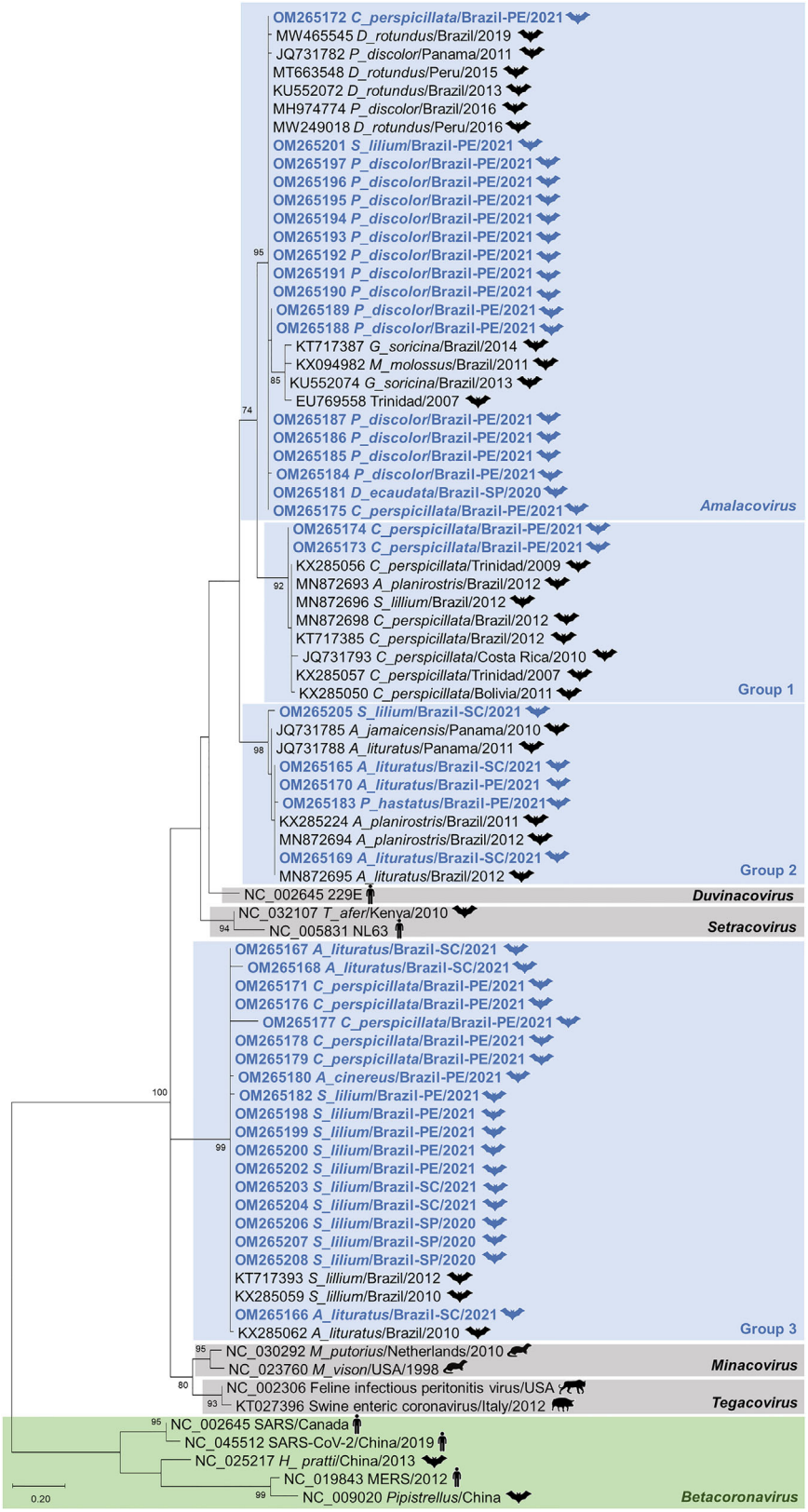

FIGURE 3 Phylogenetic tree of the Coronavirus RdRp genomic region using the Maximum Likelihood method based on the Le Gascuel model with a discrete gamma distribution, with a total of 108 amino acid positions in the final data set, based on 44 sequences from this study and 38 representative sequences of *Alpha* and *Beta* coronavirus genera, and subgenera within *Alpha* genus available in GenBank, represented by different background colours. The nodes show bootstrap values higher than 60. Images beside the tips of the tree are a graphic representation of the host. Sequences detected in this study are highlighted in blue.

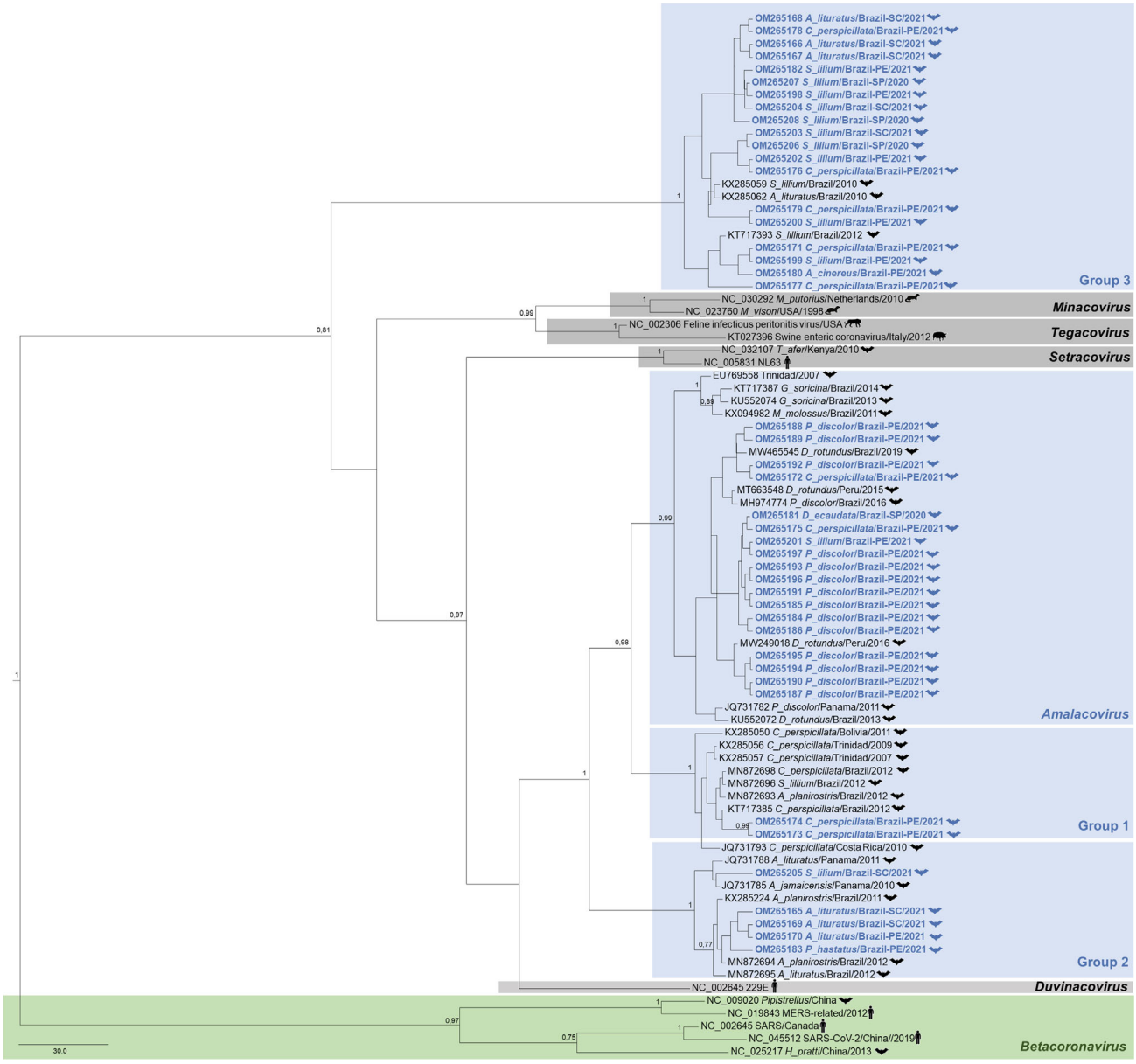

FIGURE 4 Phylogenetic tree of the Coronavirus RdRp genomic region using the Bayesian method based on 44 sequences from this study and 38 representative sequences of *Alphacoronavirus* and *Betacoronavirus* genera, and subgenera within *Alphacoronavirus* genus available in GenBank, represented by different background colours. The nodes show posterior values higher than 0.7. Images beside the tips of the tree are a graphic representation of the host. The *Alphacoronavirus* subgenera and the sequences detected in this study are highlighted in blue.

3 | RESULTS

3.1 | Virus detection based on RdRp gene

From the 456 pools of oral and rectal swabs analysed, 44 samples were positive for CoVs targeting the RdRp gene, belonging to seven different species of the Phyllostomidae family: *Artibeus lituratus* ($n = 6$), *Carollia perspicillata* ($n = 9$), *Artibeus cinereus* ($n = 1$), *Diphylla ecaudata* ($n = 1$), *Phyllostomus discolor* ($n = 14$), *Phyllostomus hastatus* ($n = 1$), and *Sturnira lilium* ($n = 12$).

The rate of detection varied according to the Brazilian region, with the highest rate in São Paulo State (12.9%), followed by Pernambuco (9.7%) and Santa Catarina (8.3%) (Figure 2), with an average of 9.6%. The detection rate in juvenile bats was 1.7-fold higher than in adults, with nine (15%) out of 60 juvenile bats positive for Bat-CoV, while 35 (8.8%) adults of 393 tested were positive for Bat-CoV. However, the statistical analysis did not significantly differ between young and adult bats ($p = 0.11$) in Bat-CoV detection.

We also tested 22 tissue samples from bats collected in São Paulo State, in which the intestine and heart from *D. ecaudata* and the intestine of one *S. lilium* were positive.

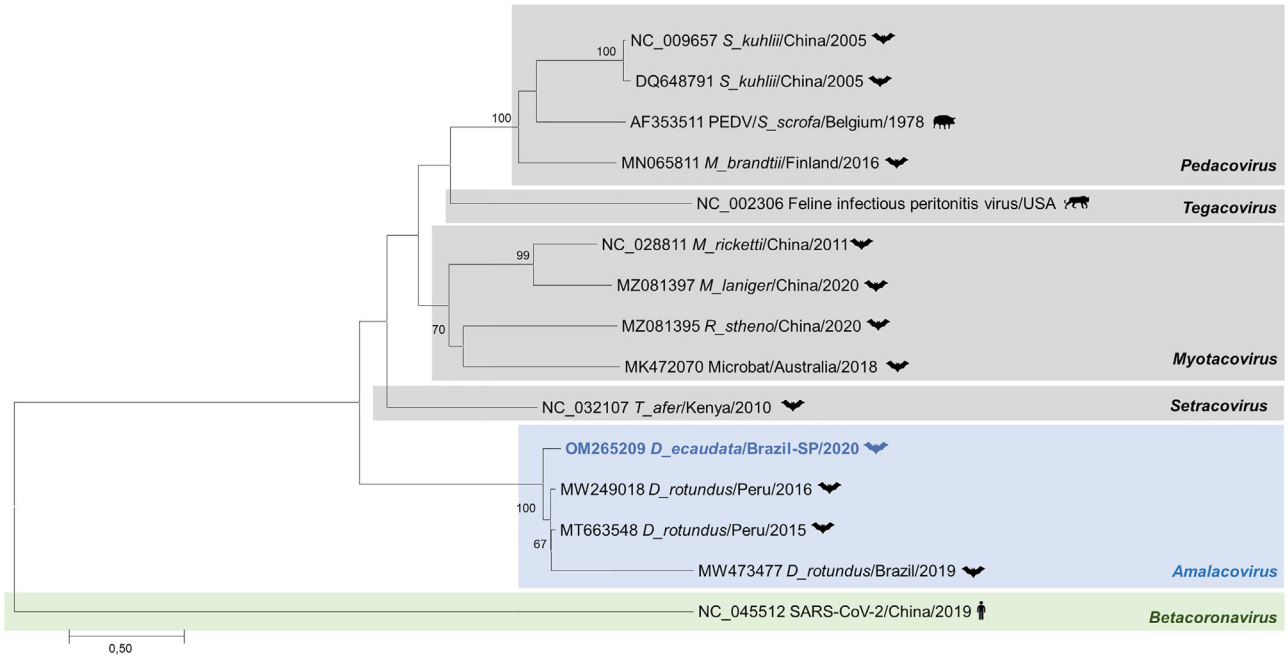

FIGURE 5 Phylogenetic analysis of the Coronavirus S-gene using the Maximum Likelihood method based on the Le Gascuel model with a discrete gamma distribution, with a total of 1627 positions in the final data set, based on a data set of 15 amino acid sequences, which includes one sequence from this study and 14 sequences representatives of *Alphacoronavirus* and *Betacoronavirus* genera, and subgenera within *Alphacoronavirus* genus available in GenBank, represented by different background colours. The nodes show bootstrap values higher than 60. Images beside the tips of the tree are a graphic representation of the host. The sequence detected in this study is highlighted in blue. The *Alphacoronavirus* subgenera and the sequences detected in this study are highlighted in blue.

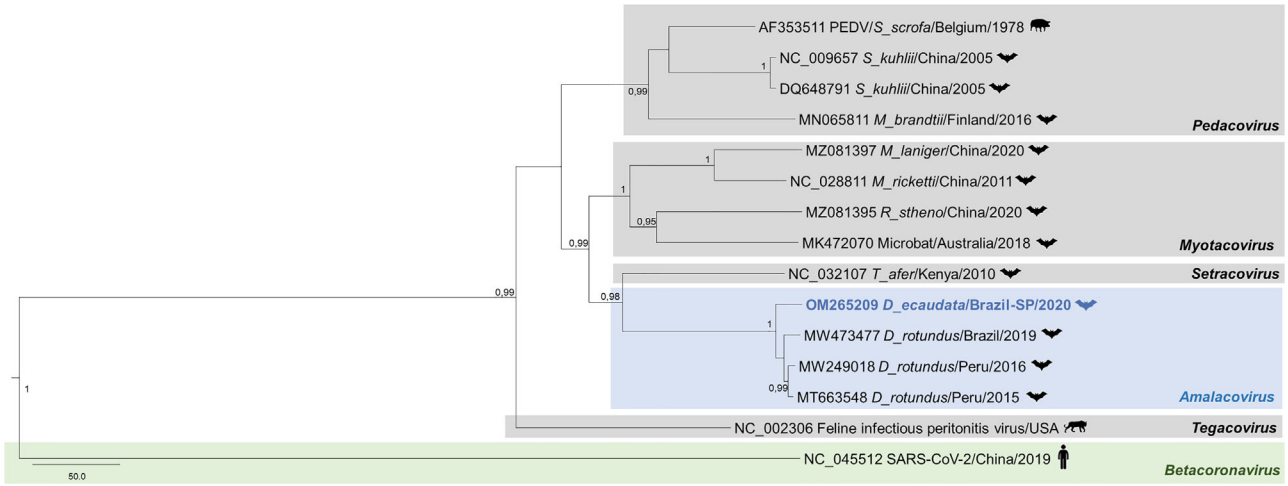

FIGURE 6 Phylogenetic tree of the Coronavirus S-gene using the Bayesian method based on one sequence from this study and 14 representative sequences of *Alpha* and *Beta* coronavirus genera, and subgenera within *Alpha* genus available in GenBank, represented by different background colours. The nodes show posterior values higher than 0.7. Images beside the tips of the tree are a graphic representation of the host. The sequence detected in this study is highlighted in blue.

3.2 | Phylogenetic analysis by coding sequence of the RdRp gene

In both Maximum Likelihood and Bayesian analyses, all 44 aa sequences formed a monophyletic group within the *Alphacoronavirus* genus, with robust bootstrap (100) and posterior probability values (0.81) (Figures 3 and 4). Both analyses showed a similar topology. We could observe that the Bat-CoV sequences grouped accordingly to the bat hosts, that is, sequences from the same bat species or genus were grouped in monophyletic branches, with a few exceptions. All pairwise aa identity values are summarized in Table S2. Sequences of alphacoronaviruses could be divided into genetic groups based on the partial

RdRp gene using a cut-off of aa identities below 90%. Our sequences belonged into four subgenus/groups, including the subgenus *Amalacovirus*. The other three subgenera were named here as groups 1–3, as our Bat-CoVs sequences were clustered with previously reported sequences that remained unclassified by ICTV.

All sequences from *P. discolor* ($n = 14$) CoV collected in PE grouped with CoV sequences of subgenus *Amalacovirus*, which have sequences identified in the same bat species previously described in different locations, such as Panama and in Southeast Brazil from 2011 and 2016, (JQ731782 and MH974774), with pairwise aa identities ranging from 99.1% to 100%. *Amalacovirus* subgenus also has the Bat-CoV sequences previously detected in the hematophagous bat species *D. rotundus* clustered with the CoV sequence from *D. ecaudata*, another hematophagous bat species. Pairwise aa identities of *D. ecaudata* Bat-CoV sequence ranged from 99.3% to 100% compared to *D. rotundus* CoV sequences from Peru and Brazil from 2013 to 2019 (MT663548, MW249018, KU552072, and MW465545).

The first new proposed subgenus (Group 1) had Bat-CoV sequences from *C. perspicillata* and from other bat species (*S. lilium*, and *Artibeus planirostris*). Two CoV sequences out of nine obtained from *C. perspicillata*, collected in PE, grouped with six CoV sequences previously described in the same species, captured in Trinidad, Costa Rica, and Brazil from 2009 to 2012 (KX285056, JQ731793, KX285057, KX285050, KT717385, and MN872698), with a pairwise aa identity varying from 97.6% to 99.2%.

Bat-CoV sequences from *P. hastatus* were clustered into Group 2 with CoV sequences from species of *Artibeus* genus, although they were collected in different capture campaigns and regions. Pairwise aa identities of the CoV sequences from *A. lituratus* and *P. hastatus* varied from 98.2% to 100% when compared to sequences previously detected in 2010–2012 in different *Artibeus* species (*A. lituratus*, *A. jamaicensis*, and *A. planirostris*) from Brazil and Panama (MN872695, MN872694, KX285224, JQ731785, and JQ731788).

Group 3 had 10 out of 12 obtained Bat-CoV sequences from *S. lilium*, which were collected in the three regions of Brazil grouped with other Bat-CoV sequences previously detected in the same species in Southeast Brazil captured in 2010 and 2012 (KX285059 and KT717393), with pairwise aa identity ranging from 98.8% to 100%. Also, the sequences obtained from *S. lilium* from the three regions showed variation in the pairwise aa identity, varying from 72.1% to 100%. The group 3 also had Bat-CoV sequences from the genus *Artibeus*. The Bat-CoV sequence obtained from *A. cinereus* captured in PE grouped close to three other sequences from the congeneric species *A. lituratus* that were captured in another state (SC), with pairwise identity varying from 97.3 to 98.1%. The CoV sequences obtained from *A. lituratus* from SC and PE were also classified into Group 3, with an aa identity varying from 65.2 to 68.4%.

3.3 | Virus detection based on the S gene

We selected samples from 18 animals that had Bat-CoVs classified into the subgenus *Amalacovirus* (*D. ecaudata*, *C. perspicillata*, *P. discolor*, and *S. lilium*) grouped with the MT663548 sequence in the phylogenetic analysis based on the aa sequences coding the RdRp gene. Selected samples were tested for the nested-PCR targeting the S gene using primers designed based on the MT663548 sequence. We tested tissue samples (heart, kidney, liver, lungs, and intestine) from *D. ecaudata* and four *S. lilium*, and placenta and foetus for one *S. lilium*, and we could amplify the S gene in all tested samples from *D. ecaudata*, but none from *S. lilium* samples. Also, from the 18 tested tissues, we could amplify the S gene in the *D. ecaudata* swab sample but none from the other species. We obtained the partial sequence of the S gene from the samples from *D. ecaudata*, using the primers designed with the Primal Scheme tool, which was deposited on GenBank (OM265209).

3.4 | Phylogenetic analysis based on the coding sequence of the S gene

In both Maximum Likelihood and Bayesian analyses (Figures 4 and 5), the aa sequence from the complete S gene obtained in *D. ecaudata* grouped within the *Alphacoronavirus* genus, subgenus *Amalacovirus*, with the other sequences from *D. rotundus* previously described. A high bootstrap (100) and posterior probability (0.99) values support the branch with Bat-CoV detected in Peru (MT663548 and MW249018) in 2015 and 2016, and Brazil (MW473477) in 2019. Pairwise identity of *D. ecaudata* was 73.6%, 88.8%, and 88.8% when compared to MW473477, MW249018, and MT663548, respectively. The Brazilian Bat-CoV (MW473477) detected in *D. rotundus* was grouped with Bat-CoV detected in the same species, but it had a low aa identity varying from 73.6% to 79.9% (Table S3).

4 | DISCUSSION

Since the outbreaks of the three most severe human pathogenic CoV—SARS-CoV, MERS, and SARS-CoV-2—bats have been the main target for coronavirus surveillance. They are referred to as natural reservoirs of coronaviruses (Banerjee et al., 2019). Surveillance of these mammals is critical to detect any virus with zoonotic potential for the preparedness of outbreaks (Morse et al., 2012).

Previous reports of Bat-CoV in Brazil (Alves et al., 2022; Asano et al., 2016; Brandão et al., 2008; Góes et al., 2013, 2016) were done with passive surveillance for rabies virus, that is, suspected animals, being dead or alive, found by health professionals or the population during daytime or at atypical locations are submitted to the municipalities' centre to control zoonotic diseases (Duarte et al., 2020). In our study, active surveillance, that is, sampling of healthy animals in the field aiming to detect disease cases (Hattendorf et al., 2017), was carried out in three Brazilian regions (Northeast, Southeast, and South) of the Atlantic Forest during 7 months from November 2020 to May 2021, with a detection rate average of 9.6%. The number of samples varied from each region, with the Northeast having more samples collected, followed by the South and Southeast, as reflected by the sampling effort for each region. All Bat-CoV sequences detected here belong

to *Alphacoronavirus* genus, grouping together with previously reported Bat-CoV sequences, which reiterates the high circulation of this Bat-CoV genus in bats (Ruiz-Aravena et al., 2021). We did not detect any *Betacoronavirus* circulating in our bat samples, showing that the *Betacoronavirus* diversity in New World bats might be lower than in Old World bats (Anthony et al., 2017).

A higher detection rate of Bat-CoV in juvenile bats was described in other studies (Osborne et al., 2011). Here, we also found a higher detection rate in juvenile bats, reinforcing the evidence that juvenile bats could be more susceptible to Bat-CoV than adults, probably due to a naïve immune response (Drexler et al., 2011; Gloza-Rausch et al., 2008), and they may play a major role in maintaining and spreading these viruses in a bat colony (Osborne et al., 2011). As some bat species maintain a maternity roost during the breeding season, maternity roosts are important to amplify the Bat-CoV in temperate zones (Drexler et al., 2011). This event is also observed with a Filoviridae virus, the Marburg virus, infecting the Old World bat species *Rousettus aegyptiacus*. During the birth season and, consequently, the high number of juvenile bats, an increase in the detection rate of infected juvenile bats is observed, which coincides with spillover events in humans (Amman et al., 2012).

The detection rate average of 9.6% in our study was comparable with some prior studies in Brazil, in which our detection rate was higher than three studies that varied from 2.7% to 3.7% (Asano et al., 2016; Corman et al., 2013; Góes et al., 2016). This could be explained by the type of samples analysed, as swab samples seem to be the most suitable type of sampling for Bat-CoV detection (Anthony et al., 2017). These previous studies analysed tissue samples collected from bats in active surveillance (Corman et al., 2013) and passive surveillance of rabies (Asano et al., 2016; Góes et al., 2016). Our samples were collected from bats, immediately conserved in dry ice during the fieldwork and transportation and stored in −80°C freezers once they arrived in the laboratory, which could have preserved the viral genome and, consequently, increased the Bat-CoV detection rate.

In some cases, a low detection rate correlates to a limited number of samples analysed (Anthony et al., 2017). However, the highest Bat-CoV prevalence in our samples from São Paulo State (12.9%) was not correlated to the highest number of samples analysed. We hypothesize that the positive individuals from *S. lilium*, representing 60% of positive samples at this location, belong to the same colony. They were captured for two consecutive days in the same area and approximately at the same time. Our detection rate was lower than that in two previous studies, which varied from 17.2% (Bittar et al., 2019) to 19.3% (Lima et al., 2013). However, these studies sampled two bat species from the same maternity roost (Lima et al., 2013), or the same site in São José do Rio Preto, a city in the northwest of São Paulo State (Bittar et al., 2019).

In our phylogenetic analysis, in the group 1, Bat-CoV sequences detected from different bat species, for example Bat-CoV sequences found in *P. hastatus*, an omnivorous bat, were closely related to the frugivorous genus *Artibeus*, and not with sequences from the congeneric species *P. discolor*. Similar conditions were observed for the Bat-CoV sequence obtained from *A. cinereus*, which were closely related to Bat-CoV sequences obtained from *C. perspicillata* and *S. lilium in the group 3*, all three being frugivorous species. Different species of bats can cohabitate within the same colony, even species from other bat families (Costa et al., 2010, 2015). This cohabitation between other bat species can also be a factor that facilitates the spread of pathogens (Nziza et al., 2020).

The co-evolution between bats and Bat-CoVs has been proven with significant statistics support of a co-evolution relationship (Liang et al., 2021). The origin of coronaviruses dates around 325 million years ago, and they may have been infecting bats since their origin, dating from 50 million years ago (Teeling et al., 2005; Wertheim et al., 2013). Demarcation criteria for the family members of *Coronaviridae* are based on the aa identity in the conserved replicase domains, such as RdRp gene. Sequences with greater than 90% aa sequence identity are considered to be the same species (de Groot et al., 2011). Bat-CoVs identified in our study were split into four subgenera based on the conserved RdRp aa sequences. With a few exceptions, most of the Bat-CoV sequences from the same bat species belonged to the same virus subgenus. Bat-CoV sequences from *S. lilium* collected in three locations belonged to the same subgenus (Group 3) based on the RdRp analysis. We selected samples from four bat species classified into the subgenus *Amalacovirus* to sequence the S gene. The S gene was designed based on the reference sequence of subgenus from a virus detected in *D rotundus*. In our study, no sample was amplified, except for the sample from *D. ecaudata*, which belongs to the same bat subfamily (*Desmodontinae*) of *D. rotundus*. Phylogenetic analysis based on the complete S gene sequence split them into different branches, with aa identities below 90% compared to sequences from other species *D. rotundus*. The data suggest a new Bat-CoV species and reinforce the co-evolution among bats and Bat-CoVs, although there is a lack of sequences to confirm this hypothesis. The genetic diversity of the S gene sequences would be essential to distinguish the Bat-CoVs when more data are available from more studies. Virus sharing among bat species within the same genus or between closely related bat species found in our study is similar to that previously described in Latin America (Anthony et al., 2017). The Bat-CoV sequences obtained in different bat species were grouped with identical or congeneric species. In general, Bat-CoVs seem to be host specific, suggesting a co-evolution between bats and coronaviruses (Góes et al., 2016; Osborne et al., 2011).

The S gene of the Bat-CoV detected in *D. ecaudata* was successfully amplified in all tested tissue and swab samples from this species. The virus was detected in oral/rectal swabs, kidneys, lungs, intestine, heart, and liver, suggesting a systemic replication. The Bat-CoV sequences from *D. ecaudata* and *D. rotundus*, both hematophagous bats, were closely related when comparing the RdRp, but a lower nucleotide identity was observed when analysing the S gene. The RdRp gene of Bat-CoV was detected in the intestine of *S. lilium* and two tissues of the *D. ecaudata*, which could be due to the low sensitivity of the RdRp nested-PCR and higher viral loads in the intestine.

The novelty of our study is the discovery of Bat-CoV in three new Neotropical bat species (*D. ecaudata*, *P. hastatus*, and *A. cinereus*) (Hernández-Aguilar et al., 2021). Additionally, we identified the co-circulation of four *Alphacoronavirus* subgenera in Brazil. The Bat-CoV detection in tissue samples suggests variable pathogenesis depending

on the bat species and viruses, which should be better investigated. Our data also reinforce the susceptibility of juvenile bats to coronavirus infection due to the naïve immune responses, as observed in other studies (Drexler et al., 2011; Gloza-Rausch et al., 2008). Altogether, our data highlight the limited knowledge of the coronavirus epidemiology in Neotropical bat species.

Finally, although our data show a high detection rate of coronavirus in bat samples, our study does not intend to villainize or to promote any culling of bats. Bats play important ecological roles in forest regeneration, acting in pollination and seed dispersion, and also in insect and pest control (Kasso & Balakrishnan, 2013); thus, the conservation of these mammals is crucial for the One Health aspects.

ACKNOWLEDGEMENTS

We thank the support of Brazilian Post and Telegraph Company (Correios) for transporting our field work supplies and samples. This work is funded by the Ministry of Science, Technology, and Innovation (MCTI-Brazil) and by the National Council for Scientific and Technological Development (CNPq: 403761/2020-4, 400172/2022-4). HLF, CWA, LMB, JCB, GOS, INC, RM, IFA, RCR, IMSP, FF, ADR, DP, AG, ACSB, JT, and TO are recipient of CNPq scholarship. LMNS and MVSM are recipient of CAPES scholarship. We also thank the National Network for Virus Surveillance in Wild Animals (PREVIR-MCTI Network).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and performed the research. Data collection, analysis, and interpretation of data were performed by all authors. The first draft of the manuscript was written by Larissa Mayumi Bueno, Laís Santos Rizotto, Amanda de Oliveira Viana, and Helena Lage Ferreira and all authors commented on previous versions of the manuscript. The funding acquisition was done by Clarice Weis Arns, Edison Luiz Durigon, and Helena Lage Ferreira. All authors read and approved the final manuscript.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All the sample collections were authorized by the Brazilian Institute of Environment and Natural Renewable Resources (SISBIO/IBAMA: 76621-1, 12475-3, 10698-3, 76814-1, 11597-4) and by the Ethics Committee on the Use of Animals of the University of São Paulo (CEUA/FZEA-USP: 1128141120).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov.

ORCID

Larissa Mayumi Bueno https://orcid.org/0000-0003-4869-3697
Laís Santos Rizotto https://orcid.org/0000-0003-3582-5160
Amanda de Oliveira Viana https://orcid.org/0000-0002-5586-7491
Laura Morais Nascimento Silva https://orcid.org/0000-0002-4019-4044
Maria Vitória dos Santos de Moraes https://orcid.org/0000-0001-6309-1657
Julia Cristina Benassi https://orcid.org/0000-0002-9778-3528
Guilherme Pereira Scagion https://orcid.org/0000-0003-0160-9515
Erick Gustavo Dorlass https://orcid.org/0000-0001-8169-3133
Iolanda Maria da Silva Pereira https://orcid.org/0000-0002-4573-7562
Lilia D'ark Nunes dos Santos https://orcid.org/0000-0003-3833-7453
Fabiane Fisch https://orcid.org/0000-0002-9011-7020
Alana Drielle Rocha https://orcid.org/0000-0002-4970-432X
Dagoberto Port https://orcid.org/0000-0003-3909-7957
Gabriela Stahelin Pereira https://orcid.org/0000-0001-9661-6560
Junko Tsukamoto https://orcid.org/0000-0003-4064-2385
Erika Hingst-Zaher https://orcid.org/0000-0001-9547-6666
Wallace Rodrigues Telino Jr. https://orcid.org/0000-0002-5570-1993
Joaquim Olinto Branco https://orcid.org/0000-0002-3521-1671
Tatiana Ometto https://orcid.org/0000-0002-7219-329X
Jansen de Araujo https://orcid.org/0000-0002-7250-3024
Clarice Weis Arns https://orcid.org/0000-0002-7308-460X
Helena Lage Ferreira https://orcid.org/0000-0002-3261-3712
Edison Luiz Durigon https://orcid.org/0000-0003-4898-6553

REFERENCES

Abreu, E. F., Casali, D., Costa-Araújo, R., Garbino, G. S. T., Libardi, G. S., Loretto, D., Loss, A. C., Marmontel, M., Moras, L. M., Nascimento, M. C., Oliveira, M. L., Pavan, S. E., & Tirelli, F. P. (2021). Lista de Mamíferos do Brasil. *Zenodo*, , . https://doi.org/10.5281/ZENODO.5802047

Alves, R., do Canto Olegário, J., Weber, M., da Silva, M., Canova, R., Sauthier, J., Baumbach, L., Witt, A., Varela, A., Mayer, F., da Fontoura Budaszewski, R., & Canal, C. (2022). Detection of coronavirus in vampire bats (*Desmodus rotundus*) in southern Brazil. *Transboundary and Emerging Diseases*, *69*(4), 2384–2389. https://doi.org/10.1111/TBED.14150

Amman, B. R., Carroll, S. A., Reed, Z. D., Sealy, T. K., & Balinandi, S. (2012). Seasonal pulses of Marburg virus circulation in juvenile *Rousettus aegyptiacus* bats coincide with periods of increased risk of human infection. *PLoS Pathogens*, *8*(10), e1002877. https://doi.org/10.1371/journal.ppat.1002877

Anthony, S., Johnson, C., Greig, D., Kramer, S., Che, X., Wells, H., Hicks, A., Joly, D., Wolfe, N., Daszak, P., Karesh, W., Lipkin, W., Morse, S., Mazet, J., & Goldstein, T. (2017). Global patterns in coronavirus diversity. *Virus Evolution*, *3*(1), vex012. https://doi.org/10.1093/VE/VEX012

Anthony, S., Ojeda-Flores, R., Rico-Chávez, O., Navarrete-Macias, I., Zambrana-Torrelio, C., Rostal, M., Epstein, J., Tipps, T., Liang, E., Sanchez-Leon, M., Sotomayor-Bonilla, J., Aguirre, A., Ávila-Flores, R., Medellín, R., Goldstein, T., Suzán, G., Daszak, P., & Lipkin, W. (2013). Coronaviruses in bats from Mexico. *The Journal of General Virology*, *94*(5), 1028–1038. https://doi.org/10.1099/VIR.0.049759-0

Asano, K. M., Hora, A. S., Scheffer, K. C., Fahl, W. O., Iamamoto, K., Mori, E., & Brandão, P. E. (2016). Alphacoronavirus in urban Molossidae and

Phyllostomidae bats, Brazil. *Virology Journal*, *13*(1), 110. https://doi.org/10.1186/S12985-016-0569-4

Banerjee, A., Kulcsar, K., Misra, V., Frieman, M., & Mossman, K. (2019). Bats and coronaviruses. *Viruses*, *11*(1), 41. https://doi.org/10.3390/v11010041

Barbosa, C. M., Durigon, E. L., Thomazelli, L. M., Ometto, T., Marcatti, R., Nardi, M. S., de Aguiar, D. M., Pinho, J. B., Petry, M. V., Neto, I. S., Serafini, P., Rodrigues, R. C., de Azevedo, S. M., Jr., Góes, L. G. B., & de Araujo, J. (2019). Divergent coronaviruses detected in wild birds in Brazil, including a central park in São Paulo. *Brazilian Journal of Microbiology*, *50*(2), 547–556. https://doi.org/10.1007/S42770-019-00065-7

Barnabé, C., Durães-Carvalho, R., Neto, D., Caserta, L., Artacho, L., Jacomassa, F., Martini, M., Bianchi Dos Santos, M., Felippe, P., Ferreira, H., & Arns, C. (2015). Bat coronavirus in Brazil related to appalachian ridge and porcine epidemic diarrhea viruses. *Emerging Infectious Diseases*, *21*(4), 729–731. https://doi.org/10.3201/EID2104.141783

Bittar, C., Machado, R. R. G., Comelis, M. T., Bueno, L. M., Beguelini, M. R., Morielle-Versute, E., Nogueira, M. L., & Rahal, P. (2019). Alphacoronavirus detection in lungs, liver, and intestines of bats from Brazil. *Microbial Ecology*, *79*(1), 203–212. https://doi.org/10.1007/S00248-019-01391-X

Brandão, P. E., Scheffer, K., Villarreal, L. Y., Achkar, S., Oliveira, R. de N., Fahl, W. de O., Castilho, J. G., Kotait, I., & Richtzenhain, L. J. (2008). A coronavirus detected in the vampire bat *Desmodus rotundus*. *Brazilian Journal of Infectious Diseases*, *12*(6), 466–468. https://doi.org/10.1590/S1413-86702008000600003

Chu, D. K. W., Leung, C. Y. H., Gilbert, M., Joyner, P. H., Ng, E. M., Tse, T. M., Guan, Y., Peiris, J. S. M., & Poon, L. L. M. (2011). Avian coronavirus in wild aquatic birds. *Journal of Virology*, *85*(23), 12815–12820. https://doi.org/10.1128/jvi.05838-11

Corman, V., Rasche, A., Diallo, T., Cottontail, V., Stöcker, A., Souza, B., Corrêa, J., Carneiro, A., Franke, C., Nagy, M., Metz, M., Knörnschild, M., Kalko, E., Ghanem, S., Morales, K., Salsamendi, E., Spínola, M., Herrler, G., Voigt, C., … Drexler, J. (2013). Highly diversified coronaviruses in neotropical bats. *The Journal of General Virology*, *94*(9), 1984–1994. https://doi.org/10.1099/VIR.0.054841-0

Costa, L., Lourenço, E., Esbérard, C., & Silva, R. (2010). Tamanho da colônia, proporção sexual e coabitação em refúgios de *Phyllostomus hastatus* (Pallas) (Chiroptera: Phyllostomidae). *Brazilian Journal of Biology*, *70*(4), 1047–1053. https://doi.org/10.1590/S1519-69842010000500019

Costa, L., Lourenço, E., Esbérard, C., & Silva, R. (2015). Morcegos (mammalia, chiroptera) em refúgios diurnos artificiais na região sudeste do Brasil. *Mastozoología Neotropical*, *22*(2), 239–253.

de Groot, R. J., Baker, S. C., Baric, R., Enjuanes, L., Gorbalenya, A. E., Holmes, K. V., Perlman, S., Poon, L., Rottier, P. J. M., Talbot, P. J., Woo, P. C. Y., & Ziebuhr, J. (2011). Coronaviridae. ICTV 9th Report. https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/222/coronaviridae

Drexler, J. F., Corman, V. M., Wegner, T., Tateno, A. F., Zerbinati, R. M., Gloza-Rausch, F., Seebens, A., Müller, M. A., & Drosten, C. (2011). Amplification of emerging viruses in a bat colony. *Emerging Infectious Diseases*, *17*(3), 449–456. https://doi.org/10.3201/EID1703.100526

Duarte, N. F. H., Alencar, C. H., Cavalcante, K. K., de S., Correia, F. G. S., Romijn, P. C., Araujo, D. B., Favoretto, S. R., & Heukelbach, J. (2020). Increased detection of rabies virus in bats in Ceará State (Northeast Brazil) after implementation of a passive surveillance programme. *Zoonoses and Public Health*, *67*(2), 186–192. https://doi.org/10.1111/ZPH.12670

Fischer, K., Zeus, V., Kwasnitschka, L., Kerth, G., Haase, M., Groschup, M. H., & Balkema-Buschmann, A. (2016). Insectivorous bats carry host specific astroviruses and coronaviruses across different regions in Germany. *Infection, Genetics and Evolution*, *37*, 108–116. https://doi.org/10.1016/j.meegid.2015.11.010

Ge, X.-Y., Wang, N., Zhang, W., Hu, B., Li, B., Zhang, Y.-Z., Zhou, J.-H., Luo, C.-M., Yang, X.-L., Wu, L.-J., Wang, B., Zhang, Y., Li, Z.-X., & Shi, Z.-L. (2016). Coexistence of multiple coronaviruses in several bat colonies in an abandoned mineshaft. *Virologica Sinica*, *31*(1), 31. https://doi.org/10.1007/S12250-016-3713-9

Gloza-Rausch, F., Ipsen, A., Seebens, A., Göttsche, M., Panning, M., Drexler, J. F., Petersen, N., Annan, A., Grywna, K., Müller, M., Pfefferle, S., & Drosten, C. (2008). Detection and prevalence patterns of group i coronaviruses in bats, northern Germany. *Emerging Infectious Diseases*, *14*(4), 626–631. https://doi.org/10.3201/EID1404.071439

Góes, L., Campos, A., Carvalho, C., Ambar, G., Queiroz, L., Cruz-Neto, A., Munir, M., & Durigon, E. (2016). Genetic diversity of bats coronaviruses in the Atlantic Forest hotspot biome, Brazil. *Infection, Genetics and Evolution*, *44*, 510–513. https://doi.org/10.1016/J.MEEGID.2016.07.034

Góes, L., Campos, A., Durigon, E., Dávalos, L., & Dominguez, S. (2013). Novel bat coronaviruses, Brazil and Mexico. *Emerging Infectious Diseases*, *19*(10), 1711–1713. https://doi.org/10.3201/EID1910.130525

Hattendorf, J., Bardosh, K. L., & Zinsstag, J. (2017). One Health and its practical implications for surveillance of endemic zoonotic diseases in resource limited settings. *Acta Tropica*, *165*, 268–273. https://doi.org/10.1016/J.ACTATROPICA.2016.10.009

Hernández-Aguilar, I., Lorenzo, C., Santos-Moreno, A., Naranjo, E. J., & Navarrete-Gutiérrez, D. (2021). Coronaviruses in bats: A review for the Americas. *Viruses*, *13*(7), 1226. https://doi.org/10.3390/V13071226

Hingst-Zaher, E., & Brandão, M. V. (2021). Atlas Craniano: Mamíferos da mata Atlântica e lista de espécies. *Repositório Butantan*, https://doi.org/10.32673/9786588932018

IBGE. (2022). *Portal do IBGE*. Portal Do IBGE - Instituto Brasileiro de Geografia e Estatística. https://www.ibge.gov.br/pt/inicio.html

Jones, B. A., Grace, D., Kock, R., Alonso, S., Rushton, J., Said, M. Y., McKeever, D., Mutua, F., Young, J., McDermott, J., & Pfeiffer, D. U. (2013). Zoonosis emergence linked to agricultural intensification and environmental change. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(21), 8399–8404. https://doi.org/10.1073/pnas.1208059110

Kasso, M., & Balakrishnan, M. (2013). Ecological and economic importance of bats (Order Chiroptera). *International Scholarly Research Notices*, *2013*, 187415. https://doi.org/10.1155/2013/187415

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/MOLBEV/MSW054

Latinne, A., Hu, B., Olival, K. J., Zhu, G., Zhang, L., Li, H., Chmura, A. A., Field, H. E., Zambrana-Torrelio, C., Epstein, J. H., Li, B., Zhang, W., Wang, L. F., Shi, Z. L., & Daszak, P. (2020). Origin and cross-species transmission of bat coronaviruses in China. *Nature Communications*, *11*(1), 1–15. https://doi.org/10.1038/s41467-020-17687-3

Lau, S. K. P., Woo, P. C. Y., Li, K. S. M., Huang, Y., Tsoi, H. W., Wong, B. H. L., Wong, S. S. Y., Leung, S. Y., Chan, K. H., & Yuen, K. Y. (2005). Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(39), 14040–14045. https://doi.org/10.1073/PNAS.0506735102

Letko, M., Seifert, S. N., Olival, K. J., Plowright, R. K., & Munster, V. J. (2020). Bat-borne virus diversity, spillover and emergence. *Nature Reviews Microbiology*, *18*(8), 461–471. https://doi.org/10.1038/s41579-020-0394-z

Liang, J., Zhu, C., & Zhang, L. (2021). Cospeciation of coronavirus and paramyxovirus with their bat hosts in the same geographical areas. *BMC Ecology and Evolution*, *21*(1), 148. https://doi.org/10.1186/S12862-021-01878-7

Lima, F., Campos, F., Kunert Filho, H., Batista, H., Carnielli, P., Cibulski, S., Spilki, F., Roehe, P., & Franco, A. (2013). Detection of alphacoronavirus in velvety free-tailed bats (*Molossus molossus*) and Brazilian free-tailed bats (*Tadarida brasiliensis*) from urban area of Southern Brazil. *Virus Genes*, *47*(1), 164–167. https://doi.org/10.1007/S11262-013-0899-X

Mihindukulasuriya, K. A., Wu, G., Leger, J. St., Nordhausen, R. W., & Wang, D. (2008). Identification of a novel coronavirus from a beluga whale by using a panviral microarray. *Journal of Virology*, *82*(10), 5084–5088. https://doi.org/10.1128/JVI.02722-07

Moreira-Soto, A., Taylor-Castillo, L., Vargas-Vargas, N., Rodrıguez-Herrera, B., Jimenez, C., & Corrales-Aguilar, E. (2015). Neotropical bats from Costa Rica harbour diverse coronaviruses. *Zoonoses and Public Health*, *62*(7), 501–505. https://doi.org/10.1111/ZPH.12181

Morse, S. S., Mazet, J. A. K., Woolhouse, M., Parrish, C. R., Carroll, D., Karesh, W. B., Zambrana-Torrelio, C., Lipkin, W. I., & Daszak, P. (2012). Prediction and prevention of the next pandemic zoonosis. *The Lancet*, *380*(9857), 1956–1965. https://doi.org/10.1016/S0140-6736(12)61684-5

Nziza, J., Goldstein, T., Cranfield, M., Webala, P., Nsengimana, O., Nyatanyi, T., Mudakikwa, A., Tremeau-Bravard, A., Byarugaba, D., Tumushime, J. C., Mwikarago, I. E., Gafarasi, I., Mazet, J., & Gilardi, K. (2020). Coronaviruses detected in bats in close contact with humans in Rwanda. *Ecohealth*, *17*(1), 152–159. https://doi.org/10.1007/S10393-019-01458-8

Osborne, C., Cryan, P. M., Shea, O., Oko, T. J., & Ndaluka, L. M. (2011). Alphacoronaviruses in New World bats: Prevalence, persistence, phylogeny, and potential for interaction with humans. *PLoS ONE*, *6*(5), 19156. https://doi.org/10.1371/journal.pone.0019156

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F., Beutler, N. A., Burton, D. R., Lewis-Ximenez, L. L., De Jesus, J. G., Giovanetti, M., Hill, S. C., Black, A., Bedford, T., Carroll, M. W., Nunes, M., … Loman, N. J. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature Protocols*, *12*(6), 1261–1266. https://doi.org/10.1038/NPROT.2017.066

Reis, N., Peracchi, A., Batista, C., Lima, I., & Pereira, A. (2017). *História Natural dos Morcegos Brasileiros Chave de identificação de Espécies* (1st ed.). Technical Books Editora Ltda.

Rosa, M. R., Brancalion, P. H. S., Crouzeilles, R., Tambosi, L. R., Piffer, P. R., Lenti, F. E. B., Hirota, M., Santiami, E., & Metzger, J. P. (2021). Hidden destruction of older forests threatens Brazil's Atlantic Forest and challenges restoration programs. *Science Advances*, *7*(4), eabc4547. https://doi.org/10.1126/sciadv.abc4547

Ruiz-Aravena, M., McKee, C., Gamble, A., Lunn, T., Morris, A., Snedden, C. E., Yinda, C. K., Port, J. R., Buchholz, D. W., Yeo, Y. Y., Faust, C., Jax, E., Dee, L., Jones, D. N., Kessler, M. K., Falvo, C., Crowley, D., Bharti, N., Brook, C. E., … Plowright, R. K. (2021). Ecology, evolution and spillover of coronaviruses from bats. *Nature Reviews Microbiology*, *20*, 1–16. https://doi.org/10.1038/s41579-021-00652-2

Shirato, K., Maeda, K., Tsuda, S., Suzuki, K., Watanabe, S., Shimoda, H., Ueda, N., Iha, K., Taniguchi, S., Kyuwa, S., Endoh, D., Matsuyama, S., Kurane, I., Saijo, M., Morikawa, S., Yoshikawa, Y., Akashi, H., & Mizutani, T. (2011). Detection of bat coronaviruses from *Miniopterus fuliginosus* in Japan. *Virus Genes*, *44*(1), 40–44. https://doi.org/10.1007/S11262-011-0661-1

Subudhi, S., Rapin, N., Bollinger, T., Donaldson, M., Davy, C., Warnecke, L., Kyle, C., Willis, C., & Misra, V. (2017). A persistently infecting coronavirus in hibernating *Myotis lucifugus*, the North American little brown bat. *The Journal of General Virology*, *98*(9), 2297–2309. https://doi.org/10.1099/JGV.0.000898

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., & Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution*, *4*(1), vey016. https://doi.org/10.1093/VE/VEY016

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(30), 11030–11035. https://doi.org/10.1073/pnas.0404206101

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O'Brien, S. J., & Murphy, W. J. (2005). A molecular phylogeny for bats illuminates biogeography and the fossil record. *Science*, *307*(5709), 580–584. https://doi.org/10.1126/SCIENCE.1105113/SUPPL_FILE/TEELING.SOM.FINAL.PDF

Wertheim, J. O., Chu, D. K. W., Peiris, J. S. M., Kosakovsky Pond, S. L., & Poon, L. L. M. (2013). A case for the ancient origin of coronaviruses. *Journal of Virology*, *87*(12), 7039–7045. https://doi.org/10.1128/JVI.03273-12/ASSET/50C6DE3A-82C1-4D82-85ED-22F9C938BA39/ASSETS/GRAPHIC/ZJV9990977490003.JPEG

Wong, A., Li, X., Lau, S., & Woo, P. (2019). Global epidemiology of bat coronaviruses. *Viruses*, *11*(2), 174. https://doi.org/10.3390/V11020174

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Bueno, L. M., Rizotto, L. S., Viana, A. O., Silva, L. M. N., de Moraes, M. V. S., Benassi, J. C., Scagion, G. P., Dorlass, E. G., Lopes, B. L. T., Cunha, I. N., Melinski, R., de Alvarenga, I. F., Leitão, G. L., Rodrigues, R. C., Pereira, I. M. S., Santos, L. D. N., Fisch, F., Rocha, A. D., Port, D., … Durigon, E. L. (2022). High genetic diversity of alphacoronaviruses in bat species (Mammalia: Chiroptera) from the Atlantic Forest in Brazil. *Transboundary and Emerging Diseases*, *69*, e2863–e2875. https://doi.org/10.1111/tbed.14636