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Novel herpesviruses in neotropical bats and their relationship with other members of the Herpesviridae family

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ABSTRACT

In the past decade, a large number of studies have detected herpesvirus sequences from many bat species around the world. Nevertheless, the discovery of bat herpesviruses is geographically uneven. Of the various bat species tested to date, only a few were from the New World. Seeking to investigate the distribution and diversity of herpesviruses circulating in neotropical bats, we carried out molecular screening of 195 blood DNA samples from 11 species of three bat families (Phyllostomidae, Mormoopidae, and Molossidae). Using polymerase chain reaction amplification, with degenerate consensus primers targeting highly conserved amino acid motifs of the herpesvirus *DNA polymerase* and *Glycoprotein B* genes, we characterized novel viral sequences from all tested species. BLAST searches, pairwise nucleotide and amino acid sequence comparisons, as well as phylogenetic analyses confirmed that they all belonged to the *Herpesviridae* family, of the *Beta-* and *Gammaherpesvirinae* subfamilies. Fourteen partial *DNA polymerase* gene sequences, of which three beta- and 11 gamma-herpesviruses, were detected. A total of 12 partial *Glycoprotein B* gene sequences, all gamma-herpesviruses, were characterized. Every sequence was specific to a bat species and in some species (*Desmodus rotundus*, *Carollia perspicillata*, and *Pteronotus rubiginosus*) multiple viruses were found. Phylogenetic analyses of beta- and gammaherpesvirus sequences led to the identification of bat-specific clades. Those composed of sequences obtained from different bat species belonging to distinct subfamilies follow the taxonomy of bats. This study confirms the astonishing diversity of bat herpesviruses and broadens our knowledge of their host range. Nevertheless, it also emphasizes the fact that, to better appreciate the evolutionary history of these viruses, much remains to be done at various taxonomic levels.

Keywords: Chiroptera, Betaherpesvirus, Gammaherpesvirus, *DNA polymerase*, *Glycoprotein B*, evolution, phylogeny.

HIGHLIGHTS

- Molecular screening was carried out on 11 bat species from French Guiana and Martinique (French West Indies).
- Partial *DNA polymerase* gene sequences of 14 viruses were characterized, three beta- and 11 gammaherpesviruses, as well as 12 *Glycoprotein B* sequences, all of gamma-herpesviruses.
- Genetic characterization of these sequences reveals a high degree of genetic divergence.
- On a phylogenetic viewpoint, most of the newly discovered herpesvirus sequences fall within bat-specific clades well correlated with the taxonomy of their hosts.
- This study is the largest conducted to date in terms of species diversity from the New World.

1. INTRODUCTION

With over 1,200 species, representing more than 20% of all registered mammal species, Chiroptera is the second-largest species-rich mammalian order (Fenton, 1997). Bats are present on all continents except the poles and a few isolated oceanic islands. They are highly diverse in terms of their anatomy and lifestyles, and have different diets (insectivorous, frugivorous, nectarivorous, carnivorous, piscivorous, or hematophagous). Thanks to their biological traits, bats provide key ecosystem services (Kunz et al., 2011; López- Baucells et al., 2018). At the same time, as a natural reservoir of many viruses, their global distribution, abundance, ability to fly and migrate over large distances, and the diversity of their diets and sociality are all factors that favor the acquisition and spread of viruses (Calisher et al., 2006; Drexler et al., 2012; Mackenzie, 2005; Wong et al., 2007). They are thus considered to play a major role in the emergence and transmission of zoonotic viruses such as lyssaviruses, coronaviruses, paramyxoviruses, or filoviruses (Baker et al., 2013b; Calisher et al., 2006; Davis et al., 2006; Freuling et al., 2011; Halpin et al., 2000; Kupferschmidt, 2013; Leroy et al., 2005; Li, 2005; Luby et al., 2009; Smith and Wang, 2013; Towner et al., 2009; Wong et al., 2007; Yob et al., 2001; Zaki et al., 2012). Bats are also carriers of other viruses, including adeno-, astro-, polyoma-, picorna-, or herpesviruses (Drexler et al., 2011; Fagrouch et al., 2012; Jánoska et al., 2011; Lau et al., 2011; Y. Li et al., 2010).

Within the *Herpesvirales* order, viruses of mammals all belong to the *Herpesviridae* family. Members of this viral family are grouped into three subfamilies (*Alpha*-, *Beta*-, and *Gammapherpesvirinae*) and 13 genera (Davison et al., 2009; Pellett et al., 2011). This repartition into subfamilies and genera reflects the diversity of the different known viral species. This diversity concerns the nature of the host and the cellular tropism as well as the functional

organization of the viral genome and the pathogenic properties. In addition, each mammal species can carry multiple herpesvirus species that are host specific. However, there are examples of cross-species transmission (Ehlers et al., 2008). The first description of bat herpesvirus sequences dates back to 2007 (Wibbelt et al., 2007). Over the past decade, dozens of herpesvirus sequences have been described from different bat species on every continent (Anthony et al., 2013; Baker et al., 2013a; Dacheux et al., 2014; Dietrich et al., 2018; Donaldson et al., 2010; Escalera-Zamudio et al., 2016; Ge et al., 2012; Geldenhuys et al., 2018; He et al., 2013; Holz et al., 2018; Hu et al., 2017; Jánoska et al., 2011; L. Li et al., 2010; Molnár et al., 2008; Mühlendorfer et al., 2011; Noguchi et al., 2018; Paige Brock et al., 2013; Pozo et al., 2016; Razafindratsimandresy et al., 2009; Salmier et al., 2017; Sano et al., 2015; Sasaki et al., 2014; Shabman et al., 2016; Subudhi et al., 2018; Wada et al., 2018; Watanabe et al., 2010, 2009; Wray et al., 2016; Wu et al., 2012; Yang et al., 2013; Zhang et al., 2012; Zheng et al., 2016, 2018). Most of them were characterized from apparently healthy animals sampled during trapping campaigns in the frame of random surveillance programs. To date, two bat herpesviruses, fruit bat alphaherpesvirus 1 and bat gammaherpesvirus 8 (FBAHV1 and BGHV8, respectively) have been recognized as species by the International Committee for Taxonomy of Viruses (ICTV) according to the latest master species list (MSL# 34) released on March 8, 2019 (<https://talk.ictvonline.org/files/master-species-lists/m/msl/8266>). Their ICTV official names are *Pteropodid alphaherpesvirus 1* (PtAHV1) and *Vespertilionid gammaherpesvirus 1* (VeGHV1). They were classified in the *Simplexvirus* and *Percavirus* genera, respectively. As of November 2019, when it was last updated, the DBatVir (<http://www.mgc.ac.cn/DBatVir/>) database reported 233 bat herpesvirus sequences (Chen et al., 2014). A few others have not yet been included (Holz et al., 2018; Noguchi et al., 2018; Wada et al., 2018). They are distributed among the three *Herpesvirinae* subfamilies, but more than half of them (144 sequences) are unclassified *Herpesviridae*. In total, they were derived from 58 species

of seven bat families (Vespertilionidae, Pteropodidae, Rhinolophidae, Molossidae, Phyllostomidae, Miniopteridae, and Hipposideridae). Nevertheless, the discovery of bat herpesviruses is geographically uneven. Most sequences are from Asian, African, and European bat species. Comparatively few data are available regarding herpesviruses of New World species. Indeed, of the 233 recorded sequences, only eight are from New World bat species, six from North and Central America and two from South America (Donaldson et al., 2010; Escalera-Zamudio et al., 2016; Razafindratsimandresy et al., 2009; Salmier et al., 2017; Shabman et al., 2016; Subudhi et al., 2018). The ones from North and Central America were obtained from two insectivorous and two hematophagous bat species of the Vespertilionidae and Phyllostomidae families, respectively, and corresponded to beta- and gammaherpesvirus sequences (Donaldson et al., 2010; Escalera-Zamudio et al., 2016; Shabman et al., 2016; Subudhi et al., 2018). Those from South America, one alpha- and one gammaherpesvirus, were derived from bats belonging to the Phyllostomidae and Molossidae families, respectively (Razafindratsimandresy et al., 2009; Salmier et al., 2017). In addition, Wray *et al.* reported the characterization of two other herpesvirus sequences, DrHV-1 and DrHV-2, described as gamma- and betaherpesvirus, respectively, from *Desmodus rotundus* individuals from Guatemala (Wray et al., 2016). Unfortunately, these last sequences have not been released in the databases.

Strikingly, with the exception of the two aforementioned sequences, there has been no prior organized effort to discover herpesviruses in neotropical bat species from South America and in particular from Amazonia, which hosts one of the highest diversity of bat species in the world (López-Baucells et al., 2016; Paglia et al., 2012). To ascertain the distribution and diversity of herpesviruses circulating in neotropical bats, additional investigations were required. Taking advantage of a unique collection of bat samples collected during the past 10 years in French

Guiana (South America) and Martinique (French West Indies), we addressed the presence of herpesviruses in the different bat species collected by analyzing two distinct informative partial genes. The partial characterization of *DNA polymerase* and *Glycoprotein B* sequences constitutes a powerful tool in the search for new herpesviruses, which in addition allows unambiguous classification of the newly detected sequences within the different *Herpesvirinae* subfamilies. Here, we report finding sequences of herpesviruses in all New World bat species tested and describe their phylogenetic relationships.

2. RESULTS

2.1. Screening and characterization of partial sequences of herpesvirus *DNA polymerase* gene in bat samples.

To look for the presence of herpesvirus sequences in our collection of bats, we attempted to amplify a fragment of the highly conserved herpesvirus *DNA polymerase* (*DPol*) gene from the PBMC DNA of each wild-caught bat using two different sets of degenerate primers and PCR conditions, as described previously (Ehlers et al., 1999; Rose et al., 1997; VanDevanter et al., 1996). A total of 195 samples from 11 bat species were tested (**Table 1**). DNA samples (39/195 = 20%) from 10 species scored positive after the nested PCRs (nPCRs) (**Table 1**). No bat belonging to the *Pteronotus rubiginosus* species scored positive. BLAST searches demonstrated that all of the sequences identified belonged to the *Herpesviridae* family. In addition, cloning of all PCR products and sequencing of multiple clones of each amplicon allowed detecting different herpesvirus sequences for six of them, for a total of 45 sequences. Then, sequence comparison and phylogenetic analyses revealed the presence of 14 distinct sequences. Three sequences belonged to the *Betaherpesvirinae* subfamily while the other 11 belonged to the *Gammaherpesvirinae*. Three distinct sequences, all gamma, were obtained from the *D. rotundus*

individuals as well as two distinct gammaherpesvirus sequences from the *Carollia perspicillata* individuals. Informal virus names and abbreviations were given to the 14 distinct viruses, as explained in the *Materials and Methods* section (**Table 1**).

All obtained sequences were species-specific and all but the two sequences from *Molossus molossus* viruses, MmolBHV1 and MmolGHV1, and the one from *Pteronotus alitus*, PaliBHV1, were characterized from at least two individuals (**Table 1**). Thus, DrotGHV1, DrotGHV2, and DrotGHV3 were detected from 10, four, and three out of 22 *D. rotundus* individuals, respectively while DyouGHV1, AplaGHV1, and SangGHV1 were each identified from five individuals. We also characterized a few cases of co-infections: two *D. rotundus* individuals were co-infected with DrotGHV1 and DrotGHV2 and three with DrotGHV1 and DrotGHV3 as well as one *C. perspicillata* individual infected with CperGHV1 and CperGHV2.

We then designed species-specific antisense primers to extend each of the newly identified sequences in upstream direction by about 300 bp with a one round of semi-nested semi-specific PCR (**Supplementary Table 1**). This resulted in concatenated nucleotide sequences of 439 and 454 bp in size for betaherpesviruses and of 472 bp in size for gammaherpesviruses.

2.2. Screening and characterization of partial sequences of herpesvirus *Glycoprotein B* gene in bat samples.

We then attempted to amplify a 500-bp fragment of the *Glycoprotein B* (*Gb*) gene (**Table 1**). An amplification product of the expected size was obtained from 23 samples (23/195=11.8%) belonging to nine different bat species (**Table 1**). We did not obtain any amplification product from *Artibeus planirostris*, *P. alitus*, and *M. molossus* (from French Guiana) individuals.

BLAST searches of the obtained sequences followed by sequence comparison demonstrated that they all belonged to the *Herpesviridae* family and revealed the presence of 12 distinct sequences, all from the *Gammaherpesvirinae* subfamily. Among them, three different sequences were found in *D. rotundus* (DrotGHVA, DrotGHVB, and DrotGHVC) and two in *P. rubiginosus* (PrubGHVA and PrubGHVB). Each viral sequence detected was specific to a bat species and, even though some viruses were found in different individuals of the same species, we did not identify any case of co-infection (**Table 1**).

2.3. Pairwise sequence comparison of *DPol* sequences.

Sequence identity was determined by comparing nucleotide and amino acid sequences of all herpesviruses detected in the different bat species with viruses of the same subfamily described in several mammalian orders (**Supplementary Table 2 and Supplementary Table 3**). For clarity, comparison of the percentage of identity between the different newly identified bat viruses with those of other bat species is reported by grouping viral sequences at the host family level and with those of other mammals according to the viral genus. All sequences obtained from the various bat species differed from each other at the nucleotide and amino acid levels. Sequences obtained from different specimens of the same species were 100% identical, with the exception of DrotGHV1 sequences showing 99.6–100% identity at the nucleotide level and DrotGHV3 sequences showing 99.2–100% nucleotide identity and 97.5–100% amino acid identity (**Supplementary Table 3**).

For betaherpesviruses, the three new sequences exhibited among themselves from 53.6% (StilBHV1 vs. PaliBHV1) to 65.4% (StilBHV1 vs. MmolBHV1) nucleotide identity and from 63.7% to 66.9% amino acid identity (**Supplementary Table 2**). They showed 48.8–67.3%

nucleotide identity and 43.5–75% amino acid identity with the other bat betaherpesviruses. The three viruses were more distantly related to betaherpesviruses of Rhinolophidae than to those of Miniopteridae, Molossidae, and Vespertilionidae. Finally, they were more closely related to viruses of the *Cytomegalovirus* and *Muromegalovirus* genera than to those of the two other betaherpesvirus genera (**Supplementary Table 2**).

Considering gammaherpesviruses, the new sequences exhibited among themselves from 48.6% (DrotGHV2 vs. DyouGHV1) to 95.5% (McoiGHV1 vs. MmolGHV1) nucleotide identity and from 43.2% (DrotGHV1 vs. DrotGHV2) to 97.5% (McoiGHV1 vs. MmolGHV1) amino acid identity (**Supplementary Table 3**). The percentage of identity between the three viruses detected in *D. rotundus* ranged from 49% to 55.1% at the nucleotide level and from 43.2% to 58% at the amino acid level. The two sequences detected in *C. perspicillata* showed 62.9% and 64.2% identity in nucleotides and amino acids, respectively. Comparison with the other bat gammaherpesviruses yielded similar results, ranging from 44.1% to 86.9% nucleotide identity and from 40.7% to 91.4% amino acid identity. In addition, the level of nucleotide and amino acid identity with the other mammal gammaherpesviruses ranged from 42% to 69.4% and from 35.8% to 69.1%, respectively.

2.4. Pairwise sequence comparison of *Gb* sequences.

Sequence identity of *Gb* sequences was determined by comparing sequences of all bat gammaherpesviruses identified as well as with those of other mammal gammaherpesviruses representative of the different gammaherpesvirus genera (**Supplementary Table 4**). All sequences obtained here from the different bat species differed from each other at the nucleotide and amino acid levels. Sequences obtained from several individuals of the same species were

100% identical with the exception of DrotGHVA sequences, showing 99.1–100% sequence identities at the nucleotide and amino acid levels, and PrubGHVB sequences showing 99.4% nucleotide identity and 98.3% amino acid identity (**Supplementary Table 4**). The percentage of identity between the three viruses detected in *D. rotundus* ranged from 62.1% to 85.5% at the nucleotide level and from 59.1% to 93% at the amino acid level, while the two distinct sequences detected in *P. rubiginosus* showed 56.4% and 54.8–55.7% nucleotide and amino acid identities, respectively.

Overall, the percentages of nucleotide and amino acid identities between the newly obtained sequences with those already published from bats or other mammals were higher or on the same order of magnitude as for *DPol* sequences (**Supplementary Table 3** vs. **Supplementary Table 4**). Thus, the new sequences exhibited among them from 56.4% (PrubGHVA vs. PrubGHVB) to 92% (McoiGHVA vs. MmolGHVA) nucleotide identity and from 51.3% (CperGHVA vs. PrubGHVB) to 95.7% (McoiGHVA vs. MmolGHVA) amino acid identity (**Supplementary Table 4**). Comparison with the other chiropteran gammaherpesviruses showed from 48.7% to 93.7% nucleotide identity and from 41.7% to 98.3% amino acid identity. Finally, the level of nucleotide and amino acid identity with the other mammal gammaherpesviruses ranged from 49.3% to 69.8% and from 45.2% to 74.8%, respectively.

2.5. Phylogenetic analyses of betaherpesvirus *DPol* sequences.

Phylogenetic analyses performed on nucleotide sequences of the newly characterized betaherpesvirus partial *DPol* sequences with those of other betaherpesviruses available in the databases grouped the bat viral sequences in three well-supported monophyletic clades only composed of bat virus sequences (**Figure 1**). These monophyletic groups are distinct from the

other groups of *Betaherpesvirinae* corresponding to the genera *Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus*, and *Proboscivirus*. The first group of bat sequences is composed of two viral sequences detected in *Rhinolophus ferrumequinum* (Rhinolophidae) (Pozo et al., 2016, Wu et al., 2012). These viruses are closely related to viruses of the *Roseolovirus* genus. The two other lineages are close to the *Cytomegalovirus* genus. The first one is only composed of sequences derived from Vespertilionidae bats. In addition, this clade is divided into two different groups, (a) one containing sequences detected in bats belonging to the genus *Myotis* and the one identified from *Tylonycteris robustula* and (b) the second one containing viral sequences detected from the other Vespertilionidae genera (*Nyctalus*, *Pipistrellus*, *Eptesicus*, etc.). The third clade is composed of sequences of Molossidae, Phyllostomidae, Mormoopidae, and Miniopteridae. It is further subdivided into four well-supported lineages corresponding to the different families of the bats studied. The three betaherpesvirus sequences we characterized belong to this third clade. The sequence from *M. molossus*, MmolBHV1, is grouped with sequences from *Tadarida* in a clade of Molossidae viruses. The two others, StilBHV1 and PaliBHV1, obtained from bats of the Phyllostomidae and Mormoopidae families, respectively, are the only representative viruses of these bat families. StilBHV1 and PaliBHV1 belong to well-supported monophyletic lineages of Molossidae + Phyllostomidae and of Mormoopidae + Miniopteridae viruses, respectively.

2.6. Phylogenetic analyses of gammaherpesviruses *DPol* sequences.

The phylogenetic analysis based on *DNA polymerase* sequences of *Gammaherpesvirinae* shows that bat viral sequences are distributed all over the tree (**Figure 2**). Based on this sequence dataset, all that could be obtained is a multifurcating tree with low support for the deepest nodes. Therefore, resolution of the branching pattern is incomplete and phylogenetic relationships

between the different *Gammaherpesvirinae* genera are not supported. Nevertheless, in the terminal branchings, certain highly supported clades can be recognized. Two of them correspond to the *Lymphocryptovirus* (LCV) and Old World primate *Rhadinovirus* (OWPRHV) genera. Three others are bat-specific (clades I, II, and III). Eight of the 11 new sequences reported here fall within two clades (clades I and II). These two clades only comprise viral sequences of Phyllostomidae. Clade I consists of five novel sequences (AgeoGHV1, DrotGHV3, AplaGHV1, SangGHV1, and CperGHV1 from *A. geoffroyi*, *D. rotundus*, *A. planirostris*, *S. angeli*, and *C. perspicillata*, respectively) representative of the four different subfamilies of Phyllostomidae tested. Clade II comprises three viral sequences infecting Desmodontinae bats: two new sequences, DrotGHV1 and DyouGHV1, from *D. rotundus* and *Diaemus youngi*, as well as the one previously described from *Diphylla ecaudata* (*Diphylla ecaudata* gammaherpesvirus SD12, accession number KU942403). Clade II also includes, at its basis, CperGHV2 from *C. perspicillata*. Clades I and II are highly supported with posterior probability (pp) values of 0.93 and 1, respectively. The third clade, clade III, supported by a pp of 0.93, is constituted of previously published viral sequences detected from Vespertilionidae of different species and from one Pteropodidae species. Regarding the three other sequences we identified that do not belong to these well-supported clades, the third sequence from *D. rotundus*, DrotGHV2, clusters with sequences of the *Lymphocryptovirus* genus, although with a low support, while the two sequences from *Molossus*, MmolGHV1 and McoiGHV1, are grouped together in a separate lineage.

2.7. Phylogenetic analyses of gammaherpesviruses *Gb* sequences.

The phylogenetic analysis between the newly characterized bat gammaherpesvirus *Gb* sequences and that of already published sequences from bats and of representative

gammaherpesvirus sequences available in the databases is presented in **Figure 3**. Almost all nodes of the tree are highly supported ($pp > 0.7$) and the main clades of *Lymphocryptovirus*, *Rhadinovirus*, *Macavirus*, and *Percavirus* genera can be recognized. This analysis clearly places the bat viral sequences into 10 well-supported (pp over 0.91) and bat-specific clades (named a to j) scattered over the entire tree. Five clades are only composed of sequences obtained from bat species of the same family (clades c, e, g, h, and j) while the other five comprise sequences from bats belonging to different families (clades a, b, d, f, and i). Some sequences do not belong to these clades: They correspond to previously published viral sequences from *Scotophilus kuhlii* and *Rhinolophus blythi* closely associated with bovine herpesvirus sequences (Bovine herpesvirus 6 and Bovine lymphotropic herpesvirus) that belong to the *Macavirus* genus and to the viral sequence derived from *Hipposideros pomona* that is associated with *Sorex araneus* gammaherpesvirus (SaraGHV1) and viral sequences from Carnivora (MusHV1, FcatGHV1 and LrufGHV1) but with a pp value of less than 0.7 (Watanabe et al., 2009; Zheng et al., 2016). This last sequence and those of clades a and b from bat species of the Vespertilionidae, Rhinolophidae, and Miniopteridae families belong to a monophyletic well-supported ($pp=0.96$) lineage of Percaviruses.

All new sequences detected here fall within four distinct clades (d, e, g, and j). Clade d is composed of sequences of Mormoopidae (PrubGHVA) and of Phyllostomidae of the Desmodontinae subfamily that includes viral sequences from *D. rotundus*, *D. youngi*, and *D. ecaudata*. Two distinct sequences from *D. rotundus*, DrotGHVB and DrotGHVC, belong to this clade. These two sequences are closely associated with the *D. rotundus* gammaherpesvirus sequence previously described from Mexico (Escalera-Zamudio et al., 2016). In addition, the sequence from *D. youngi*, DyouGHVA, is associated with the one from *D. ecaudata* (Escalera-

Zamudio et al., 2016). Within this clade, viruses of Desmodontinae form a highly supported (pp=1) subclade associated with PrubGHVA from *P. rubiginosus*. In addition, clade d belongs to a highly supported monophyletic lineage (pp=0.88) also composed of two other clades, clade c of Pteropodidae bats and the *Rhadinovirus* clade of Old World primates (OWPRHV). Clade e comprises five newly identified Phyllostomidae viruses, AgeoGHVA from *A. geoffroyi*, CperGHVA from *C. perspicillata*, SangGHVA and StilGHVA, from *S. angeli* and *S. tildae*, respectively, and DrotGHVA from *D. rotundus*. The phylogenetic relationships between the different viral sequences of clade e are well correlated with the taxonomy of the bats and the different well-supported subclades are differentiated according to the subfamilies (Glossophaginae, Carollinae, Stenodermatinae, and Desmodontinae) to which the infected bats belong. Indeed, viruses from *Sturnira* species (SangGHVA and StilGHVA) group together and are related to CperGVHA from *C. perspicillata* in a monophyletic clade with a pp value of 0.96. These two clades are related to AgeoGHVA with a pp value of 0.95. Finally, these Stenodermatinae, Carollinae, and Glossophaginae clades group together with DrotGHVA of Desmodontinae in a monophyletic clade (clade e) supported with a pp value of 1. The same phylogenetic clustering that correlated with the bat subfamilies from which the viruses have been detected is also observed for clade h of Vespertilionidae viruses. Clade e is located at the basis of a highly supported monophyletic lineage comprising *Percavirus* and *Rhadinovirus* sequences, with the exception of rhadinoviruses of Muridae (BsavRHHV1, McerRHHV1 and MmusRHHV1), bat sequences of clades a–d, as well as the unclassified *Tapirus terrestris* and *Saimiri sciureus* gammaherpesviruses, TterGHV1 and SsciGHV2. From its side, clade g is composed of sequences from the two Molossidae species tested, *M. molossus* and *M. coibensis*. It possesses a basal position relative to a large clade comprising all percaviruses and rhadinoviruses including rhadinoviruses of Muridae as well as the unclassified *Procapra capensis* gammaherpesvirus,

PcapGHV2, and clade f. Finally, clade j, composed of two sequences of PrubGHVB from *P. rubiginosus*, is located at the basis of the *Gammaherpesvirinae* subfamily.

3. DISCUSSION

This is the largest study conducted to date to assess the occurrence and diversity of herpesviruses in New World bat species. A viral sequence was obtained for every tested species. Each obtained sequence was novel, species-specific and no case of cross-transmission between bat species was identified. Except for *P. rubiginosus*, for which we did not generate any *DPol* sequence, and *A. planirostris* and *P. alitonus*, for which no *Gb* sequence was amplified, the two types of sequences were obtained for all other species. Nevertheless, for the species from which both sequences were obtained, PCR targeting *Gb* was less sensitive than the one targeting *DPol* with a lower number of positive samples (**Table 1**). In addition, for *Gb*, only gammaherpesvirus sequences were obtained. These results prove that, to screen samples, the combinations of primers targeting *DPol* are a better tool both in terms of sensitivity and diversity of amplified sequences. This also suggests that for *Gb* other combinations of primers should be used to allow for amplification of sequences of other subfamilies. Nevertheless, the fact that we preferentially amplified gammaherpesvirus sequences is not only a question of primer degeneracy, but also depends on the type of samples tested. Indeed, of all the bat herpesviruses currently described in the literature, most alpha- and betaherpesviruses (globally underrepresented compared with gammaherpesviruses) were amplified from oral swabs (Pozo et al., 2016; Razafindratsimandresy et al., 2009). It will therefore be important for future studies to test not only other bat species but also different types of samples from the same species.

From a phylogenetic perspective, in view of the available data, it appears that differential clustering exists between bat betaherpesvirus sequences according to their host families (**Figure 1**). In addition, these clusters are also congruent with the evolutionary relationships of most large groups within families (Hoofer and Bussche, 2003; Simmons, 2005; Teeling, 2005). Nevertheless, the phylogenetic relationships between them are not concordant with the current interpretations of the host pattern of diversification at the family level (Agnarsson, 2011). Even though the different clades are all well supported, interpretations are currently limited by the fact that only a few bat betaherpesvirus sequences are available and that only one betaherpesvirus sequence is available for certain families while others are not represented.

Regarding *Gammaherpesvirinae*, the phylogeny based on *Gb* sequences is well supported with well-defined clades corresponding to the different known genera. Comparatively, the topology of the *DPol* tree is less reliable and exhibits poor overall support. The two phylogenies are based on alignments of similar size, and thus the lower support observed for *DPol* can be attributed to an overall higher level of sequence divergence. Nevertheless, the two phylogenies show that bat gammaherpesviruses are scattered over the entire tree (**Figures 2 and 3**). They also demonstrate that viral sequences from Phyllostomidae are distributed in two distinct clades, while those of Molossidae fall together on a separate branch. Based on the *Gb* phylogeny, which is in agreement with the known phylogeny of *Gammaherpesvirinae*, we further observe that viruses of Phyllostomidae are close to the *Rhadinovirus* and *Percavirus* genera, while those of Molossidae possess a basal position relative to these genera. Finally, within the different clades (c, d, e, and h), composed of viral sequences obtained from bat species belonging to different subfamilies, the phylogenetic relationships of the different viral sequences demonstrate a good correlation with the taxonomy of the host species (**Figure 3**) (Agnarsson, 2011). These results are in support of a

co-evolutionary scenario. Nevertheless, each bat-specific clade is only represented by a few sequences from different species ($2 < n < 5$). Owing to the paucity of the available data, considering the number of bat species, genera, subfamilies, and families that remain to be tested, this assumption will only be confirmed when more sequences become available.

This study greatly expands our knowledge on the distribution and genetic variation of bat herpesviruses. It adds new insights into the viral diversity hosted by bats from French Guiana and Martinique and, as may be expected, confirms the astonishing diversity of bat herpesviruses. However, we are still far from having deciphered the in-depth details of this diversity. Indeed, the number of New World bat species tested still accounts for only a tiny part. In French Guiana alone, 107 bat species are currently recognized, with the number evolving steadily, mostly due to the splitting of taxa on the basis of new genetic evidence (Catzefflis, 2017, 2015; Simmons and Voss, 1998). Therefore, the number of tested species accounts for just 10% of the local bats, suggesting a wide diversity of herpesviruses awaiting discovery. These results emphasize the crucial need for a better assessment of herpesvirus distribution in bats. Analysis of other species at a wider geographical scale, as well as of the same species but on other types of samples, should maximize our chances of detecting the whole diversity of bat herpesviruses and thereby expanding our understanding of the diversification processes and evolutionary history of these viruses.

4. MATERIALS AND METHODS

4.1. Ethical and legal statements. All animals were captured, handled, and sampled following ASM guidelines under the supervision of researchers granted the French animal experimentation level 1 diploma (Sikes and the Animal Care and Use Committee of the

American Society of Mammalogists, 2016). For bats captured in Martinique, ad hoc authorization was received (# 2014 140-008) delivered by the Préfecture de Martinique. In French Guiana, bats are not protected by law, but captures that occurred within protected areas (nature reserves) received ad hoc authorizations (# 35 from 03/21/2013 and # 59 dated 04/17/2013 delivered by the Préfecture de la Guyane). In compliance with the Access and Benefit Sharing procedure implemented by the Loi pour la Reconquête de la Biodiversité, the use of the genetic resources was declared to the French Ministry of Environment under reference # TSP 128 316, and received an internationally recognized certificate of compliance reference # ABSCH-IRCC-FR-246973-1.

4.2. Collection of specimens and biological material and DNA extraction. All bats examined in this study were collected as part of an investigation program on rabies virus circulation. In French Guiana, captures were implemented in bat communities during a 10-year period (de Thoisy et al., 2016). In Martinique, bats were captured in January 2015. All captures were performed at night with Japanese mist nets erected near breeding sites, roosts, at forest edges, around livestock, or through putative foraging courses. Animals were kept in individual bags before being sampled for blood with a sterile needle and capillary at the brachial vein. Before release, external pressure was exerted on the vein with a sterilized absorbent hemostatic sponge to prevent bleeding and facilitate healing. Blood samples were preserved at 4°C until arrival at the laboratory and centrifuged at 6500 g for 10 min to separate sera. Sera and buffy coat samples were stored at -80°C for later use in the laboratory. The sex and age of all animals were recorded, and the animals were identified morphologically in the field. When possible errors of species identification were suspected, identification was molecularly confirmed by sequencing a fragment of the mitochondrial *Cytochrome oxidase I* or *Cytochrome b* genes (Borisenko et al.,

2008). Nucleic acids were extracted using the NucliSENS easyMAG® bio-robot (bioMérieux®, Marcy l'Etoile, France). A total of 195 DNA samples from 11 bat species belonging to three families (Phyllostomidae, Mormoopidae, and Molossidae) were obtained (**Table 1**).

4.3. Screening of samples. Molecular screening was done by semi-nested or nested PCR amplifications with degenerate consensus primers targeting highly conserved amino acid (aa) motifs of the herpesvirus *DNA polymerase* and *Glycoprotein B* genes (**Supplementary Table 1**) (Prepens et al., 2007; Rose et al., 1997; VanDevanter et al., 1996). For the *DNA polymerase* amplification, two sets of primers targeting the same region of the gene but with different levels of degeneracy were used on each DNA sample in separate reactions for the first-round PCR (DFASA/GDTD1B or DFA+ILK/KG1) and second-round PCR (VYGA/GDTD1B or TGV/IYG) (**Supplementary Table 1**) (Rose et al., 1997; VanDevanter et al., 1996). For the *Glycoprotein B* amplification, one set of primers was used: 2759s/2762as for the first-round PCR and 2760s/2761as for the second-round PCR (66). All amplicons of approximately the expected size were purified, cloned *via* TA cloning, and sent for sequencing to Genewiz®, Takeley, UK (<https://www.genewiz.com/>). To help increase the possibility of identifying different herpesvirus sequences from each amplicon obtained, five to eight clones of the “screening amplicons” were sequenced on both strands.

4.4. Partial *DNA polymerase* gene amplification. To obtain the nucleotide sequence upstream of the VYGA or TGV motif, species-specific non-degenerate primers were derived from the complementary sequences of the small fragments and used in an nPCR amplification with the DFASA or DFA primer pools using the initial PCR products as templates (**Supplementary Table 1**). Overlapping amplicons were generated, cloned, and sequenced as

described above. Each sequence corresponds to at least three independent clones sequenced on both strands. Contig sequences were then assembled using MEGA 5.05 software (Tamura et al., 2011). The concatenated nucleotide sequences are between 439 and 472 bp in size depending on the strain.

4.5. Phylogenetic analysis. Raw sequences were analyzed and edited in MEGA 5.05 (Tamura et al., 2011). Sequence homology analyses were performed using the BLAST program at the National Center of Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (48). Then, multiple sequence alignments were constructed using ClustalW with all other previously published bat herpesvirus sequences extracted from GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>) or DBatVir (<http://www.mgc.ac.cn/DBatVir/>) as well as representative sequences for each genus or subfamily. Alignments were checked manually. Nucleotide and amino acid sequences were checked for gaps. Pairwise sequence identity (at the nucleotide and amino acid levels) of the partial coding sequences was calculated with MEGA version 5.05 using uncorrected p-distances.

Phylogenetic trees were inferred from the aligned nucleotide sequences. The best-fitted model of nucleotide substitution (GTR + I + G) was determined for each dataset using MEGA 5.05 under corrected Akaike information criteria (AICc) and used for the Bayesian approach with Mr. Bayes 3.2.2 (49, 50). Markov Chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations, with four simultaneous chains, using a sample frequency of 100 and a burn-in of 25,000. Majority rule consensus trees were obtained from the output. Validation of the inference was assessed based on the standard deviation of split frequencies, which was less than

the expected threshold value of 0.01. Trees obtained were visualized using the FigTree v1.4 program (<http://tree.bio.ed.ac.uk/software/figtree/>) (Rambaut, 2009).

4.6. Informal naming of novel herpesviruses. For the readability of the paper, the novel viruses were named trinomially according to the host species from which they were detected and the viral subfamily they were assigned to. The first two words designate the name of the bat species in italics, while the third word designates the tentative assignment of the novel virus to a herpesvirus subfamily within the *Herpesviridae*. Then, a number (for *DNA polymerase* sequences) or a letter (for *Glycoprotein B* sequences) was given to differentiate different viruses obtained from the same host species and belonging to the same herpesvirus subfamily. Example: *Desmodus rotundus* Gammaherpesvirus 1. Abbreviations use the first letter of the generic host name in uppercase and the first three letters of the specific host name, followed by three uppercase letters corresponding to the viral subfamily (BHV or GHV for Betaherpesvirus or Gammaherpesvirus, respectively) to which they were then assigned the Arabic numeral (1, 2, etc.) for the *DNA polymerase* sequences or a letter in uppercase following alphabetical order (A, B, etc.) for the *Glycoprotein B* sequences. Example: *Desmodus rotundus* GammaHerpesVirus 1, DrotGHV1.

4.7. Accession numbers. The sequences reported in this paper were deposited in the GenBank database under accession numbers **MN850443** to **MN850483**.

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AUTHORS' CONTRIBUTION

SJ and VL conceived the project; AL and BdT provided samples; SJ and DD performed the molecular and genetic analyses; SJ, AL, and VL performed the sequence and phylogenetic analyses; SJ, AL, and VL analyzed the data; SJ and VL wrote the paper. All authors read and commented on the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing financial interests.

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FIGURE LEGEND

FIG. 1. Phylogenetic tree of betaherpesvirus *DNA polymerase* sequences. The phylogenetic tree was derived from the partial nucleotide sequences of the *DNA polymerase* gene (471 bp) of 84 representatives of betaherpesviruses using the Bayesian method with the GTR + I + G model of nucleotide evolution. Human herpesvirus 4 sequence (HHV4 NC_009334) served as outgroup. The tree is shown as a majority rule consensus tree. Support for nodes was provided by the posterior probabilities of the corresponding clades. All resolved nodes have posterior probability greater than 0.75. A scale indicating divergence, as substitutions per site, is at the foot. Sequences generated in this study are in boldface. The virus names are associated with their accession numbers. The *Proboscivirus*, *Roseolovirus*, *Muromegalovirus*, and *Cytomegalovirus* genera are collapsed for clarity, and the size of the collapsed clade is arbitrary. The collapsed *Proboscivirus* clade comprises sequences of Elephant endotheliotropic herpesviruses: EEHV1 (NC_020474), EEHV1B (HM568550), EEHV2 (HM568558), EEHV3 (JQ300065), EEHV4 (EU658934), EEHV5 (NC_024696), EEHV6 (HM060765) and EEHV7A (JQ300083). The *Roseolovirus* clade comprises sequences of MndHV β (AF282942), PanHV6 (AY359407), HHV6A and HHV6B (NC_001664 and NC_000898, respectively), HHV7 (NC_001716), PtroHV7 (KJ843227 and KJ843228), PpanHV7 (KJ843230), GgorHV7 (KJ843231), and MneHV7 (NC_030200). The *Muromegalovirus* clade is composed of RatCMV/MuHV2/MuHV8 strains (AY728086, KP967684 and NC_019559), RexuCMV1 (EF125071), BindCMV3 (EF125067), MmusCMV2 (GU017485), MCMV/MuHV1 (AM886412 and NC_004065), MarvCMV1 (EF125059), MglacMV1 (EF125061), AflaCMV2 (EF125063), and RatCMV Maastricht (NC_002512). Finally, viruses of the *Cytomegalovirus* collapsed clade are: AoHV1 (FJ483970), PpitCMV1

(KU963229), AsenCMV1 (KU963225), AmacCMV1 (KU963227), ApanCMV1 (KU963228), SalbCMV1 (KU963231), SaHV4 (FJ483967), CapeCMV1 (KU963230), CebHV1 (JQ264772), MndCMV (AY129399), BaCMV (NC_027016), CeHV5 strains (JQ264771 and FJ483968), MfasCMV1 strains (KP796148 and AY728171), McHV3 (AF033184 and DQ120516), CgueCMV1.1 (AY129397), GgorCMV2.1 (FJ538490), PnHV2 (AF480884), and HHV5 strains (M14709, AC146905 and AY315197).

FIG.2. Phylogenetic tree of Gammaherpesvirus *DNA polymerase* sequences. The phylogenetic tree was derived from the partial nucleotide sequences of the *DNA polymerase* gene (489 bp) of 80 representatives of gammaherpesviruses using the Bayesian method with the GTR + I + G model of nucleotide evolution. Herpes simplex virus type 1 sequence (HHV1 M10792) served as outgroup. The tree is shown as a majority rule consensus tree. Support for nodes was provided by the posterior probabilities of the corresponding clades. All resolved nodes have posterior probability greater than 0.75. A scale indicating divergence, as substitutions per site, is at the foot. Sequences generated in this study are in boldface. The virus names are associated with their accession numbers. For bat viruses, the host species from which the virus has been detected is indicated after the virus name. In addition, they are color-coded according to the bat families. Abbreviations of virus names use the first letter of the generic host name in uppercase and the first three letters of the specific host name followed by either GHV (Gammaherpesvirus), LCV (*Lymphocryptovirus*), RHV (*Rhadinovirus*), or HV (Herpesvirus) and an Arabic numeral (1, 2, 3, 4) depending on the virus. Therefore, Rfer stands for *Rhinolophus ferrumequinum*, Ccro for *Crocota crocata*, Ezeb for *Equus zebra*, Pvam for *Pteropus vampyrus*, Sara for *Sorex araneus*, Lruf for *Lynx rufus*, Fcat for *Felis catus*, Tbel for *Tupaia belangeri*, Emax for *Elephas maximus*, Deca for *Diphylla ecaudata*, Pham for *Papio hamadryas*, Ggor for *Gorilla gorilla*, Mfas for

Macaca fascicularis, Asyl for *Apodemus sylvaticus*, Bind for *Bandicota indica*, MglA for *Myodes glareolus*, Bbab for *Babyrousa babyrussa*, Sbar for *Sus barbatus*, Dbic for *Diceros bicornis*, Ssci for *Saimiri sciureus*, Tter for *Tapirus terrestris*, Mmus for *Mus musculus*, Mcer for *Mus cervicolor*, Bsav for *Bandicota savilei*, Pcon for *Puma concolor*, Pleo for *Panthera leo*, Ptro for *Pan troglodytes*, Mfus for *Macaca fuscata*, Pcap for *Procavia capensis*, Rrup for *Rupicapra rupicapra*, and Hlib for *Hexaprotodon liberiensis*. Others that do not exactly follow this nomenclature are listed: HDHV1 stands for *Hipposideros diadema* herpesvirus 1, MrGHV1 and MrGHV2 for *Myotis ricketti* gammaherpesvirus 1 and 2, respectively, EHV2 and EHV5 for Equid herpesvirus 2 and 5, respectively, VeGHV1 for *Vespertilionid* Gammaherpesvirus 1, MusHV1 for Mustelid herpesvirus 1, SaHV2 for Saimiriine herpesvirus 2, AtHV3 for Ateline herpesvirus 3, PLHV2 and 3 for Porcine lymphotropic herpesvirus 2 and 3, respectively, MGHV for Megabat gammaherpesvirus, CalHV3 for Callitrichine herpesvirus 3, HHV4 and 8 for Human herpesvirus 4 and 8, MHV68 for Murine herpesvirus 68, BoHV4 and 6 for Bovine herpesvirus 4 and 6, respectively, OvHV2 for Ovine herpesvirus 2, AlHV1 for Alcelaphine herpesvirus 1, T2rRHV for Type 2 ruminant rhadinovirus, BLHV for Bovine lymphotropic herpesvirus, SuHV1 for Suid herpesvirus 1. Latin numerals I, II, and III on the right-hand side indicate the three well-supported bat gammaherpesvirus clades. LCV and OWPRHV on the right-hand side stand for *Lymphocryptovirus* and Old World primate *Rhadinovirus*.

FIG.3. Phylogenetic tree of Gammaherpesvirus *Glycoprotein B* sequences. The phylogenetic tree was derived from the partial nucleotide sequences of the *Glycoprotein B* gene (464 bp) of 101 representatives of gammaherpesviruses using the Bayesian method with the GTR + I + G model of nucleotide evolution. Herpes simplex virus type 1 sequence (HHV1 JN555585) served as outgroup. The tree is shown as a majority rule consensus tree. Support for nodes was provided

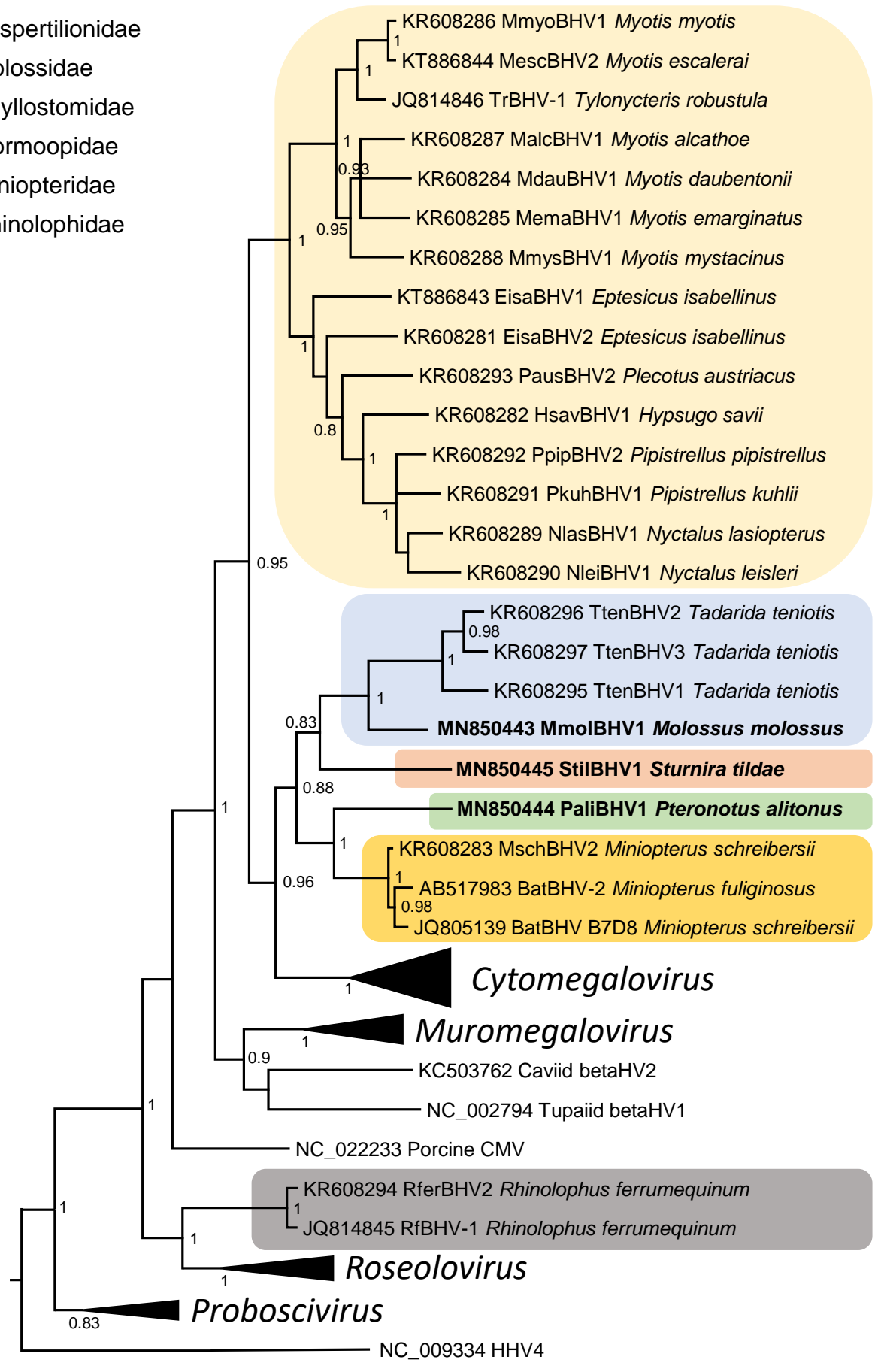
by the posterior probabilities of the corresponding clades. All resolved nodes have posterior probability greater than 0.7. A scale indicating divergence, as substitutions per site, is at the foot. Sequences generated in this study are in boldface. The virus names are associated with their accession numbers. For bat viruses, the host species from which the virus has been detected is indicated after the virus name. They are further color-coded according to bat families. Abbreviations of virus names follow the same system as for Figure 2. SsynLCV2 stands for *Symphalangus syndactylus* lymphocryptovirus 2, CpRHV2 for Caprine herpesvirus 2, MusHV1 for Mustelid herpesvirus 1 and RGHV1 for *Rhinolophus* gammaherpesvirus 1. The different well-supported bat gammaherpesvirus clades are indicated by letters (a, b, ..., j) on the right-hand side. OWPRHV on the right-hand side stands for Old World primate *Rhadinovirus*.

TABLE 1. New World bat species tested for herpesviruses using molecular methods and survey results

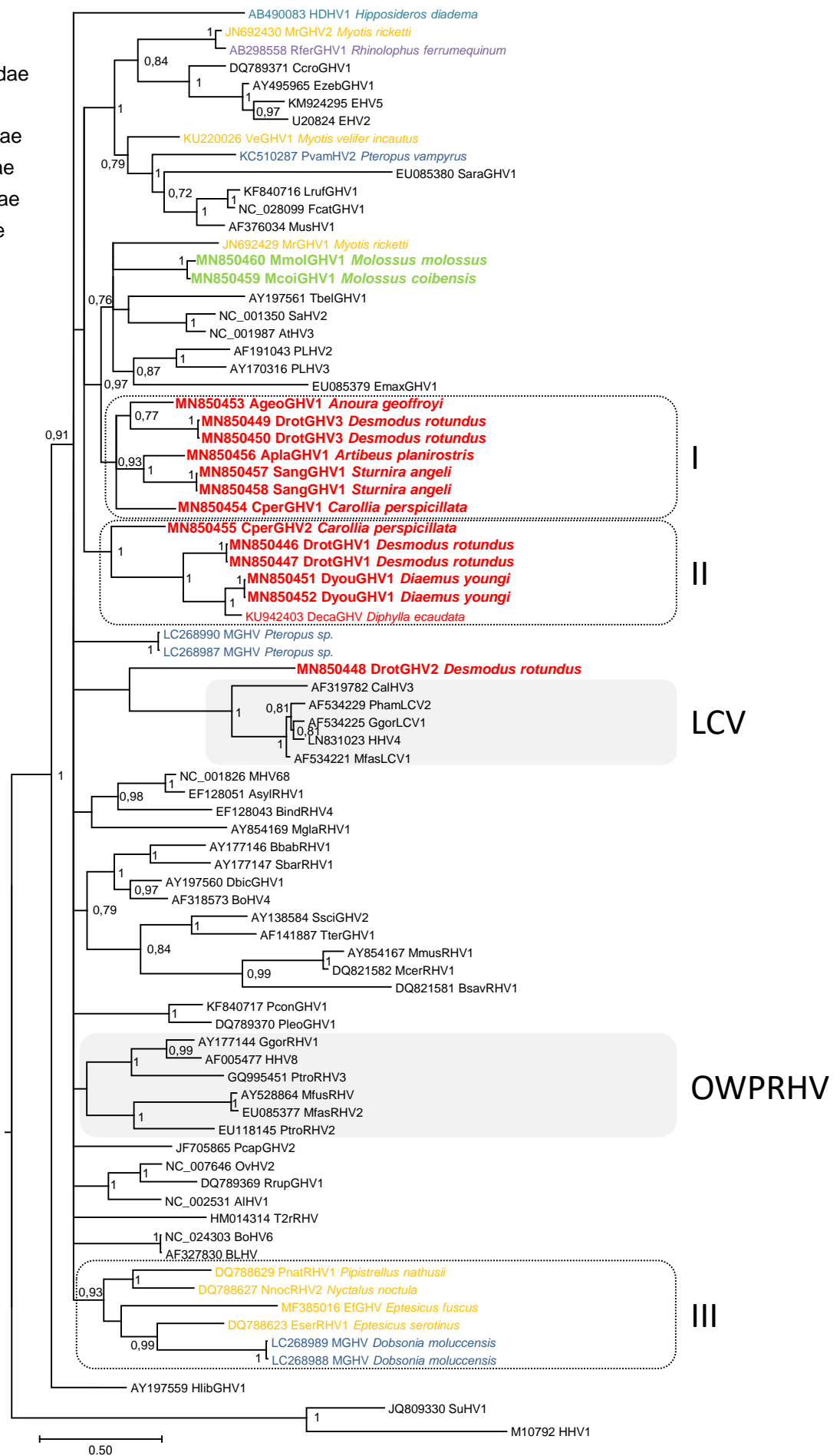
O	Family	Subfamily	Species	Common name	Origin	N	DNA Polymerase			Glycoprotein B		
							Informal name	Acronym	n	Informal name	Acronym	n
Chiroptera	Phyllostomidae	Desmodontinae	<i>Desmodus rotundus</i>	Common vampire bat	French Guiana	22	<i>Desmodus rotundus</i> Gammaherpesvirus 1	DrotGHV1	10	<i>Desmodus rotundus</i> Gammaherpesvirus A	DrotGHVA	5
							<i>Desmodus rotundus</i> Gammaherpesvirus 2	DrotGHV2	4	<i>Desmodus rotundus</i> Gammaherpesvirus B	DrotGHVB	3
							<i>Desmodus rotundus</i> Gammaherpesvirus 3	DrotGHV3	3	<i>Desmodus rotundus</i> Gammaherpesvirus C	DrotGHVC	1
		Glossophaginae	<i>Diaemus youngi</i>	White-winged vampire bat	French Guiana	8	<i>Diaemus youngi</i> Gammaherpesvirus 1	DyouGHV1	5	<i>Diaemus youngi</i> Gammaherpesvirus A	DyouGHVA	1
			<i>Anoura geoffroyi</i>	Geoffroy's tailless bat	French Guiana	20	<i>Anoura geoffroyi</i> Gammaherpesvirus 1	AgeoGHV1	2	<i>Anoura geoffroyi</i> Gammaherpesvirus A	AgeoGHVA	1
		Carollinae	<i>Carollia perspicillata</i>	Seba's short-tailed bat	French Guiana	20	<i>Carollia perspicillata</i> Gammaherpesvirus 1	CperGHV1	2	<i>Carollia perspicillata</i> Gammaherpesvirus A	CperGHVA	2
							<i>Carollia perspicillata</i> Gammaherpesvirus 2	CperGHV2	2			
		Stenodermatinae	<i>Artibeus planirostris</i>	Flat-faced fruit-eating bat	French Guiana	20	<i>Artibeus planirostris</i> Gammaherpesvirus 1	AplaGHV1	5			
			<i>Sturnira tildae</i>	Tilda's yellow-shouldered bat	French Guiana	18	<i>Sturnira tildae</i> Betaherpesvirus 1	StilBHV1	2	<i>Sturnira tildae</i> Gammaherpesvirus A	StilGHVA	1
			<i>Sturnira angeli</i>		Martinique	18	<i>Sturnira angeli</i> Gammaherpesvirus 1	SangGHV1	5	<i>Sturnira angeli</i> Gammaherpesvirus A	SangGHVA	4
	Mormoopidae		<i>Pteronotus rubiginosus</i>		French Guiana	15				<i>Pteronotus rubiginosus</i> Gammaherpesvirus A	PrubGHVA	1
			<i>Pteronotus alitonus</i>		French Guiana	5	<i>Pteronotus alitonus</i> Betaherpesvirus 1	PaliBHV1	1	<i>Pteronotus rubiginosus</i> Gammaherpesvirus B	PrubGHVB	2
	Molossidae		<i>Molossus coibensis</i>	Coiban mastiff bat	French Guiana	17	<i>Molossus coibensis</i> Gammaherpesvirus 1	McoiGHV1	2	<i>Molossus coibensis</i> Gammaherpesvirus A	McoiGHVA	1
			<i>Molossus molossus</i>	Pallas's mastiff bat	French Guiana	12	<i>Molossus molossus</i> Gammaherpesvirus 1	MmolGHV1	1			
			<i>Molossus molossus</i>	Pallas's mastiff bat	Martinique	20	<i>Molossus molossus</i> Betaherpesvirus 1	MmolBHV1	1	<i>Molossus molossus</i> Gammaherpesvirus A	MmolGHVA	1

Abbreviations: O, order; N: number of tested animals; n: number of herpesvirus-positive animals (by PCR, cloning and sequencing) for either *DNA polymerase* or *Glycoprotein B*.

- Figure(s)**
- Vespertilionidae
 - Molossidae
 - Phyllostomidae
 - Mormoopidae
 - Miniopteridae
 - Rhinolophidae

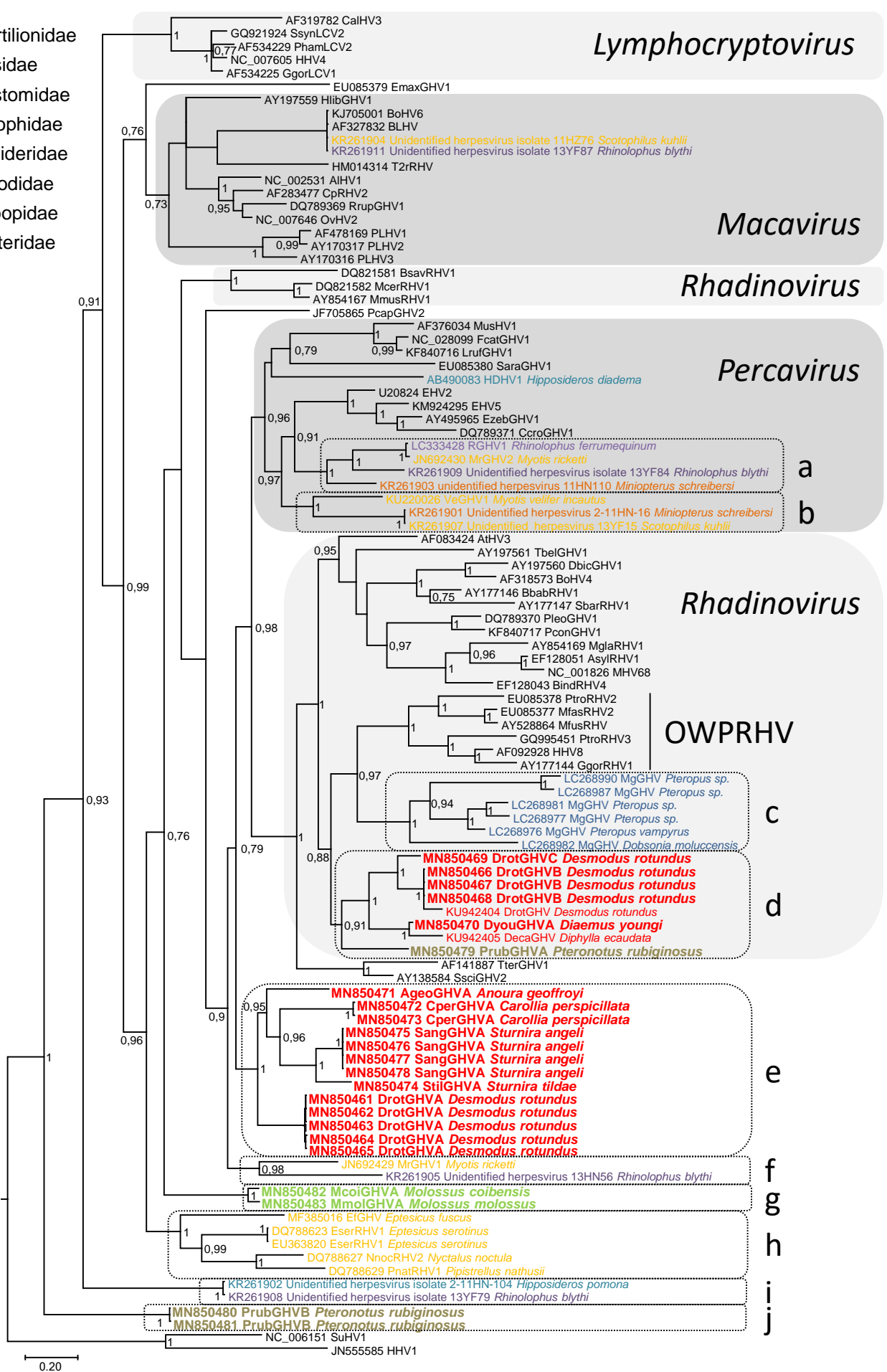


Figure(s)



Figure(s)

-  Vespertilionidae
-  Molossidae
-  Phyllostomidae
-  Rhinolophidae
-  Hipposideridae
-  Pteropodidae
-  Mormoopidae
-  Miniopteridae



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