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Communication

First Genomic Evidence of a Henipa-like Virus in Brazil

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Abstract: The viral genus *Henipavirus* includes two highly virulent zoonotic viruses of serious public health concern. *Hendra henipavirus* and *Nipah henipavirus* outbreaks are restricted to Australia and Southeast Asia, respectively. Henipavirus genus comprises mostly bat-borne viruses, but exceptions have already been described with novel viruses having rodents and shrews as reservoir animals. In the Americas, scarce evidence supports the circulation of these viruses. In this communication, we report a novel henipa-like virus from opossums (*Marmosa demerarae*) from a forest fragment area at Peixe-Boi municipality, Brazil, after which the virus was named the Peixe-Boi virus (PBV). The application of next-generation sequencing and metagenomic approach led us to discover the original evidence of a henipa-like virus genome in Brazil and South America and the original description of henipaviruses in marsupial species. These findings emphasize the importance of further studies to characterize PBV and clarify its ecology, impact on public health and its relationship with didelphid marsupials and other henipaviruses.

Keywords: metagenomics; henipavirus; marsupialia; opossums

1. Introduction

Environmental changes associated with socioeconomic factors contribute to reduce barriers between wildlife and human populations. Deforestation causes natural habitats destruction and changes ecosystems dynamics, creating an imbalance in enzootic cycles already well defined in nature, exposing humans and other animals, including domestic and livestock, to emerging pathogens [1].

Emerging viruses have represented severe public health issues in the past decades, with most of them originating in wild animals such as bats, rodents, non-human primates, and mosquitoes [2,3]. Arboviruses (Dengue, Zika, Chikungunya and Crimean-Congo hemorrhagic fever viruses), coronaviruses (SARS-CoV, MERS-CoV and SARS-CoV-2), filoviruses (Ebola and Marburg viruses), arenaviruses (Sabiá and Lassa fever viruses) and paramyxoviruses (*Hendra henipavirus* – HeV, and *Nipah henipavirus* - NiV) all emerged since the 1990s [3,4].

Paramyxoviruses have a wide host range among vertebrates and some of them already emerged in humans: measles virus, human mumps virus, human parainfluenza viruses, HeV and NiV. In addition, novel mammalian paramyxoviruses have been frequently identified, some of which may be pathogenic to humans, and under propitious conditions could transpose interspecies barriers and spillover, eventually causing outbreaks [5].

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Paramyxoviridae is a viral family currently comprising four subfamilies, 17 genera and 86 species [6]. It is a large group of RNA viruses with non-segmented, negative-sense genomes ranging in length about 14.296 to 20.148 nt. The genomes encode at least six proteins: a nucleocapsid protein (N), a matrix protein (M), a fusion protein (F), a receptor-binding protein (RBP) and a phosphoprotein (P) that associates to a polymerase protein (L) to compose the RNA-dependent RNA polymerase complex [7,8].

Henipavirus is a *Paramyxoviridae* genus that comprises some emerging viruses of serious public health concern. HeV and NiV, the main representatives of the viral genus, are highly virulent zoonotic viruses that cause neurological and respiratory diseases and have been reported to be responsible for outbreaks in humans since being first described in Australia and Malaysia in 1995 and 1998, respectively [5,8].

The zoonotic transmission of HeV occurs through contact with infected body fluids from horses, which act as amplifying hosts [9]. On the other hand, transmission of NiV is primarily related to contact with the saliva or urine of infected bats, consumption of meat derived from infected animals and from person-to-person via the respiratory route during outbreaks, which justifies the increasing concern of the emergence of not only NiV but also other henipaviruses [8].

Henipavirus outbreaks are currently restricted to Southeast Asia and Australia. For this reason, studies involving viruses of the genus outside of these geographic regions are less frequent. There are also important serological surveys and phylogenetic studies in both humans and animals conducted in Africa that indicate henipavirus is circulating on the continent. Moreover, a complete genome of a henipa-like virus was recently classified as a novel henipavirus species, *Ghanaian bat henipavirus* (GhV), with a still unknown zoonotic potential [10,11].

Almost all recognized species in the genus are bat-borne viruses. Bats of the the *Pteropus* genus are the primary hosts on Asia and Australia, with *Eidolon helvum*, the fruit bat from which GhV genome was first detected, possibly having an important role in Africa [5,10,12]. However, the *Mojiang henipavirus* (MojV), suspected to have caused human disease in China, was considered a possible exception due to its association with rodents of the *Rattus flavipectus* [13]. Recently, a novel rodent-borne henipavirus sequence, closely related to MojV, from a *Apodemus agrarius* host became available at the NCBI in September 2021 [14].

Furthermore, novel henipaviruses were described or had sequences submitted on GenBank database referring to being found in shrews, mostly of the genus *Crocidura* from China, South Korea, Belgium, and Guinea [14,15,16]. Besides, a presumably shrew-borne novel henipavirus, phylogenetically related to MojV, referred as Langya henipavirus, was associated to a febrile illness in patients from China, mostly farmer workers. The limited epidemiological data analysis indicated that human-to-human transmission was unlikely, and the human cases probably emerged from multiple spillover events [17]. These new data on the diversity of henipavirus in shrews reinforced the possibility that other mammals may harbor henipaviruses instead of only bats and rodents and highlight their potential of emerging as novel human pathogens despite the taxonomic classification of their reservoir animals.

In the Americas, scarce evidence supports the circulation of these viruses [10], possibly because of the few henipavirus surveillance studies on the continent. Here we report the first genomic finding of a henipa-like virus in Brazil and South America, a novel virus named the Peixe-Boi virus (PBV), which corroborates the circulation of henipaviruses far from Africa, Asia, and Australia. It is also the first description of henipa-like viruses in marsupials worldwide, indicating the importance of including these animals in surveillance studies.

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2. Materials and Methods

2.1. Samples Collection

In September 2015, tissue samples (spleen, lymph nodes, heart, and lungs) were collected from three specimens of *Marmosa demerarae* opossums (Figure 1) captured using Tomahawk and Sherman traps. Captured animals were anesthetized and euthanized by cervical dislocation and tissues were stored at -20°C prior to transport to the Department of Arbovirology and Hemorrhagic Fevers of Evandro Chagas Institute, where they were stored at -80°C.

Figure 1. *Marmosa demerarae* digital illustration.

The three opossums were captured in a fragmented forest area adjacent to the rural community of Ananin village in the municipality of Peixe-Boi, Pará State, Brazil (Figure 2). Traps were disposed 10 meters distant from each other in three distinct locations: trails inside the forest (A1 to A3), the forest edge (B1 to B3), and in the peripheral area of the community (C1 to C3). This region was chosen because of the high deforestation rate, one of the highest in the eastern Amazon (80.24% of its territory as of 2021).

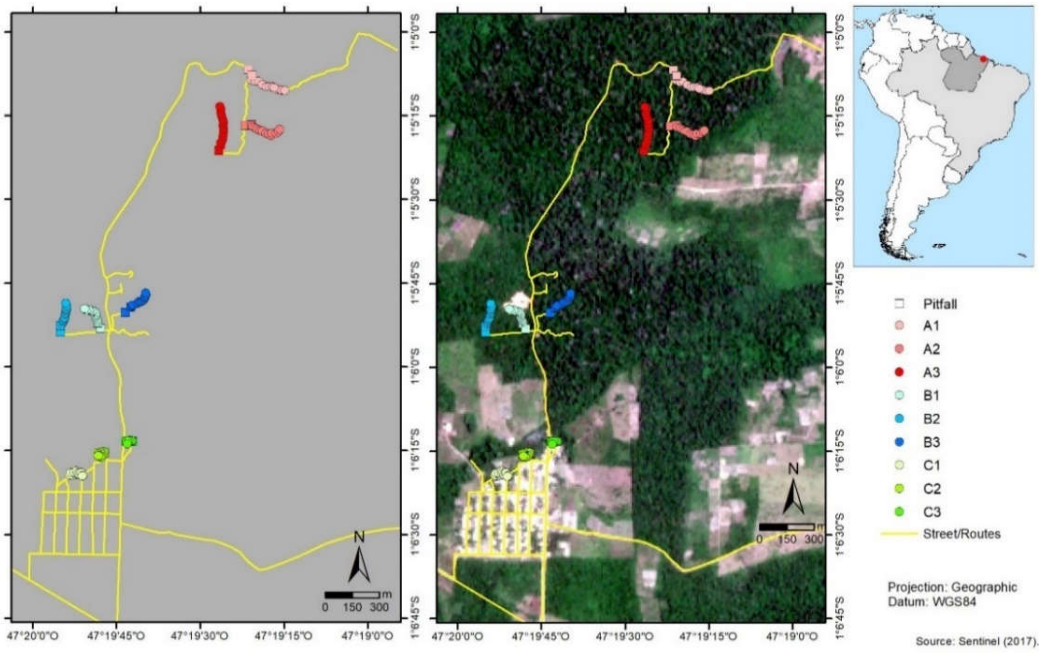

Figure 2. Sample collection sites in the proximities of Vila Ananin, municipality of Peixe-Boi, Pará State, Brazil.

Animal species were morphologically identified during sampling and confirmed by mitochondrial DNA analysis in laboratory (Figure S1, Table S1). This study was

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authorized by the Ethics Commission on Animal Use of the Evandro Chagas Institute under protocol no. 40/2019.

2.2. RNA Extraction and cDNA Synthesis

Samples from the three individuals were pooled since they were from the same species. A total of 5 mg fragment of the pooled tissue was homogenized in a tube filled with 600 µl of 1-Thioglycerol/Homogenization Solution and one 5 mm tungsten bead using the TissueLyser II system (Qiagen, Hilden, Germany) for 2 min at 25 Hz. RNA extraction was performed with a Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI, USA) in the Maxwell® 16 System (Promega) according to the manufacturer's protocol and was followed by double strand cDNA (complementary DNA) synthesis applying the SuperScript™ VILO™ Master Mix (Thermo Fischer Scientific, Waltham, MA, USA) for first strand synthesis and the NEBNext® mRNA Second Strand Synthesis Module (New England BioLabs, Ipswich, MA, USA) for second strand.

2.3. Sequencing and Sequence Assembly

The cDNA library was prepared with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Quantification of cDNA was assessed using Qubit 2.0 Fluorometer (Thermo Fischer Scientific) and fragments size range was evaluated using 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on the NextSeq 500 System (Illumina) based on 150 bp paired-end technology [18]. The generated results were assembled *de novo* by IDBA-UD (k-mers 20, 40, 60, 80 and 100) [19], and aligned against the non-redundant protein database by DIAMOND [20] with a 10^{-3} e-value threshold. The contigs were inspected at MEGAN6 [21] to identify those corresponding to viral sequences and then were mapped to reference at Geneious v.9.1.8 [22].

2.4. Phylogenetic Analysis

Multiple alignment of 86 sequences was performed using Clustal W analysis [23]. Of all these sequences, 72 were from *Paramyxoviridae* RefSeq genomes currently available, nine complete genomes of novel rodent and shrew-borne henipaviruses, four sequences of Brazilian jeilongviruses and PBV partial genome. The phylogenetic tree was built at IQ-TREE v.2 [24] rooted on the midpoint using the GTR+F+R7 nucleotide substitution model, which was defined by the software, and maximum likelihood (ML) analysis with 1000 bootstrap iterations [25]. The obtained trees were edited for graphical display using Inkscape v.1.1 [26].

3. Results and Discussion

One of the assembled contigs was classified as an unclassified paramyxovirus at MEGAN6. The sequence of 2.377 nt in length showed 54.2% of pairwise identity with the L gene (RNA polymerase) in the NiV reference genome (12.420 to 14.933 nt), with 137 gaps and corresponding to 13.02% of the viral genome and 34.14% of the L gene. The sequence was aligned to a set with all reference genomes of the *Paramyxoviridae* viral family deposited in the RefSeq database, except for *Cedar henipavirus*, which presented molecular differences in the aligned region with other sequences. Genomes of novel rodent and shrew-borne henipaviruses and jeilongviruses recovered from Brazilian bats (Amazon and Atlantic Forest) [27] were also included in the alignment, the latter due to their proximity with *Henipavirus* clade and their Brazilian origin.

Since *Henipavirus* genus is classified in the *Orthoparamyxovirinae* subfamily and a set of 85 sequences were included in the phylogenetic analysis, the other subfamilies were collapsed in the tree to highlight the results. All sequences included in the analysis are available at Table S2. The partial genome sequence of PBV was deposited in GenBank under the accession number MZ615319.

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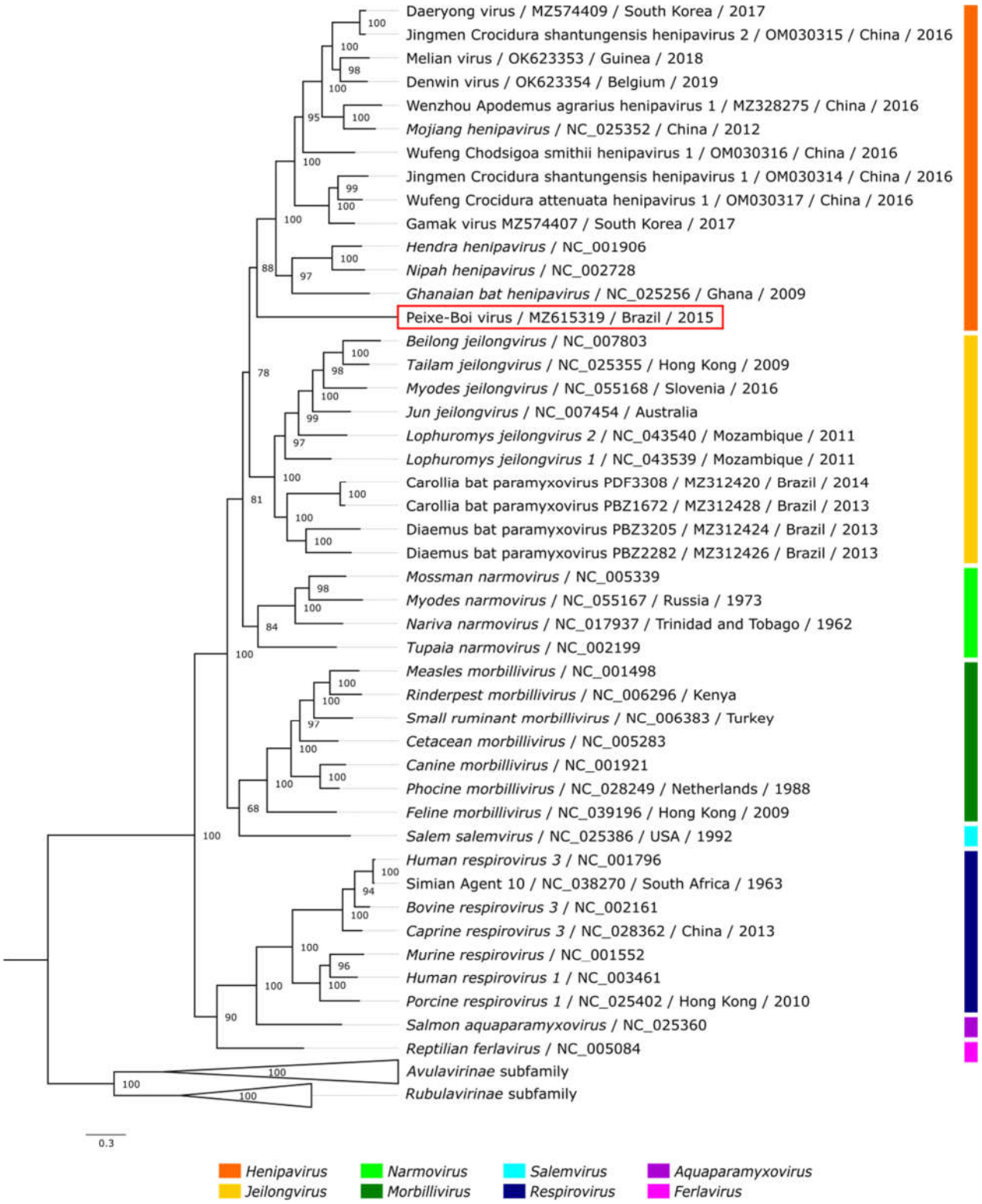

Figure 3. Phylogenetic tree based on the partial nucleotide sequence *Paramyxoviridae* L gene RefSeq sequences (except *Cedar henipavirus*), novel rodent and shrew-borne henipaviruses and Brazilian jeilongviruses sequences. PBV (red box) partial genome was detected in a pooled tissue sample of *Marmosa demerarae*. Each record consists of the virus species/name, accession number, and year of detection/isolation. *Orthoparamyxovirinae* subfamily is represented with genera indicated by color. The other two subfamilies are collapsed.

In our phylogenetic analysis, the sequence was clustered within the henipavirus clade and clearly separated from the novel Brazilian jeilongviruses clade (Figure 3), indicating that PBV is a henipa-like virus. Inside the henipavirus clade, PBV sequence stood

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on a sister clade of the bat-borne and the rodent/shrew-borne henipaviruses subclades, which could imply the existence of other lineages of henipaviruses. PBV partial L genomic sequence showed low nucleotide identity with NiV (54.72%) and HeV (54.76%) in the alignment, which was also observed between both and MojV [13] L sequence. Interestingly, similar to PBV, MojV was recovered from non-bat host (rodents).

The identification of a genome fragment related to henipavirus sequences is the original genomic evidence of the circulation of henipaviruses in Brazil and South America. Previously, just two other sequences were registered in the Americas. The only one available is a 559 bp sequence found in a feces sample of an insectivorous bat of the species *Pteronotus parnelli* collected in Costa Rica in 2010. This sequence corresponds to a fraction of the L gene and was classified, by phylogenetic analysis, as a henipa-like sequence of a distinct lineage from henipaviruses circulating in Africa, Southeast Asia, and Australia. The other henipa-like sequence was found in a frugivorous bat of the species *Carollia perspicillata*, also collected in Costa Rica, although it is unavailable for analysis. Both bat species are found in the Brazilian Amazon [10], and *Carollia perspicillata* bats are common in the municipality of Peixe-Boi. The attempt to include the available sequence to the phylogenetic inference failed to reproduce reliable results.

The two other findings in the Americas are from serological studies in bats. One was conducted in Trinidad and Tobago and found 28 bat serum samples positive for NiV glycoprotein (a type of RBP protein) or fusion protein on ELISA. These bats were of the species *Artibeus lituratus*, *Artibeus planirostris trinitatis*, and *Carollia perspicillata* [28], which were also identified with antibodies reactive to NiV in a Brazilian study [29].

The other study was performed in a Brazilian tropical savannah biome (Cerrado) in southeastern region of Brazil, in which sera from 76 bats were tested for the presence of cross-reactive NiV antibodies through an in-house developed NiV nucleoprotein ELISA and an IFA on NiV-infected Vero cells. They found positivity in 13 of 76 samples, including from the three species already cited in the Trinidad and Tobago study and *Desmodus rotundus* species [29], all of which are endemic in the Brazilian Amazon. Although none of the studies provided genomic detection, they support the hypothesis of henipavirus circulation in Latin America.

The detection of PBV is also the original description of henipa-like virus affecting marsupials worldwide. The remaining serological and molecular henipavirus findings from Latin America were found in bats, similar to most studies conducted in Africa, Southeast Asia, and Australia [10,28,29].

This finding suggests to the possibility of interspecies transmission of PBV to *Marmosa demerarae*. These opossums were previously classified as *Micoureus demerarae*, but after recent taxonomic reorganization based on phylogenetic data, the whole *Micoureus* genus is now considered a subgenus of *Marmosa* [30]. The species is restricted to South America and can be found in French Guyana, Guyana, Venezuela, Peru, and Brazil, including the Brazilian Amazon, and are known as specialized arboreal and nocturnal species with insectivorous and frugivorous diet [31,32], which could occasionally allow the sharing of habitat and food with some bat species, including the species cited in the studies of Trinidad and Tobago and Brazil [28,29]. If *Marmosa demerarae* is just an accidental host, a hypothetical transmission cycle for the reported henipa-like virus is a feasible possibility since bats are usually the primary hosts of important henipaviruses.

Another possibility is that PBV is a marsupial-borne henipavirus. Recently, it was demonstrated the existence of rodent-borne henipavirus [10,14], from China, and shrew-borne henipaviruses, from China, South Korea, Belgium, and Guinea [14-17], which further indicates the possibility of other mammals harboring henipaviruses. Although this matter remains unclear, due to lack of data about PBV genomic organization and ecological features, the marsupial-borne origin hypothesis is still supported by the high divergence of the nucleotide sequence and the isolated position of PBV in a sister clade to the bat-borne and rodent/shrew-borne subclades in the phylogenetic reconstruction. Further investigations on the *Henipavirus* diversity among didelphid marsupials might support more robust inferences about this possible new subclade.

The vulnerability of deforested environments such as those at the study site, where wild habitats overlap with rural areas, resembles the conditions in which HeV and NiV outbreaks arose [5]. This emphasizes the importance of further surveillance studies in the research area in an attempt to isolate and characterize the proposed virus and clarify its nature, the relationship with other henipaviruses and impact on public health. Furthermore, the role of didelphid marsupials, mainly the *Marmosa demerarae* species, in the hypothetical transmission cycle must be elucidated. This finding also instigates a wider inclusion of this group of mammals in *Henipavirus* surveillance studies.

Supplementary Materials: Figure S1: Structural representation of *Marmosa demerarae* mitochondrial DNA. Internal values indicate the content of the nucleotide bases. Yellow, green, and red blocks indicate rRNAs, PCGs, and tRNAs, respectively; Table S1: Description of *Marmosa demerarae* mitochondrial genes; Table S2: Genomes included in the multiple alignment and phylogenetic analysis.

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Data Availability Statement: The consensus sequence is deposited in GenBank under accession number MZ615319.

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