

Distinct Lineage of Vesiculovirus from Big Brown Bats, United States

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We identified a novel rhabdovirus, American bat vesiculovirus, from postmortem tissue samples from 120 rabies-negative big brown bats with a history of human contact. Five percent of the tested bats were infected with this virus. The extent of zoonotic exposure and possible health effects in humans from this virus are unknown.

Bats are reservoirs for many emerging viral pathogens, including Ebola viruses, Marburg viruses, henipaviruses, and severe acute respiratory syndrome coronaviruses; >80 bat virus species have been characterized (1,2). The diversity of these viruses and their high infection rates in bats may be attributed to multiple factors that facilitate virus transmission and maintenance, including bats' large social group size, high species diversity, long life, long-distance migration, roost sharing by multiple species, and social habits such as mutual grooming and biting (1,2).

Rabies virus (family *Rhabdoviridae*, genus *Lyssavirus*) is commonly detected in bats from the United States. Analyses of several cases of human rabies infections have reported insectivorous bats as the source (3). The *Rhabdoviridae* family contains 6 formally approved genera, but most bat rhabdoviruses belong to the *Lyssavirus* genus (Figure). Nonrabies lyssaviruses have been characterized from bats in other parts of the world, including Australia, Europe, Africa, and Asia (4–9). In contrast to the known diversity in bats of the extensively analyzed *Lyssavirus*

genus, the diversity of other *Rhabdoviridae* genera in bats remains largely undetermined. Vesiculoviruses (genus *Vesiculovirus*), such as vesicular stomatitis virus, cause fever and vesicular diseases in animals such as cattle, horses, and pigs. Some vesiculoviruses, including Chandipura virus and vesicular stomatitis virus, are also zoonotic and cause acute diseases in humans.

The bat virome has not been fully characterized. Most bat virome studies have been conducted by analyzing fecal, anal swab, or pharyngeal swab specimens from healthy bats (10–13). These studies have revealed a variety of viruses but no new rhabdoviruses. However, viruses in fecal and pharyngeal samples could include ingested and inhaled viruses that originated from insects and plants (10–13). To focus specifically on viruses infecting the bats themselves, we performed unbiased metagenomic sequencing of RNA viruses purified from the lungs and livers of 120 rabies-negative big brown bats (*Eptesicus fuscus*) collected in Maryland, USA.

The Study

During 2008, more than 500 bats associated with possible human exposure were submitted to the Maryland Department of Health and Mental Hygiene State Laboratory for postmortem diagnosis of rabies by direct fluorescent antibody assay (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/12/12-1506-Techapp1.pdf). For this study, virus particles were purified from the lungs and livers of 120 rabies-negative bats with good carcass condition, and viral nucleic acids were extracted, randomly amplified, and sequenced by using 454 pyrosequencing (Roche, Mannheim, Germany) and Solexa Illumina sequencing (Illumina, San Diego, CA, USA). A total of ≈100,000 pyrosequences and 13.5 million Solexa Illumina sequences were generated and then assembled to form contigs. More than 30 contigs showed low protein identities to known vesiculoviruses (BLASTx; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), indicating a possible novel virus. PCR and rapid amplification of cDNA ends were performed to obtain the complete genome of this virus (primers shown in online Technical Appendix Figures 1, 2). We proposed the name American bat vesiculovirus (ABVV) for this virus.

The full-length ABVV genome (GenBank accession no. JX569193) consists of 10,692 nt of negative-sense, single-stranded RNA beginning with a 103-nt 5' untranslated region, followed by open reading frames encoding for the nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase protein genes (Figure, panel A). Phylogenetic analyses showed that ABVV is related to vesiculoviruses including Chandipura virus and Isfahan virus, both of which are associated with encephalitic illness in humans. ABVV is located close to the root of vesiculoviruses in the Bayesian analysis of the nucleoprotein gene

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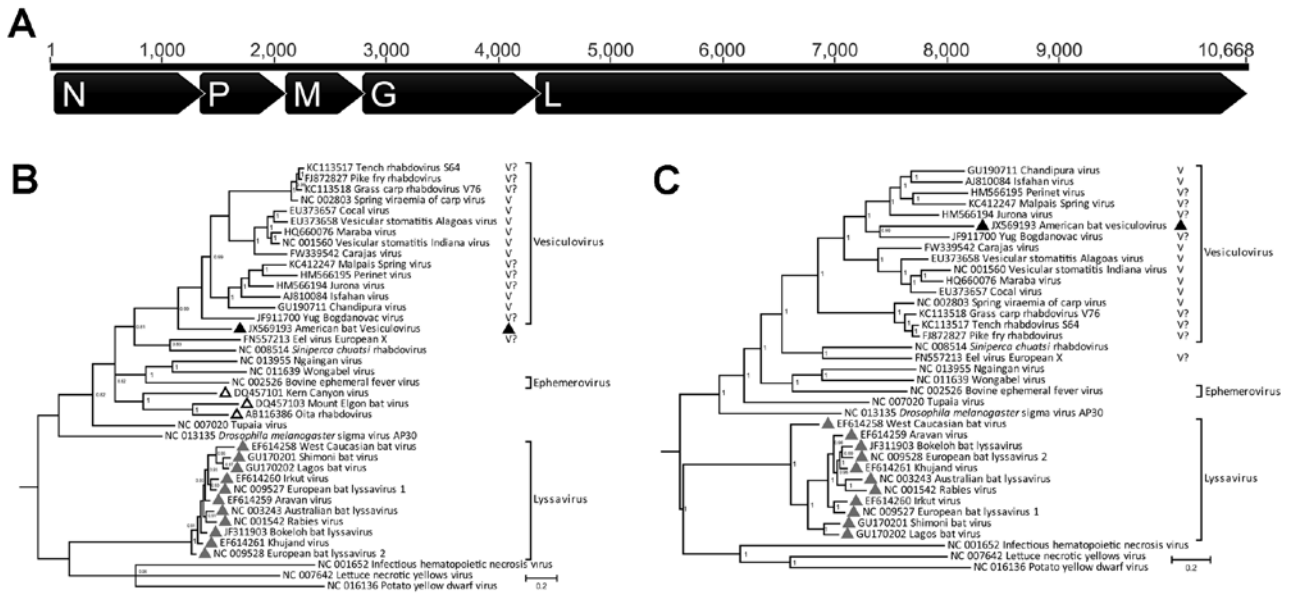


Figure. Analyses of American bat vesiculovirus (ABVV) compared with other members of the family *Rhabdoviridae*. A) Genome organization of ABVV; B) Bayesian inference tree of the ABVV N gene; C) Bayesian inference tree of the 5 concatenated ABVV genes (N, P, M, G, L). For the Bayesian analyses, sequences from the entire gene were used, except for a few partially sequenced genomes for which only ≈ 100 aa were publicly available. Posterior probabilities ($>75\%$) of the Bayesian analysis are shown next to each node. Formally classified vesiculoviruses are labeled with "V," whereas potential vesiculoviruses not formally recognized by the International Committee on Taxonomy of Viruses are labeled with "V?." Distinct clades of bat rhabdoviruses are labeled with triangles of different colors: black, vesiculovirus; gray, lyssavirus; white, unclassified. N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, polymerase protein. Scale bar indicates amino acid substitutions per site.

(Figure, panel B) and shares 41%–49% aa identity with known vesiculoviruses, similar to the vesiculovirus interspecies identities reported (47.9%–72.5%) and higher than the intergenera identities between vesiculoviruses, lyssaviruses, and ephemeroviruses (17.0%–33.1%) (14). Analyses of the polymerase gene alone (online Technical Appendix Figure 3) and of a concatenation of all 5 genes (Figure, panel C) suggested that ABVV lies within the vesiculovirus clade, more closely related to mammalian than fish vesiculoviruses. Combined, these analyses indicate that ABVV is likely to belong to the *Vesiculovirus* genus, rather than representing a novel genus. The basal phylogenetic position of ABVV suggests early divergence from other mammalian vesiculovirus species.

Lung and liver tissues from 60 of the bats used for the pooled metagenomic analyses were screened individually for ABVV by using reverse transcription PCR targeting the polymerase gene (online Technical Appendix). Three (5%) bats tested positive for ABVV: 1 adult female, 1 adult male, and 1 juvenile male. Viral RNA was found in liver tissue from the 2 male bats and in lung and liver tissues from the female adult bat.

Considering the extensive lyssavirus diversity in bats, we hypothesize that bat vesiculoviruses are similarly diverse. To facilitate characterization of diverse vesiculoviruses

in bats, we designed 2 pairs of degenerate PCR primers (VesiConAF-KCDGAYAARAGYCAITCVATGA; VesiConAR-TGNGCNACDGTNARDGCATT; VesiConBF-GGNMGRITTYTCHYTDATGTC; VesiConBR-TCHGCNGAYTGATNGTYTCA) on the basis of a sequence alignment of the polymerase gene of ABVV and the formally classified mammalian vesiculoviruses. When the ABVV-positive bat liver cDNA was used as a control, the nested PCR yielded an amplicon of 704 bases, and its sequence was confirmed by cloning and Sanger sequencing. Future studies may use these pan-vesiculovirus PCR primers to investigate vesiculovirus diversity in other bat species and in other regions.

Conclusions

Big brown bats are prevalent in North America, where their geographic range overlaps extensively with that of humans, and considerable interactions occur between big brown bats and humans and their pets. Big brown bats from this region are a known reservoir of rabies virus; our analysis shows that these bats also constitute a sylvatic mammalian reservoir of vesiculoviruses.

The characterization of ABVV sheds light on vesiculovirus diversity in bats. The other bat rhabdoviruses—Mount Elgon bat virus, Oita rhabdovirus, and Kern Canyon

virus—do not belong to the vesiculovirus clade but cluster together in a separate clade (14) (Figure, panel B). A recent report described several rhabdoviruses in oropharyngeal swab specimens from Spanish bats, but the short reads (100 bases) precluded a detailed phylogenetic analysis (15). Of the bats tested in our study, 5% were infected with ABVV, a finding that suggests vesiculoviruses are prevalent in bats. The characterization of a novel rhabdovirus in bats with a history of human contact raises questions for further research, including health effects on the virus' hosts, seroprevalence, possible transmission by insect vectors, and the extent of zoonotic exposure in humans. ABVV-specific and vesiculovirus-consensus PCRs, as well as future endeavors to culture this virus, will help address these questions.

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Dr Ng is a postdoctoral fellow at the Blood System Research Institute and University of California, San Francisco. His current research focuses on metagenomic discovery of novel human and animal viruses using deep sequencing, including rhabdoviruses, caliciviruses, picornaviruses, picaliviruses, hepeviruses, and astroviruses.

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Technical Appendix

Materials and Methods

Sample collection and viral metagenomic sequencing

In 2008, >500 bats associated with possible human exposure were submitted to the Maryland Department of Health and Mental Hygiene State Laboratory for the postmortem diagnosis of rabies by direct fluorescent antibody assay. The carcasses of the bats negative for rabies were kept frozen at -80°C . A total of 120 bats were selected for necropsy, including 30 individuals for each of the following categories: juvenile male, juvenile female, adult male and adult female. The lungs and livers were dissected from the bats and pooled for virus purification and metagenomic sequencing according to previously described protocols (1,2). In short, virus particles were purified from the tissues using homogenization, filtration and nuclease treatment. Purified viral RNA was amplified with random primers using the TransPlex Whole Transcriptome Amplification kit (Sigma-Aldrich) according to manufacturer's instructions. The resulting shotgun libraries were sequenced using 454 pyrosequencing with GS FLX+ Titanium, as well as Solexa Illumina sequencing. A total of 100 thousand pyrosequences and 13.5 million Illumina sequences were generated. Sequences were trimmed and those sharing at least 95% nucleotide identities over 35 bases were assembled into contigs (3). Assembled contigs were compared to the GenBank non-redundant protein database using BLASTx with an E-value cutoff of 10^{-4} (4).

Complete genome sequencing

The complete genome of the American bat vesiculovirus (ABVV) was obtained by Sanger dideoxy sequencing of PCR products obtained using combinations of primers designed throughout the genome (Technical Appendix Figures 1 and 2) and the 5' and 3' extremities were obtained using Rapid Amplification of cDNA Ends (RACE). PCR primers were designed from metagenomic contigs using PRIMER3 (5). PCRs were performed using LA Taq (Clontech) with reagent concentrations according to the manufacturer's instructions. PCR reactions were carried out with a "universal touch-down PCR" suitable for the melting temperatures of all primers, as follows: 95°C for 5 min, 45 cycles of [94°C for 1 min, 58°C minus 0.2°C per cycle for 1 min, 72°C for 1 to 5 min], followed by 72°C for 10 min. Amplicons were sequenced to their entirety by Sanger sequencing. The 5' and 3' genome extremities were amplified using RACE amplification kits (Invitrogen) according to the manufacturer's instructions and previously described protocols (4).

To determine the relationship of ABVV to other rhabdoviruses, a phylogram was created based on the amino acid sequence encoded by the nucleoprotein (N), polymerase (L) gene, as well as the five gene concatenated alignment. The deduced amino acid sequences were aligned using Mafft 5.8 (6) with the E-INS-I alignment strategy and previously described parameters (4,7). Bayesian inference trees were constructed using MrBayes (8). The Markov chain was run for a maximum of 1 million generations, with a stopping rule implemented so that the analysis would halt when the average deviation of the split frequencies was less than 0.01%. Every 50 generations were sampled and the first 25% of mcmc samples were discarded as burn-in. Mid-point rooting was conducted using MEGA (9).

Specific reverse transcription PCR (RT-PCR) for ABVV and consensus RT-PCR for mammalian vesiculoviruses

RNA was extracted from the liver and lung tissues of 60 bats using the QIAamp MinElute Virus Spin kit (Qiagen). cDNA was generated from the sample RNA using the SuperScript III reverse transcription (RT; Invitrogen) with 100 pmol of random hexamer primer, 10 pmol of each dNTP, 10 µL of RNA, 1 µL buffer, 5mM DTT, 1 µL of RiboLock RNase Inhibitor (Fermentas), and 200 units of RT enzyme following the manufacturer's instruction. PCR primers ABVV-AF (5'⁶³³³CGACCTGATGAGAGTGGTGA 3') and ABVV-AR (5'⁶⁷⁹⁵AGTCGGGAGTTGATCATTGG 3') were used in PCR reactions targeting the polymerase gene of ABVV, producing an amplicon of 463 nt. The PCR reaction (containing 1 µM of each primer, 200 µM dNTPs, 1 U RedTaq DNA Polymerase (Sigma-Aldrich), 1X Red Taq Reaction Buffer, and 5 µl of target DNA in a 50 µl reaction) was carried out with the touch-down PCR conditions described above for genome completion. Amplicons were analyzed by ethidium bromide gel electrophoresis.

For the mammalian-vesiculovirus-consensus PCR, two pairs of degenerate PCR primers were designed based on the sequence alignment of the polymerase gene of formally classified mammalian vesiculoviruses (Figure 1 in main text; Technical Appendix Figure 3). Primers VesiConAF (5'KCDGAYAARAGYCAITCVATGA 3') and VesiConAR (5'TGNGCNACDGTNARDGCATT 3') were used for the first round of PCR. VesiConBF (5'GGNMGRTTYTTYTCHYTDATGTC 3') and VesiConBR (5'TCHGCNGAYTGCATNGTYTCA 3') were used for the second round of PCR with 2.5 µl of the first round PCR product. The LA Taq reaction composition and touch-down PCR was performed as described above.

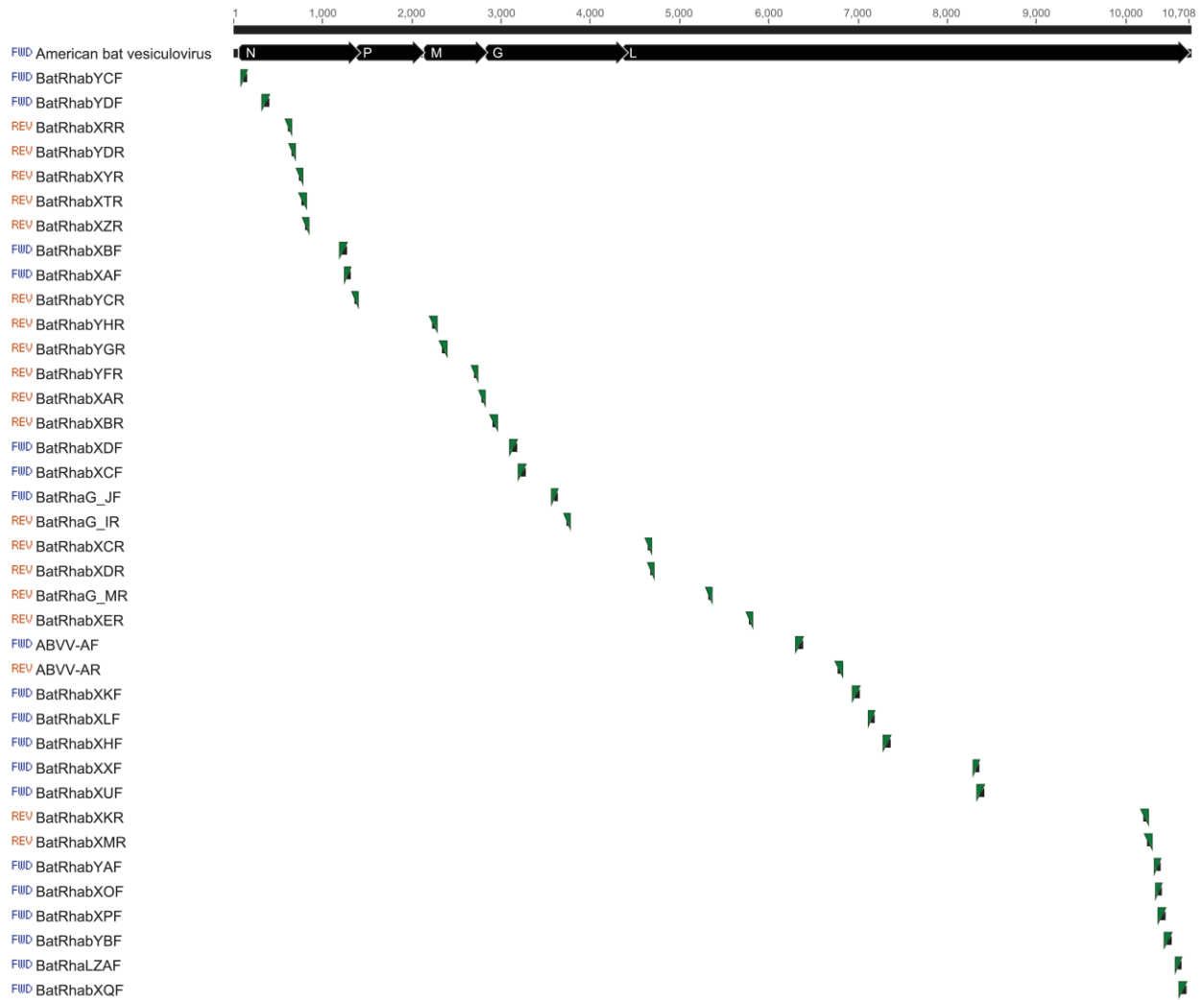
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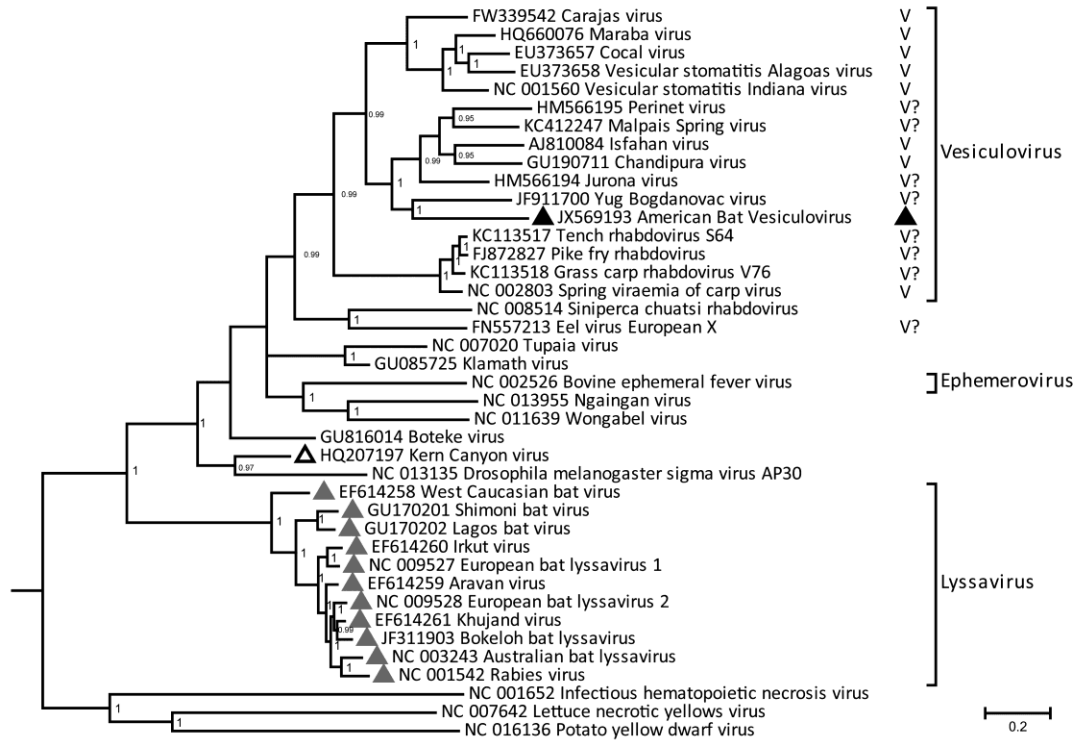
Technical Appendix Figure 1. Location of the primers used in this study relative to the American bat vesiculovirus.


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1          10          20          27
BatRhabYCF  TCTCGCGTAAAGAGACGTTGA
BatRhabYDF  AGACCCAACGGTGAGAAATGG
BatRhabXRR  GAGCGACAGCTCTATGAGAAAGA
BatRhabYDR  TTTCCCCATCCGTCATAAAA
BatRhabXYR  CAAATCGAATGACTGCCCGGTC TGA
BatRhabXTR  GCTGCACAAATCCTTGATTCG
BatRhabXZR  CCGGTGACCTTGTTGAGATGGCAA
BatRhabXBF  GCAGAGACTGTGCAGAAATGG
BatRhabXAF  GTATCCCGACTGCCGATATG
BatRhabYCR  CCTCAAAAATAAGCCTTAGCAA
BatRhabYHR  CAAC TCCCGGGGCTCCTTTGGTCTC
BatRhabYGR  CGAAACAAATCCTGAGGGATCCCGAAA
BatRhabYFR  TGGACGGGTTGAGGATTTGATCCAAGG
BatRhabXAR  TAGCTTGTTCGCCATCTTCCCT
BatRhabXBR  TGACCGGCAGTATGACTTTTG
BatRhabXDF  AAAGAGTCCGCCACAGTGT
BatRhabXCF  GGATTTCCACCAACCATCTTG
BatRhaG_JF  TCCAGGAGGATCTTGGTCA
BatRhaG_IR  CCTCTCCAAGTCCCATACCA
BatRhabXCR  TCAGCAGATTTGTGCCAGTC
BatRhabXDR  TGATCCAAGACCCAAACCAT
BatRhaG_MR  TTC TTGACGTGCTCTCCAAA
BatRhabXER  CGGTTCAT TGAA TGGC TCTT
ABVV-AF    CGACCTGATGAGAGTGGTGA
ABVV-AR    AGTCGGGAGTTGATCA TTGG
BatRhabXKF  GACAA TTTGGCGTTCAAGGT
BatRhabXLF  CAAT TGTGCGTTTC TTTTGG
BatRhabXHF  GATCGTCCGAGATGCAGTTA
BatRhabXXF  TGTGCGAGTCGATCACATCT
BatRhabXUF  CTTGGGGAGACCC TGT TGT
BatRhabXKR  GCC TGGTCTTTT TGGAGACA
BatRhabXMR  CAGAGCAC TAGCCAT TCTCG
BatRhabYAF  CTTC CCA TCCGAA TTCGCG TGACA
BatRhabXOF  TTCGCGTGACAAAAGTGAAC
BatRhabXPF  TCATGGAGTTGTACCGGAGA
BatRhabYBF  TCGGGCAA TGGA TTAGGACCC TACACA
BatRhaLZAF  CGACTTAACAGGGATAAAGGATG
BatRhabXQF  ACAAGTCTGTGCCGTTTGCT
VesiConAF  KCDGAY AARAGYCA YTCVATGA
VesiConAR  TGNGCNACDGTNARDGCATT
VesiConBF  GGNMGR TTYTTYTCHYTDATGTC
VesiConBR  TCHGCNGA YTGCA TNGTYTCA

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Technical Appendix Figure 2. Sequences of the primers used in this study. IUPAC nucleotide ambiguity codes were used for the degenerate primers.



Technical Appendix Figure 3. Bayesian inference tree based on the polymerase (L) gene depicting relationships among the members of the family *Rhabdoviridae*.