Evolution and divergence of the genetic lineage of Rabies Virus Desmodus rotundus/Artibeus lituratus in São Paulo State

Débora Nunes de Souza (deboradns.unesp@gmail.com)
Pasteur Institute, Brazil https://orcid.org/0000-0002-6316-8308
Rafael Novaes Oliveira
Pasteur Institute, Brazil
Fabiana Bettoni
Hospital Sirio-Libanes
Paula Fontes Asprino
Hospital Sirio-Libanes
Carla Isabel Macedo
Pasteur Institute, Brazil
Samira Maria Achkar
Pasteur Institute, Brazil
Willian de Oliveira Fahl
Pasteur Institute
Paulo Eduardo Brandão
USP: Universidade de Sao Paulo
Juliana Galera Castilho
Pasteur Institute, Brazil

Research Article

Keywords: epidemiological surveillance, frugivorous bat, hematophagous bat, molecular biology, next generation sequencing, rabies

Posted Date: December 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2343279/v1

Version of Record: A version of this preprint was published at Archives of Virology on October 6th, 2023. See the published version at https://doi.org/10.1007/s00705-023-05864-w.

Abstract

Since 1998, with the occurrence of the last case of rabies in dogs or cats in São Paulo State related to the dog-specific rabies virus (RABV) lineage, 55 cases of rabies in these animals have been reported until 2021, and the vast majority (51) have been genetically characterized as belonging to the *Desmodus rotundus/Artibeus lituratus* lineage of RABV, However, it has not been demonstrated so far if there is the possibility to infer which of these bats acted as a source of infection in these cases through the genetic sequencing of isolated RABV. In order to characterize the *Desmodus rotundus/Artibeus lituratus* lineage aiming at the possibility of differentiating its isolates in RABV associated with each of these reservoirs, this research performed the DNA sequencing technique by amplification on the Illumina Miseq platform in 70 RABV isolates from the State of São Paulo, sent to the virology laboratory of the Pasteur Institute of São Paulo between 2006–2015, being 33 related to the hematophagous bat *D. rotundus* and 37 to the fruit bat *A. lituratus*. A genomic approach using phylogenetic and nucleotide identity analyses demonstrated that the isolates investigated in this study can be considered as belonging to the same genetic lineage of RABV and that in São Paulo State the *D. rotundus/A. lituratus* lineage is subdivided into at least four phylogenetic sub lineages, two associated with *D. rotundus* and two with *A. lituratus*, and that these results can be practically applied to the epidemiological surveillance of rabies in this state.

Introduction

Rabies is an acute and progressive infectious disease of the central nervous system (CNS) of mammals caused by the rabies virus (RABV), which is present in the saliva of infected animals and transmitted primarily by biting (Acha and Szifres, 2003). Tens of thousands of people in Africa and Asia die of rabies each year, primarily through bites from rabid dogs (Hampson et al., 2015).

RABV has a non-segmented single-stranded RNA genome with negative polarity, making the viral RNA non-infectious because it is unable to be translated directly into proteins. The complete genome has 11,932 nucleotides based on the fixed Pasteur Virus (PV), which encode five structural proteins (N, P, M, G, and L) from monocistronic mRNAs. The five genes encoding these proteins are named as the proteins and are separated by four non-coding intergenic regions (Wunner, 2007), which play an important role in the regulation of viral expression (Finke et al., 2000).

In São Paulo state, the domestic dog-specific genetic lineage of RABV, characterized antigenically as antigenic variant 2 (AgV2) has not been detected since 1999 (Kotait et al., 2001). However, 55 cases of dogs and cats rabies have been reported by the end of 2021 as resulting from infection by bat-specific RABV genetic lineages (SVS, 2020), and in the absolute majority of these cases (51) the isolated RABV lineages was characterized as the *Desmodus rotundus/Artibeus lituratus* (characterized antigenically as variant 3), which has these two bat species as its reservoir (Castilho et al. 2018; Carnieli et al, 2009).

In recent years the species of frugivorous bat *Artibeus lituratus*, has gained importance in Public Health, because in addition to the number of these animals have increased in urban areas, they are also

reservoirs of the RABV *Desmodus rotundus /Artibeus lituratus* lineage, maintaining this epidemiological cycle independently of the cycle maintained by the hematophagous bat (Castilho et al., 2017). It is worth noting that it is not yet known which of these two bat species is the original reservoir for this lineage.

Fahl et al. (2012) studied RABV isolates from bats *Desmodus rotundus* and *Artibeus lituratus* from São Paulo state based on analysis of the G and N genes and found differentiation of this lineage from those bats, but it was not clear whether these isolates could be considered as a single lineage of RABV, nor was a way proposed to use these data to infer the source of infection in cases of rabies in domestic animals caused by the *D. rotundus/A.lituratus* lineage.

For a better understanding of the epidemiology of rabies transmitted to dogs and cats and the evolution of RABV in the bat species *Artibeus lituratus* and *Desmodus rotundus*, the study of viral genomes with a representative sample size is critical. Few RABV genome sequences with the *Desmodus rotundus/Artibeus lituratus* lineage have been characterized and are available in GenBank (Mochizuki et al. 2011, Campos et al., 2020, Oliveira et al., 2020).

Thus, the objective of this research was to determine, through a genomic approach, whether RABV isolates from *D. rotundus* and *A. lituratus* bats really belong to the same viral lineage or only represent distinct RABV lineages. These findings can contribute to the clarification of the most likely source of infection in cases of rabies in domestic animals infected with the *D. rotundus/A. lituratus* lineage.

Materials And Methods

RABV samples

Seventy first-passage RABV samples from the CNS of inoculated mice were used as established in the routine of the Rabies Diagnostic Section of the Pasteur Institute (Koprowski, 1996). RABV isolates were obtained from *Desmodus rotundus* bats (4), cats (2), cattle (23), horses (6) and *Artibeus lituratus* bats (35). All samples used were from Brazil, State of São Paulo, collected from 2006 to 2015.

RNA Preparation

Total RNA was extracted from CNS of infected mice using TRIzol (Invitrogen™), following as manufacturer's instructions. Total RNA was treated with DNAse I (Ambion), the extracted total rRNA was depleted to eliminate host rRNA using the RiboMinus Eukaryote kit (Invitrogen) followed by the RiboMinus concentration Module (Invitrogen), according to the manufacturer's instructions. Transcription to cDNA was performed using 1ug/ul of RNA with random hexamers using Superscipt III reverse transcriptase (Invitrogen) and the second strand reaction was performed with Klenow Fragment exo (Thermo Scientific).

Library preparation and NGS sequencing

Sequencing libraries were prepared with Nextera XT DNA library preparation (Illumina) according to the manufacturer's instructions. Library profile and concentration were evaluated using the bioanalyzer platform on a high sensitivity kit (Agilent). Libraries were pooled manually and sequenced at 17nM on an Illumina MiSeq (V2) with 2x150-bp paired-end reads, according to standard Illumina protocols.

The generated reads were assembled in the CLC Genomics Workbench 6 program, using a reference sequence from a RABV isolate from a *Desmodus rotundus* bat (GenBank KM594042.1). These contigs were extended by mapping reads to the reference with 95% similarity and no gaps.

Sequences with Q score ≥ 20, minimum coverage of 100 reads per site, and penalty for homopolymeric regions (30% quality reduction for each additional base) were maintained.

Data set for analysis

For the phylogenetic analyses described below, two different alignments generated with DNA sequences from the same isolates (70 from this study and 14 retrieved from GenBank) were used, one referring to the five RABV genes concatenated (10,800 nucleotides) and the other referring to a partial region of the N gene with 799 nucleotides (nucleotide 71 to 869), the same used in the routine of genetic characterization of RABV from the Laboratory of Molecular Biology of the Pasteur Institute.

The sequences were aligned with the MUSCLE method with the MEGA X program (Kumar et al., 2018), and then the alignment was manually edited with the Bioedit v. 7.0.9.0 program (Hall, 1999).

Regarding the sequences corresponding to the five RABV genes, after alignment, all the non-coding regions of the messenger RNAs of each of the five RABV genes and their intergenic regions were removed, and after this step, all the coding regions of each of the genes were concatenated in a single read step in the viral genome direction (N-P-M-G-L), totaling 10,800 nucleotides.

Estimation of nucleotide substitution rate per site per year, estimate of the time to most recent common ancestor (TACMR) and phylogeny estimates

To estimate the substitution rates per site per year and the TACMR for the *Desmodus rotundus/Artibeus lituratus*, 84 sequences of all five concatenated RABV genes were used, 70 of them generated in this study and the remaining 14 retrieved from GenBank. Phylogenetic trees with maximum clade credibility (MCC) were inferred for the five concatenated RABV genes using a Markov Chain Monte Carlo (MCMC) based approach implemented in BEAST v.1.10.4 (Drummond et al, 2012) employing the General Time Reversible (GTR) model with gamma rate and a proportion of invariant sites (GTR + Γ + I) and uncorrelated relaxed molecular clock using a lognormal probabilistic distribution (Drummond et al., 2006) with the previously estimated μ (nucleotide substitution rate) (Oliveira et al., 2020). The convergence of the MCMC was obtained after 4 independent runs with 70 million generations each, sampling every

70,000 trees, which was enough to obtain an appropriate sampling of the stationarity of the MCMC that was inspected with the aid of the program Tracer v 1.7.1 (available at http://tree.bio.ed.ac.uk/software/), considered sufficient when the effective sample sizes (ESS) of the parameters reached values greater than 200.

For the phylogenetic reconstruction of the tree based on 799 nucleotides of the N gene (nt 71 to 869 relative to fixed sample PV), the maximum likelihood method using MEGA X software (Kumar et al., 2018) with Tamura 3-parameter evolutionary model with gamma variation rate (T92 + Γ) with 1000 bootstrap repetitions was used.

Recombination analysis

For this analysis the alignment referring to the five concatenated RABV genes was used. The detection of possible homologous recombination events, identification of recombinant sequences and location of recombination points were investigated using the RDP, GenConv, Chimaera, MaxChi, Bootscan, SiScan and 3Seq methods implemented in the RDP4 beta 48 program (Martin et al., 2010), using a 95% confidence interval and Bonferroni multiple correction to avoid false positive results.

Nucleotide and amino acid identity

The average, maximum and minimum nucleotide and amino acid identity values calculated for the five concatenated RABV genes and each gene separately (N, P, M, G, and L) were calculated using the Excel program based on nucleotide and amino acid identity matrices obtained using the Bioedit v. 7.0.9.0 program (Hall, 1999).

Results

Recombination Analysis

No evidence of recombination was found among the RABV isolates of this study.

Nucleotide and Protein Similarity

The Table 1 shows the nucleotide identity values (mean, maximum and minimum) calculated for each gene separately and all the concatenated genes, using only the samples of the present study for this calculation, which ultimately reflects the degree of genetic similarity of each gene within the isolates of the *Desmodus rotundus/Artibeus lituratus* lineage circulating in the state of São Paulo.

Table 1
Mean, maximum and minimum genetic identities calculated using all DNA sequences of the isolates in this study.

Gene	Mean %	Maximum %	Minimum %
N	97,64	100	95,6
P	97,50	100	94,9
M	97,80	100	95,5
G	98,05	100	95,6
L	97,90	99,9	96,3
N-P-M-G-L	97,8	99,9	96,2

It can be observed that the P gene was more variable in the RABV *Desmodus rotundus/Artibeus lituratus* lineage, both in mean and absolute values. In mean values we have the following order of genetic similarity: G > L > M > N > P. In absolute values, the degree of similarity of genes would be in the following order L > G = N > M > P.

The Table 2 describes the values of the mean, maximum and minimum identities calculated for each RABV protein separately and for all the concatenated proteins, using for this calculation only the samples of the present study.

Table 2
Average, maximum and minimum protein identities calculated using all DNA sequences of the isolates of this study.

Protein	Mean %	Maximum %	Minimum%
N	99,5	100	98,6
P	99,02	100	95,9
M	99,3	100	97,5
G	99,05	100	96,9
L	99,5	100	98,7
N-P-M-G-L	99,4	100	98,6

The Table 2 shows that the P protein was the most variable in the RABV *Desmodus rotundus/Artibeus lituratus* lineage, both in mean and absolute values. In mean values we have the following order of similarity of the RABV proteins N = L > M > G > P. In absolute values, the degree of similarity of the proteins would be in the following order L > N > M > G > P.

Estimates of nucleotide substitution rate per site per year, time to most recent common ancestor (TACMR) and phylogeny estimates.

In Figs. 1 and 2 we have respectively the maximum credible clade phylogenetic trees obtained from the alignment of the five concatenated RABV genes and the ML tree obtained from the partial N gene alignment.

It is possible to observe in the MCC tree generated from all the RABV genes concatenated (Fig. 1) the division of the 84 isolates into nine clusters, with at least one taxon in each one, seven of them supported by high posterior values, four of these clusters related to the samples used in this study, which were arbitrarily named according to the most prevalent reservoir in each group. Thus, in the state of São Paulo there are two clusters related to the hematophagous bat *Desmodus rotundus*, named Desmodus 1 and Desmodus 2, and two clusters related to the species *Artibeus lituratus*, named Artibeus 1 and Artibeus 2.

Figure 2 shows the maximum likelihood phylogenetic tree obtained from a 799 bp fragment of the N gene, showing the recovery of the same four clusters related to the isolates of this research obtained in the MCC tree, but with a slightly different topology with respect to the origin of the groups Artibeus 1 and Desmodus 1.

According to the MCC phylogenetic trees, the most recent common ancestor time (TACMR) for the *Desmodus rotundus/Artibeus lituratus* (AgV3) lineage would have occurred around 170 years, ranging from 107 to 237 years with a 95% confidence interval (Fig. 1). The estimated average mutation rate for all concatenated RABV genes was 2.15E-4 substitutions per site per year, ranging from 1.6E-4 to 2.6E-4 with 95% confidence interval.

In the MCC phylogenetic tree (Figs. 1) it is observed that the Artibeus 1 sublineage would be the oldest among the representatives of the Desmodus rotundus/Artibeus lituratus lineage circulating in the state of São Paulo, being the first to diverge around 100 years ago, followed by the Desmodus 1 lineage, which would have diverged around 80 years ago. It is also observed that the Desmodus 2 and Artibeus 2 sublineages were the last to diverge, being groups that diverged directly from the same common ancestor, around 70 years ago. The Desmodus 1 and Artibeus 1 sublineages do not diverge directly from the same common ancestor, having their most recent common ancestor represented by the node of origin of the Artibeus 1 lineage.

In Table 3 is the description of the 70 isolates used in this research.

Table 3
Isolates used in this research, along with host, locality, underline, and GenBank number.

Sample	Host	City/State	Underline	GenBank
2990/06	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579806
5283/06	*Artibeus lituratus*	Campinas/SP	Artibeus 1	MW579825
4987/06	*Desmodus rotundus*	Ubatuba/SP	Artibeus 1	MW579824
3970/06	*Artibeus lituratus*	Piracicaba/SP	Artibeus 1	MW579814
5337/07	*Artibeus lituratus*	Campinas/SP	Artibeus 1	MW579826
6521/07	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579837
6784/07	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579839
895/09	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579785
2939/09	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579804
6901/10	Feline	Jaguariúna/SP	Artibeus 1	MW579840
4433/10	*Artibeus lituratus*	Campinas/SP	Artibeus 1	MW579817
5845/11	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 1	MW579831
5657/06	Bovine	São Carlos/SP	Artibeus 1	MW579828
161/14	*Artibeus lituratus*	Campinas/SP	Artibeus 1	MW579780
3959/10	Bovine	Pedregulho/SP	Desmodus 1	MW579813
6378/11	Bovine	Franca/SP	Desmodus 1	MW579836
113/13	Bovine	Pedregulho/SP	Desmodus 1	MW579779
8532/06	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579844
7329/08	*Artibeus lituratus*	Piracicaba/SP	Artibeus 2	MW579842
2470/09	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579801
2656/10	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579802
5731/10	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579830
1469/11	Bovino	Marília/SP	Artibeus 2	MW579787
4611/11	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579822
5517/11	*Artibeus lituratus*	Nova Odessa/SP	Artibeus 2	MW579827
4338/12	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579815
3234/12	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579808

Sample	Host	City/State	Underline	GenBank
2149/13	*Artibeus lituratus*	Barretos/SP	Artibeus 2	MW579794
2249/13	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579795
3247/13	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579810
4495/13	*Artibeus lituratus*	Itapira/SP	Artibeus 2	MW579818
2258/14	*Artibeus lituratus*	São José Do Rio Pardo/SP	Artibeus 2	MW579796
2367/14	*Artibeus lituratus*	Itapevi/SP	Artibeus 2	MW579797
2701/14	*Artibeus lituratus*	Mogi Guaçu/SP	Artibeus 2	MW579803
3235/14	*Artibeus lituratus*	Ribeirão Preto	Artibeus 2	MW579809
3251/15	Feline	Ribeirão Preto/SP	Artibeus 2	MW579811
1613/15	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579790
2430/15	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579799
3595/15	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579812
4426/15	*Artibeus lituratus*	Campinas/SP	Artibeus 2	MW579816
4507/15	*Artibeus lituratus*	Luiz Antônio/SP	Artibeus 2	MW579819
6041/11	*Artibeus lituratus*	Herculândia/SP	Artibeus 2	MW579832
2009/11	*Artibeus lituratus*	Ribeirão Preto/SP	Artibeus 2	MW579792
6371/06	Equine	Patrocínio Paulista/SP	Desmodus 2	MW579835
10357/06	Bovine	Socorro/SP	Desmodus 2	MW579846
8346/06	Bovine	Joanópolis/SP	Desmodus 2	MW579843
4548/07	Bovine	Socorro/SP	Desmodus 2	MW579821
5730/07	Bovine	Joanópolis/SP	Desmodus 2	MW579829
6077/07	*Desmodus rotundus*	Socorro/SP	Desmodus 2	MW579833
7191/08	Equine	Pedra Bela/SP	Desmodus 2	MW579841
6763/08	Bovine	São João da Boa Vista/SP	Desmodus 2	MW579838
1692/09	Bovine	São José do Rio preto/SP	Desmodus 2	MW579791
2429/09	Bovine	Divinolândia/SP	Desmodus 2	MW579798
3227/09	Bovine	São José do Rio Pardo/SP	Desmodus 2	MW579807
4731/09	Bovine	São João da Boa Vista/SP	Desmodus 2	MW579823

Sample	Host	City/State	Underline	GenBank
8622/09	Bovine	Ribeirão Preto/SP	Desmodus 2	MW579845
218/10	Bovine	Piracaia/SP	Desmodus 2	MW579781
2974/10	Muar	Socorro/SP	Desmodus 2	MW579805
2461/11	Bovine	São João da Boa Vista/SP	Desmodus 2	MW579800
2023/12	Bovine	Descalvado/SP	Desmodus 2	MW579793
1505/12	Bovine	Amparo/SP	Desmodus 2	MW579789
25/13	Bovine	Mococa/SP	Desmodus 2	MW579778
233/13	Bovine	Tambaú/SP	Desmodus 2	MW579782
6312/08	Bovine	Joanópolis/SP	Desmodus 2	MW579834
410/13	Bovine	Descalvado/SP	Desmodus 2	MW579783
4534/13	*Desmodus rotundus*	Campinas/SP	Desmodus 2	MW579820
22/14	Equine	Itatiba/SP	Desmodus 2	MW579777
1410/14	*Desmodus rotundus*	Rio Claro/SP	Desmodus 2	MW579786
1486/15	Equine	Jaguariúna/SP	Desmodus 2	MW579788
763/12	Equine	Amparo/SP	Desmodus 2	MW579784

Discussion

In São Paulo state, rabies caused by the domestic dog genetic lineage (AgV2) has not been detected in dogs and cats since 1999 (Kotait et al, 2001), but these animals continue to become infected, albeit sporadically, with lineages circulating in bats, mainly the *Desmodus rotundus/Artibeus lituratus*, (compatible with AgV3) (Castilho et al. 2017; 2018), which is endemic in São Paulo State, and probably throughout Brazil.

Desmodus rotundus are also responsible for the absolute majority of rabies cases in herbivores, which are infected by hematophagous bats with rabies at the time they feed on these animals.

Unlike herbivores, dogs and cats, due to their predatory habits, can contract rabies from bats other than hematophagous bats, including the frugivorous bat *Artibeus lituratus*, which is also a reservoir of the RABV strain *D. rotundus/A. lituratus*, and to date, it is not possible to infer, through genetic sequencing of these isolates, which of these bats acted as the most likely source of infection of these infections (Castilho et al., 2018).

Research has already demonstrated a possible genetic differentiation between RABV isolates from *D. rotundus* and *A. lituratus* but has not discussed whether these groups would be specific lineages or sub lineages within the *D. rotundus/A. lituratus* lineage, nor demonstrated the use of these results in rabies epidemiological surveillance in dogs and cats infected with this lineage (Kobayashi et al., 2007, Fahl et al., 2012).

Based on the results of research studying different Brazilian RABV strains using the N gene, it is observed that to date, isolates considered to be from the same lineages generally do not vary more than 5% in nucleotide identity (Carnieli et al., 2013; Souza et al., 2017, Oliveira et al., 2010; Macedo et al., 2010), and this value of gene identity based on the N gene is also suggested by Nadin-Davis (2020) for the separation of RABV lineage.

For all concatenated RABV genes, according to Table 2, a maximum nucleotide identity variation of 4.8% was found, while for the N gene, the maximum variation found was 4.4%. Thus, if we use the gene identity values based on the N gene, there would be no sense in classifying the clusters found in this research in distinct lineages of RABV as suggested by Fahl et al. (2012), because the variation between them based on the N gene had a maximum value of 4.4%, thus belonging to the same lineage of RABV.

As for the analysis of the calculations of genetic identities of nucleotides in relation to the samples sequenced in this study, the following result is observed in increasing order of genetic conservation in mean values: G > L > M > N > P. In absolute values, the degree of conservation of genes would decline in the following order L > N = G > M > P (Table 1).

As for the analysis of amino acids in mean values we have the following order of variability of proteins N = L < M < G < P. In absolute values, the degree of variation of proteins would be in the following order L > N > M > G > P.

Oliveira (2014) conducted a phylogenetic study of RABV based on complete genomes using a very significant sample of RABV strains distributed worldwide, where it was obtained the following result regarding the genetic identities related to the five RABV genes: N > M > L > P > G. In the same research, the author demonstrated that if RABV is divided into two large groups, in which the author called aerial and terrestrial cycles of RABV, these values varied according to the group analyzed. In this case the result of the mean identities obtained for the aerial cycle was N > M > L > P > G, while for the terrestrial cycle was N > M > L > G > P. This shows that depending on the way the data is analyzed or categorized, different values of genetic identity can be obtained.

Although the N gene was shown to be the third most divergent gene on average in the *Desmodus rotundus/Artibeus lituratus* lineage, in terms of amino acids, the N protein along with the L protein were shown to be on average the most conserved proteins among the RABVs of this lineage. Protein N is considered the most conserved protein in RABV species (Wunner & Conzelmann, 2020), but this is the first study to demonstrate that protein L may be as conserved as protein N, again showing that some features

of nucleotide and peptide identities considered to be the consensus for RABV as a whole may vary in more specific analyses.

The functional and organizational constraints imposed on RABV proteins make the same to be under intense purifying selection (Wunner & Conzelmann, 2020; Streicker & Biek, 2020; Oliveira, 2014; Kuzmina et al., 2013), which causes most of their nucleotide mutations to be synonymous, i.e. mutations encoding the same amino acid. Thus, it is possible that the third most variable gene on average in the strain studied in this research (gene N) encodes the most conserved protein (Tables 1 and 2).

Although purifying selection is the dominant type of selection in RABV proteins, studies show that there is a higher tolerance for amino acid changes in P and G proteins compared to other RABV proteins, causing nonsynonymous mutations to be fixed in larger numbers in these genes (Streicker & Biek, 2020). This could explain the fact that in this research the G gene proved on average to be the most conserved gene in the *Desmodus rotundus/Artibeus lituratus* lineage while corresponding to the second most divergent protein in this lineage.

In the recombination analysis, no evidence of this phenomenon was found in the isolates studied here, agreeing with results obtained by other authors (Kuzmin et al., 2012; Oliveira et al., 2020). Recombination between different RABV lineages is a rare event, few studies have presented events of this nature, all in China involving specific dog lineages (Liu et al., 2011; He et al., 2012; Ding et al., 2017).

As for topology, the Maximum Cluster Credibility phylogenetic tree demonstrated that the isolates sequenced in this study clustered into four clusters (Fig. 1), which were arbitrarily named according to the order of divergence in the phylogenetic tree and the main host found in each cluster. It can be suggested, according to the results found, that in São Paulo State, the *Desmodus rotundus/Artibeus lituratus* lineage is currently subdivided into at least four sub clusters named Desmodus 1, Desmodus 2, Artibeus 1 and Artibeus 2 (Fig. 1).

An interesting fact observed in the phylogeny obtained in Fig. 1 were the two independent and not directly related divergences, which gave rise to the two sub lineages associated with the species *Artibeus lituratus*. This shows that these two sublines diverged at different times and from different ancestral viruses, and according to the data of this study, the Artibeus 1 sub lineage seems to have been the first to diverge in the state of São Paulo, being perhaps the most recent common ancestor of the other 3 sub lineages circulating in the state of São Paulo. These two differentiation events can be observed in a subtle way in the research of Fahl et al. (2012), however the authors of this paper did not observe or discuss this fact.

As for the remaining groupings, it is noted that the Desmodus 1 sub lineage was probably the second to diverge, and interestingly, it is noted that the Desmodus 2 and Artibeus 2 lineages were the last to diverge directly from the same ancestral RABV.

In the analysis of each cluster, it was observed that in Desmodus 1 sub lineage only isolates associated with the hematophagous bat *Desmodus rotundus* were found.

In cluster Artibeus 1 there was a predominance of isolates from *Artibeus lituratus*. However, we found an isolate from a feline from Jaguariúna, an isolate from *Desmodus rotundus* from the municipality of Ubatuba and an isolate from a bovine from the municipality of São Carlos (Fig. 1), which can be considered very likely spillover events *Artibeus lituratus* → *D.rotundus* (Fahl et al, 2012, Oliveira et al, 2010, Menozzi et al, 2017).

In Desmodus cluster 2 isolates were found from *Desmodus rotundus*, equines and bovines, without the occurrence of probable spillover events.

In the Artibeus 2 cluster, as observed in the Artibeus 1 cluster, an isolate from a bovine was clustered that was also most likely due to an *Artibeus lituratus* → *D.rotundus* spillover event.

Although the results regarding the differentiation of the four sub lineages within the *Desmodus rotundus/Artibeus lituratus* RABV lineage circulating in the state of São Paulo found in this research are quite robust based on the high posterior and bootstrap values of the source nodes of each cluster, these results should not be extrapolated to other regions of the country due to the fact that little is known about the characterization of RABV isolates from the frugivorous bat *Artibeus lituratus* in these regions (Kobayashi et al, 2007). Furthermore, a preliminary phylogenetic analysis (data not shown) using 1281nt of the N gene from the samples in this study together with 191 other isolates from Brazil and Latin America show that several RABV isolates from *D. rotundus* and herbivores from other regions of Brazil cluster in the Artibeus 1 and Artibeus 2 sub lineages, and to infer whether this is due to spillover events or circulation of these sub lineages in *D. rotundus* in these regions, further research is needed.

In other Neotropical countries, there are no data on molecular characterization of RABV isolates from the genus *Artibeus*, however, serological studies demonstrate the existence of neutralizing antibodies to RABV in these bats, which may be indicative of the maintenance of rabies epidemiological cycles in these animals (Salmón-Mulanovich et al., 2009; Zieger et al., 2017, Seetahal et al., 2020). As such, genetic sequencing of RABV isolates from this genus of bats is critical for the characterization of lineages or sublineages associated with this genus of bats in these countries.

The divergence time of the most recent common ancestor (ACMR) of the *Desmodus rotundus/Artibeus lituratus* RABV lineage, according to the data of this research, was around 170 years, ranging from 107 to 237 years with 95% confidence interval, however this value is underestimated, since all the evolutionary signal of ancestry relationships of other sub lineages within this lineage, as well as of the different RABV lineages maintained in bats is not present in this analysis (Oliveira et al, 2020).

Oliveira et al. (2020) demonstrated that the five RABV genes evolve at different rates, a phenomenon known as heterotachy. Thus, using different RABV genes for evolutionary analysis may lead to different results regarding the rate of nucleotide substitution per year and time of most recent common ancestor.

Oliveira et al. (2020) using the concatenated G and L genes and a fairly representative sampling of the worldwide RABV distribution, arrived at an average divergence time of approximately 260 years for the *D. rotundus/A lituratus* lineage. Kuzmina et al. (2013) using the G gene, obtained the result of 309 years.

It is worth noting that the evolution of new genus or species-specific RABV lineages or the adaptation of the same lineage in different bat genera from spillover events depends on several factors, including the frequent contact of these animals and the phylogenetic proximity existing between the transmitting bat and the host (Streicker et al., 2010; Streicker et al., 2012; Mollentze et al., 2014). The bats *Desmodus rotundus* and *Artibeus lituratus*, besides belonging to the same family (*Phyllostomidae*) also share the same shelter types, a fact that corroborate the adaptation of the same lineage of RABV in these two genera (Greenhall et al., 1983; Arellano-Sota, 1988).

Thus, it can be inferred that currently in São Paulo state, the RABV lineage *D. rotundus/A. lituratus* is subdivided into 4 sub lineages, two of them associated with the hematophagous bat *D. rotundus* and the other two associated with the frugivorous bat *A. lituratus.* The use of a representative sample from a large geographic region, together with the phylogenetic signal provided by the analysis of all concatenated RABV genes provide subsidies for these findings.

In the phylogenetic analysis performed from a 799 nucleotides fragment of the N gene of RABV, the same one used in the routine molecular surveillance of rabies in the state of São Paulo, it is demonstrated the recovery of the same 4 clusters relative to the sub lineages described above, with high bootstrap values in their origin nodes, which demonstrates the possibility of differentiating these clusters without the need for modification of the RABV sequencing protocols currently used.

Interestingly, the two RABV isolated from cats present in this research clustered in the Artibeus 1 and 2 lineages, which, according to the results presented here, may suggest that the source of infection in these cases was most likely the frugivorous bat *A. lituratus*, demonstrating the great usefulness of this research for rabies molecular surveillance in São Paulo State.

Declarations

Acknowledgments

I thank Fapesp (Fundação de Amparo à Pesquisa do Estado de São Paulo) for the financial support (process no. 2018/16000-3) granted and for the scholarship (process no. 2019/04023-1).

Conflict of Interest

Authors declare that they have no conflict of interest

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.

References

1. Acha PN, & Szyfres B (2003) Rabies. Zoonosis y enfermedades transmisibles comunes al hombre y a los animales los. 3rd ed. Washington: Pan American Health Organization. Pan American Health Office, World Health Organization Regional Office 351-383.
2. Arellano-Sota C (1988) Biology, Ecology, and Control of the Vampire Bat. Reviews. Infect. Dis, 10: S615-S619. https://doi.org/10.1093/clinids/10.Supplement_4.S615
3. Campos, A. C. A, Romano, C. M., Melo, F. L., Araújo, D. B, Cunha, E, M, S, Sacramento, D. R. V, et al. (2020) Phylogenetic analysis of near-complete sequences of the Desmodus rotundus rabies virus genetic lineage. Infection Genetics and Evolution 104-179. https://doi.org/10.1016/j.meegid. 2020.104179
4. Carnieli P (2009) Genetic characterization of rabies virus isolated from cattle and horses during 1997-2002 in an epidemic area of São Paulo State, Brazil. Thesis (Doctorate in Sciences) - Graduate Program in Sciences of the Disease Control Coordination of the State Department of Health, São Paulo.
5. Carnieli P, Batista HBCR, Oliveira RN, Castilho JG, Vieira LFP (2013) Phylogeographic dispersion and diversification of rabies virus lineages associated with dogs and crab-eating foxes (Cerdocyon thous) in Brazil. Arch. Virol. 158:2307-2313. doi: 10.1007/s00705-013-1755-y. Epub 2013 Jun 8.
6. Castilho JG, De Souza DN, Oliveira RN, Carnieli P, Batista HBCR et al (2017) The epidemiological importance of bats in rabies transmission to dogs and cats in São Paulo state, Brazil, between 2005 and 2014. Zoonoses Public Health 64: 423-430. doi: 10.1111/zph.12320. Epub 2016 Nov 12
7. Castilho JG, Achkar SM, Oliveira RN, Mori E, Carnieli PJr, Macedo CI (2018). Analysis of rabies diagnosis in dogs and cats in São Paulo state, Brazil. Archives of Virology 163, 2369-2376. doi: 10.1007/s00705-018-3829-3. Epub 2018 May 8
8. Ding NZ, Xu DS, Sun YY, He HB, He CQ (2017) A permanent shift of rabies virus from Chiroptera to Carnivora associated with recombination. Sci. Rep. 7, 289.https://doi.org/10.1038/s41598-017-00395-2.
9. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. PLoS Biol. 4:e88. doi: 10.1371/journal.pbio.0040088.
10. Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian Phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29 (8), 1969-1973. doi: 10.1093/molbev/mss075. Epub 2012 Feb 25.
11. Fahl WO, Carnieli P, Castilho JG, Carrieri ML, Kotait I, Iamamoto K., et al. (2012). Desmodus rotundus and Artibeus sp. bats may have distinct rabies virus lineages. Braz. J. Infect Dis 1. 16, 545-551. https://doi.org/10.1016/j.bjid.2012.07.002.

12. Finke S, Cox HJ, Conzelmann KK. (2000) Differential attenuation of rabies virus gene transcription by intergenic regions: generation of recombinant virus on polymerase gene expression. J. Virol. 74, 7261-69. doi: 10.1128/jvi.74.16.7261-7269.2000.
13. Greenhall AM, Joermann G, Schmidt U (1983) Desmodus rotundus. Mammal Species 202: 1-6. https://doi.org/10.2307/3503895.
14. Hall TA (1999) Bioedit an easy-to-use biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 4,95-98.
15. Hampson K., Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M et al (2015) Correction: Estimating the global burden of endemic canine rabies. PLos Negt. Trop. Dis. 9:e0003709. DOI:10.1371/journal.pntd.0003786 .
16. He CQ, Meng SL, Yan HY, Ding NZ, He HB, Yan JX et al (2012) Isolation and identification of a novel rabies virus strain in China with natural recombinant nucleoprotein gene. PloS One 7(12), e49992. https://doi.org/10.1371/journal.pone.0049992.
17. Kobayashi Y, Inoue N, Sato G, Itou T, Santos HP, Brito CJ et al (2007) Phylogenetic characterization of carnivore rabies virus isolates in Brazil. J. Vet. Med. Sci. 69(7): 691-6. DOI: 10.1292/jvms.69.691
18. Koprowski H (1996) The mouse inoculation test. In: Meslin FX; KAPLAN. Laboratory techniques in rabies. 4. ed. Geneva: World Health Organization 80-87.
19. Kotait, I, Favoretto SR, Carrieri ML, Takaoka N (2001). Raiva Humana causada pela variante 3 - Desmodus rotundus - no Estado de São Paulo. In: Seminário Internacional Morcegos como transmissores da raiva; São Paulo. Programa e Resumos 70-71.
20. Kumar S, Stecher G, Li M, Knyaz, C, Tamura K. (2018) MEGA X: Molecular evolutionary genetic analysis using computer platforms. Mol. Biol. Evol 1;35(6),1547-1549. doi: 10.1093/molbev/msy096.
21. Kuzmin IV, Shi M, Orciari LA, Yager PA, Velasco-Villa A., Kuzmina N, et al (2012) Molecular inferences suggest multiple bat host shifts for mesocarnivores in Arizona during 2001-2009. PLOS Pathog. 8,e1002786. https://doi.org/10.1371/journal.ppat.1002786.
22. Kuzmina NA, Kuzmin IV, Ellison JA., Taylor ST, Bergman DL, Dew B et al (2013) A reassessment of the evolutionary time scale of bat rabies viruses based on glycoprotein gene sequences. Virus Genes 47,305-310. DOI 10.1007/s11262-013-0952-9
23. Liu W, Liu Y, Liu J, Zhai J, Xie Y (2011) Evidence for inter- and intra-clade recombinations of rabies virus. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases.11 (8), 1906-12. doi: 10.1016/j.meegid.2011.08.031. Epub 2011 Oct 24.
24. Macedo CI, Carnieli JrP, Fahl WO, Lima JYO, Oliveira RN, Achkar SM, et al (2010) Genetic characterization of rabies virus isolated from cattle and equines between 2007 and 2008 in the State of São Paulo and Minas Gerais. Revista da Sociedade Brasileira de Medicina Tropical, 43 (2),116-120. https://doi.org/10.1590/S0037-86822010000200002
25. Martin DP, Lemey P, Lott M, Molton V, Posada D, Lefeuvre P (2010) RD3P: a flexible and fast computer program for recombination analysis. Bioinformatics. Oxford, England 26 (19), 2462-2463. doi: 10.1093/bioinformatics/btq467

26. Menozzi BD, Oliveira RN, Paiz LM, Richini-Pereira VB., Langoni H (2017) Antigenic characterization of rabies virus isolated from bats (Mammalia: Chiroptera) from municipalities in São Paulo State, Southeastern Brazil. Arch. Virol. 162(5):1201-1209. doi: 10.1007/s00705-017-3220-9. Epub 2017 Jan 20.
27. Mochizuki N, Kobayashi Y, Sato G, Hirano S, Itou T, Ito FH et al (2011) Determination and molecular analysis of the complete genome sequence of two wild-type rabies viruses isolated from a hematophagous bat and a fruit bat in Brazil. J Vet Med Sci.73,759-66. doi: 10.1292/jvms.10-0238. Epub 2011 Feb 2.
28. Mollentze N, Biek R, Streicker DG (2014) The role of viral evolution in rabies host shifts and emergence. Current opinion in virology 8: 68-72. doi: 10.1016/j.coviro.2014.07.004
29. Nadin-Davis S (2020) A. Molecular epidemiology. In: Fooks AR, Jackson AC, editors. Rabies. 4th ed. ed. San Diego: Academic Press, 143-194.
30. Oliveira RN, De Souza SP, Lobo RS, Castilho JG, Macedo CI, Carnieli P et al (2010) Rabies virus in insectivorous bats: implications of the diversity of the nucleoprotein and glycoprotein genes for molecular epidemiology. Virology 405(2): 352-60. DOI: 10.1016/j.virol.2010.05.030
31. Oliveira RN (2014) Modes and times of evolution of rabies virus (RABV) strains maintained by airborne and terrestrial reservoirs based on complete genomes. Thesis (Doctor of Science) - University of São Paulo, São Paulo.
32. Oliveira RN, Freire CC, Iamarino A, Zanotto PM, Pessoa R, Sanabani SS et al (2020) Diversification of rabies virus in aerial and terrestrial mammals. Genetics and Molecular Biology 43, 3, e20190370. https://doi.org/10.1590/1678-4685-GMB-2019-0370.
33. Salmón-Mulanovich G, Vásquez A, Albújar C, Guevara C, Laguna-Torres VA, Salazar M et al (2009) Human rabies and rabies in vampire and non-vampire bat species, Southeastern Peru. Emerg Infect Dis., 15:1308-10. doi: 10.3201/eid1508.081522
34. Seetahal JFR, Greenberg L, Satheshkumar PS, Sanchez-Vazquez M, Legall G, Singh S, et al (2020) The serological prevalence of rabies virus neutralizing antibodies in the bat population on the Caribbean Island of Trinidad. Viruses 12, 178; doi:10.3390/v12020178.
35. Streicker DG, Turmelle AS, Vonhof MJ, Kuzmin IV, Mccracken GF, Rupprecht CE (2010) Host phylogeny limits cross-species emergence and establishment of rabies virus in bats. Science 329 (5992), 676-9. doi: 10.1126/science.1188836.
36. Streicker DG, Altizer SM, Velasco-Villa A, Rupprecht CE (2012) Variable evolutionary pathways for host establishment through repeated host shifts of rabies virus among bats. Proc Natl Acad SciE U S A. 109:19715-20. doi: 10.1073/pnas.1203456109.
37. Streicker DG, Biek R (2020) Evolution of rabies virus. In: Fooks AR, Jackson AC, editors. Rabies. 4th ed. San Diego: Academic Press, 83-142.
38. SVS/MS-Secretaria de Vigilância Sanitária do Ministério da Saúde, 2020. https://antigo.saude.gov.br/saude-de-a-z/raiva. Accessed: 06/01/2021.

39. Souza DN, Carnieli JP, Macedo CI, Oliveira RN, Batista HBCR, Rodrigues AC et al (2017). Phylogenetic analysis of rabies virus isolated from canids in North and Northeast Brazil. Archives of Virology, 162 (1), 71-77. DOI 10.1007/s00705-016-3079-1.
40. Wunner WH (2007) Rabies. In: Rabies. Jackson AC, Wunner WH (Eds). San Diego: Academic Press 23-68.
41. Wunner WH, Conzelmann KK (2020) Rabies virus In: Fooks AR, Jackson AC, editors. Rabies. 4th ed. San Diego: Academic Press 43-81.
42. Zieger U, Cheetham S, Santana SE, Leiser-Miller L, Matthew-Belmar V, Goharriz H et al (2017) Natural exposure of bats in Grenada to rabies virus. Ecology and Epidemiology of Infection.; 7(1):1332935. https://doi.org/10.1080/20008686.2017.1332935.

Figures

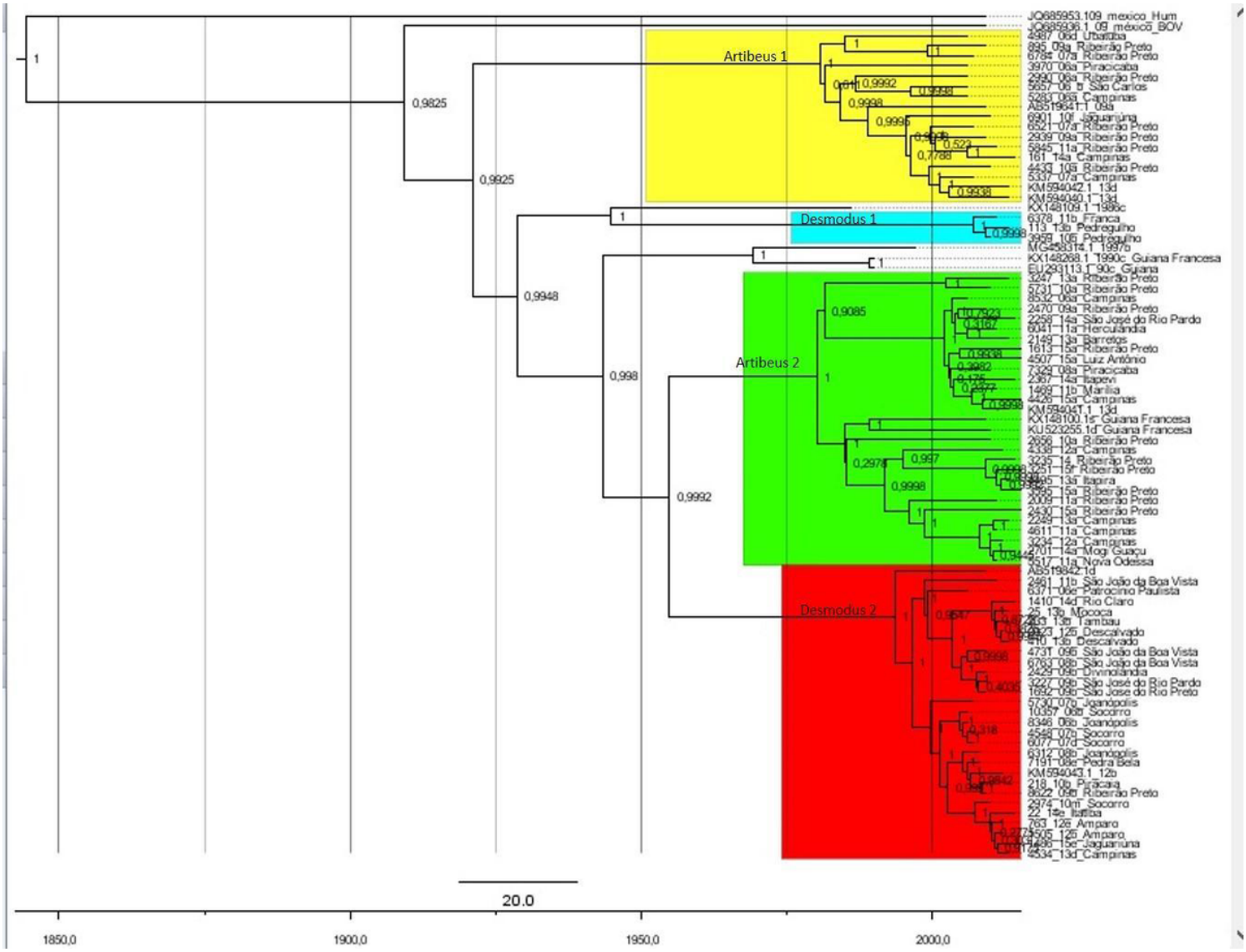

Figure 1

maximum clade credibility tree inferred from the concatenated N-P-M-G-L genes with respective posterior values obtained for each node. The branches are calibrated according to the year of isolation of the lineage. The scale at the bottom of the graph is in years and can be used to identify when RABV *Desmodus rotundus/Artibeus lituratus* underline separation events occur.

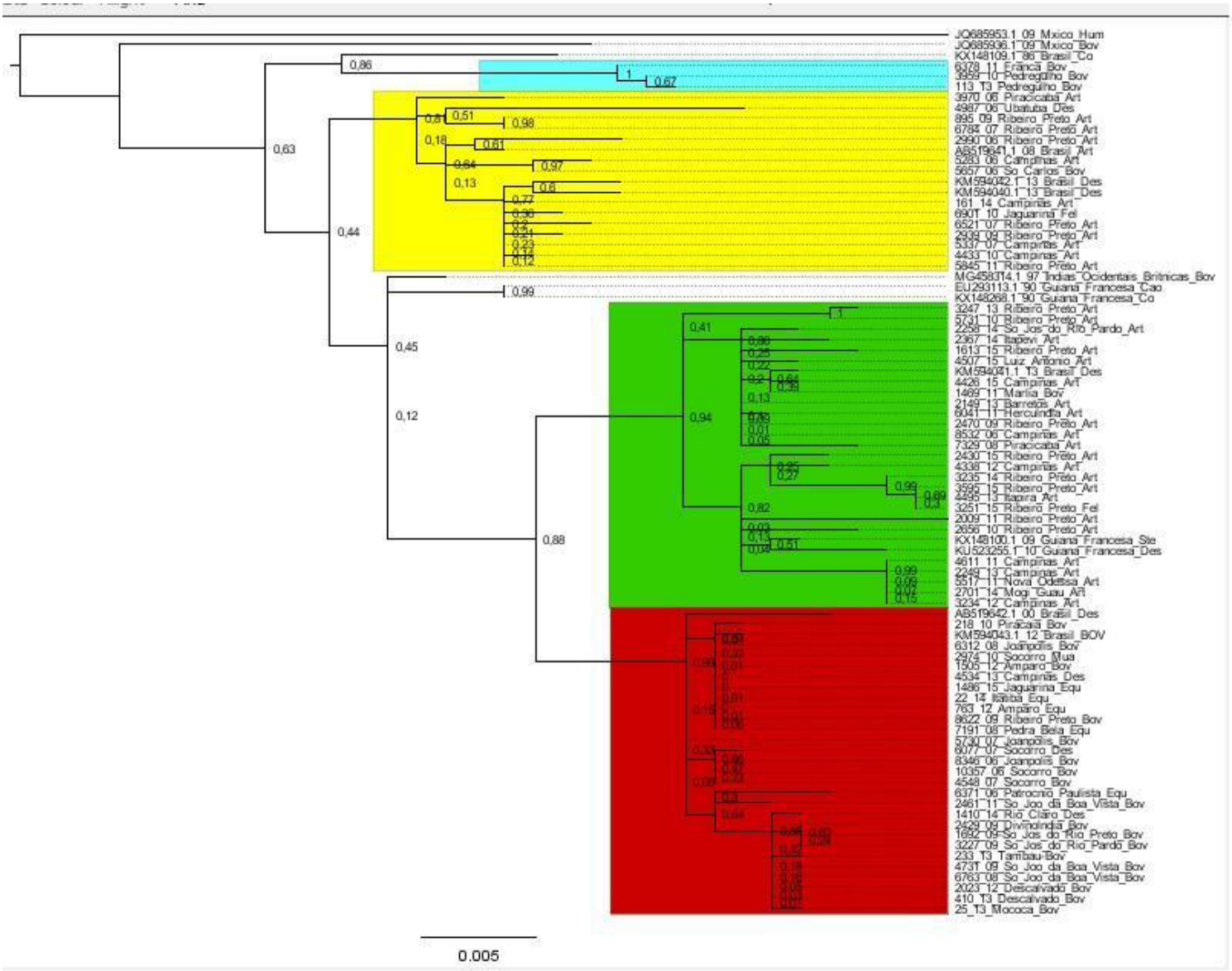

Figure 2

Maximum likelihood phylogenetic tree containing 84 sequences of rabies virus isolates (70 from this study and 14 sequences obtained from GenBank) with 799 nucleotides of the N gene. The tree was constructed using the Tamura 3-parameter evolutionary model + G (T92+G) with 1000 bootstrap repetitions.