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Ledantevirus: A Proposed New Genus in the *Rhabdoviridae* Has A Strong Ecological Association with Bats

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Abstract. The Le Dantec serogroup of rhabdoviruses comprises Le Dantec virus from a human with encephalitis and Keuriliba virus from rodents, each isolated in Senegal. The Kern Canyon serogroup comprises a loosely connected set of rhabdoviruses many of which have been isolated from bats, including Kern Canyon virus from California, Nkolbisson virus from Cameroon, Central African Republic, and Cote d'Ivoire, Kolente virus from Guinea, Mount Elgon bat and Fikirini viruses from Kenya, and Oita virus from Japan. Fukuoka virus isolated from mosquitoes, midges, and cattle in Japan, Barur virus from a rodent in India and Nishimuro virus from pigs in Japan have also been linked genetically or serologically to this group. Here, we analyze the genome sequences and phylogenetic relationships of this set of viruses. We show that they form three subgroups within a monophyletic group, which we propose should constitute the new genus *Ledantevirus*.

INTRODUCTION

Le Dantec virus (LDV) was isolated in May 1965 at the Le Dantec University Hospital in Dakar, Senegal, from the blood of a 10-year-old girl who had an acute febrile illness with signs of hepatosplenomegaly and fever.[1] The virus was subsequently identified morphologically as a rhabdovirus and found to cross-react solidly in complementation-fixation tests with Keuraliba virus (KEUV), which was isolated in 1968 from the liver of a gerbil (*Tatera kempi*) trapped in a millet and peanut plantation in Saboya, Senegal.[1–3] Surveys of human sera in Senegal detected no other evidence of either LDV or KEUV infection but KEUV antibody was detected in 1% of gerbils and other rodent species tested. However, LDV antibody was detected in a 47-year-old male in Wales who reported fever, headache, and delirium after being bitten in 1969 by an insect while unloading peanuts from a ship that had come from Nigeria.[4] The patient subsequently developed neurological symptoms diagnosed as Parkinson's disease but a clear causal relationship with LDV was never established for either of the two human cases.

Although initial studies indicated that KEUV cross-reacts weakly in complement-fixation tests with several vesiculoviruses, phylogenetic analyses using partial L protein (RdRp) sequences suggest that LDV is more closely related to Fukuoka virus (FUKV), which has been assigned to the Kern Canyon serogroup of rhabdoviruses.[5,6] The FUKV was first isolated in Japan in 1982 from mosquitoes (*Culex tritaeniorhynchus*) and biting midges (*Culicoides punctatus*), and subsequently from sentinel cattle with mild febrile illnesses and leukopenia resembling bovine ephemeral fever.[7,8] The Kern Canyon serogroup also includes Kern Canyon virus (KCV), which was isolated in 1956 from a mouse-eared bat (*Myotis yumanensis*) in California; Nkolbisson virus (NKOV), which was isolated from mosquitoes (*Eretmapodites*, *Aedes*, and *Culex* spp.) in Cameroon and Cote d'Ivoire and a human in the Central African Republic, and Barur virus (BARV), which has been isolated from ticks and a rodent (*Rattus rattus*) in India, ticks, fleas and mosquitoes of several species in Kenya and ticks in Somalia.[9–15] Phylogenetic analysis of partial N (nucleoprotein) protein sequences indicates that KCV is also related to several other viruses that have been isolated from bats in Africa and Asia.[16,17] These include Mount Elgon bat virus (MEBV) isolated in 1964 from a horseshoe bat (*Rhinolophus hildebrandtii*) in Kenya, Oita virus (OITAV) isolated in 1972 also from a horseshoe bat (*Rhinolophus cornutus*) in Japan, Kolente virus (KOLEV) isolated in 1985 from a roundleaf bat (*Hipposideros jonesi*) and a pool of ticks (*Amblyomma (Theileriella) variegatum*) in Guinea, and Fikirini virus (FKRV) isolated in 2011 from a roundleaf bat (*Hipposideros vittatus*) in Kenya.[17–20]

Surprisingly, very little further characterization has been conducted on this interesting set of rhabdoviruses, which appear to have an ecological association with bats and some of which may be of clinical significance. Genome sequences have been reported only for FKRV and KOLEV viruses,[17,20] and Nishimuro virus (NISV), which was isolated from a wild boar (*Sus scrofa*) in Japan and appears to be closely related to these viruses (Sakai and others, unpublished data, GenBank: AB609604).

MATERIALS AND METHODS

We obtained the complete genome sequence for FUKV and near complete genome sequences for NKOV, BARV, KCV, LDV, KEUV, MEBV, and OITAV, lacking only near-terminal sequences of the 3′- and 5′-UTRs. These sequences were obtained by 50 base pair (bp) paired-end sequencing on the HiSeq2000 Illumina platform. Briefly, confluent monolayers of BHK-21 cells (T25 flask) were infected with the respective viruses and harvested 5–7 days later when extensive cytopathic effect (CPE) were present. Viral RNA was prepared and processed for sequencing as described previously.[21]

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Between 0.4% and 70% of the sequence reads in the samples mapped to the corresponding viral contigs (data available on request). The published genome sequences of FKRV, KOLEV, and NISV were also included in our data set (Supplemental Table 1). Transcription profiling was performed using a previously described method and either primer LDV_U1 (CTAGTCATACCCTGTCTAATC) or KCV_U1 (GATG TCTGTCAAGGTGTCTTC) for the U1 ORFs of LDV and KCV, respectively.[22]

RESULTS

The genomes ranged in size from 10,863 nt for FUKV (the smallest rhabdovirus genome yet reported) to > 11,528 nt for KCV, which is intermediate in length to those of vesiculoviruses and lyssaviruses. The genome organizations are shown in Figure 1A. Each genome contains open reading frames (ORFs) encoding the five canonical structural proteins in the order 3′-N-P-M-G-L-5′ (in negative polarity). The length

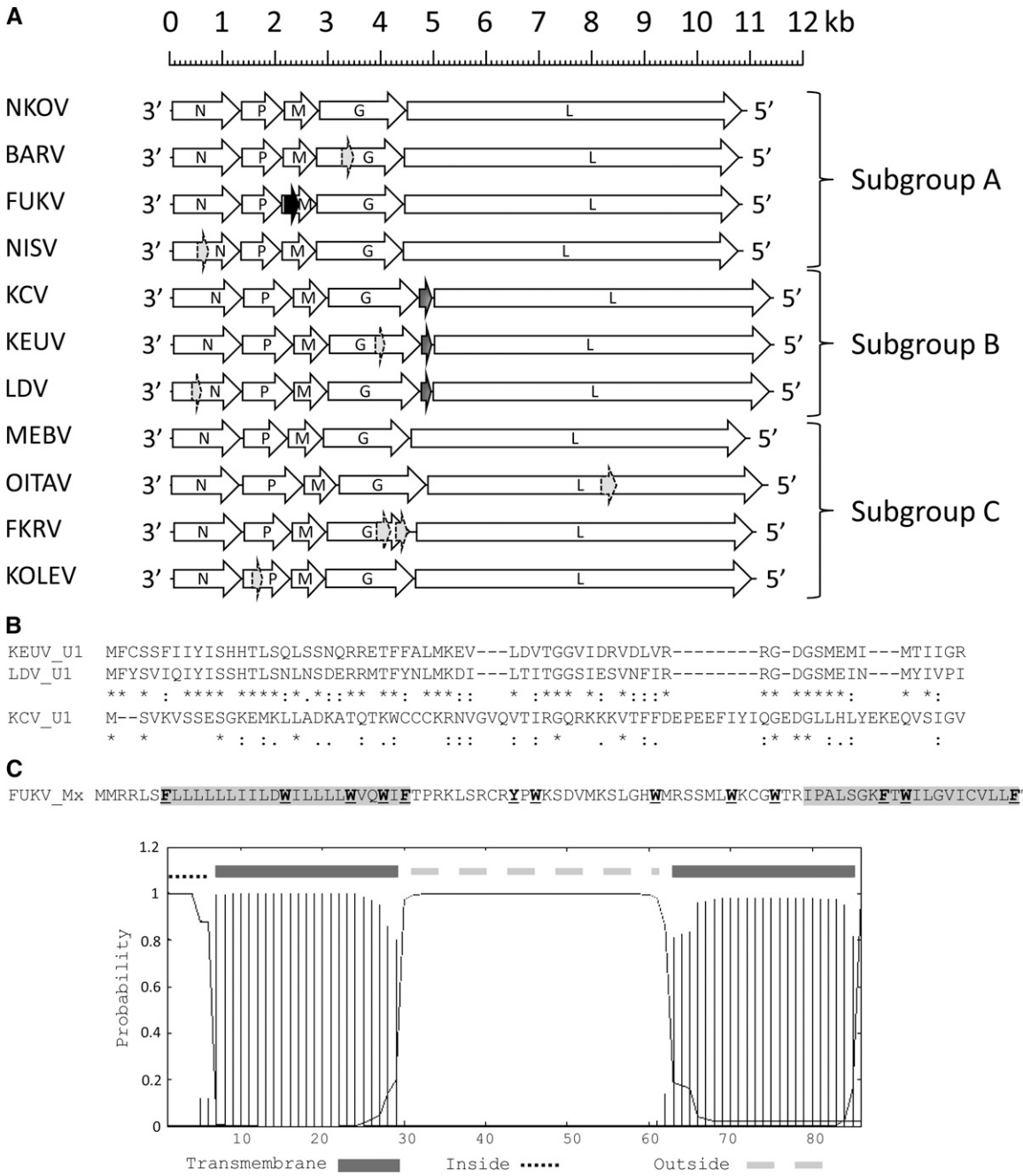

FIGURE 1. (**A**) Schematic representation of ledantevirus genome organizations showing the locations of all open reading frames (ORFs) > 180 nt, with dark gray denoting U1 ORFs, black Fukuoka virus (FUKV) Mx ORF, and pale gray with dashed outline other possible ORFs. (**B**) Alignment of the U1 proteins of Keuraliba virus (KEUV), Le Dantec virus (LDV), and Kern Canyon virus (KCV). (**C**) Sequence of the FUKV Mx protein and its predicted membrane topology (TMHMM; http://web.expasy.org).

of each ORF was observed to vary little between viruses with the exception of the P ORF, which ranges in size from 765 nt (BARV, FUKV, NISV) to 1,125 nt (OITAV). Major variations in the length of the P ORF are not unusual within rhabdoviruses. Additional short ORFs (U1) flanked by partially conserved transcription initiation (TI) and transcription termination/polyadenylation (TTP) consensus sequences are situated between the G and L genes in LDV (195 nt), KEUV (195 nt) and KCV (234 nt). Transcription profiling of LDV and KCV U1 proteins established that these genes are transcribed (Supplemental Figure 1). In some viruses, potential alternative ORFs (> 180 nt) are also present within the N (LDV, NISV), P (KEUV), M (FUKV), G (BARV, KEUV, FKRV), and L genes (BARV, OITAV, FKRV, KOLEV) but it is not known if these are expressed. Intergenic regions (IGRs) are generally very short (0–4 nt) with the exception of the [U1-L] IGR in KCV (16 nt), and in FKRV, KOLEV, MEBV and OITAV the M gene overlaps the P gene by 12–16 nt.

In pairwise comparisons of the genomes in our data set, nucleotide sequence identities ranged from 44.8% (FKRV and LDV) to 79.5% (BARV and FUKV) (Table 1). Three distinct subgroups were identified: subgroup A (KCV, LDV and KEUV) with 55.0–69.2% sequence identities; subgroup B (FKRV, KOLEV, MEBV and OITAV) with 50.0–62.0% sequence identities; and subgroup C (BARV, FUKV, NISV and NKOV) with 59.2–79.5% sequence identities. The assignment of these subgroups is supported by pairwise comparisons of amino acid (aa) sequence identities of the most highly conserved proteins (Supplemental Table 2). The BARV and FUKV consistently showed the highest pairwise aa sequence identities for all individual proteins (N = 96.4%; P = 81.1%; M = 95.7%; G = 84.4%; L = 92.2%).

The viruses assigned to subgroup A (LDV, KEUV and KCV) were the only three viruses to contain an additional gene (U1). The PSI-Blast and HHblits homology searches revealed no significant similarity between the U1 proteins and any previously identified protein sequences, and none of the proteins displayed any striking structural characteristics. The U1 proteins of LDV (7.4 kDa) and KEUV (7.3 kDa) were each predicted to be mildly acidic and a pairwise alignment indicated they are closely related (56.3% aa identity). The KCV U1 protein (8.8 kDa) is mildly basic and did not align convincingly with those of LDV and KEUV (Figure 1B). Of the alternative ORFs in the structural protein genes, only the 10.4 kDa protein encoded in the alternative ORF in the FUKV M gene (Mx protein) displayed remarkable structural characteristics with a predicted double-membrane spanning topology and a highly basic central ectodomain (Figure 1C). Like many other rhabdovirus accessory proteins, the functions of the U1 and Mx proteins remain unknown.

Maximum likelihood phylogenetic trees were generated from multiple sequence alignments of the deduced aa sequences of the N and L proteins for all viruses in the data set along with select animal rhabdoviruses. Amino acid sequences were aligned using MUSCLE[23] and ambiguously aligned regions were removed using the Gblocks program for the L protein only.[24] This resulted in alignments of 1,071 aa and 62 taxa for the L protein and 449 aa and 27 taxa for the N protein. Trees were estimated assuming the WAG+Γ model of aa substitution in the program PhyML 3.0, utilizing subtree pruning and regrafting (SPR) branch-swapping.[25] The phylogenetic robustness of each node was determined using 1,000 bootstrap replicates and nearest-neighbor branch-swapping. Analysis of each protein indicated that all viruses in our primary data set clustered within the larger dimarhabdovirus ("dipteran-mammal associated rhabdovirus") supergroup in a strongly supported monophyletic group in the L protein phylogeny (bootstrap support [BSP] = 100), hereafter referred to as the ledantevirus clade (Figure 2).[6] The ledantevirus clade comprised three subgroups that corresponded to those identified above based on pair-wise identities of genome nucleotide sequences. Each subgroup was strongly supported in both the L and N protein analyses (BSP = 100 in both N and L trees); however, the relationships between these subgroups were not well resolved (BSP = 79 in the L tree, BSP < 70 in the N tree) (Figure 2).

DISCUSSION

The *Rhabdoviridae* currently contains 11 approved genera (*Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Tibrovirus*, *Novirhabdovirus*, *Perhabdovirus*, *Sigmavirus*, *Sprivivirus*, *Tupavirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus*) and four unassigned species.[26–28] The data presented here provide a strong case for the creation of a new genus *Ledantevirus* to which the 11 viruses included in this study can be assigned. Phylogenies of the N and L proteins clearly show the monophyletic nature of this group. The full genome nt sequence identities between the members vary from 44.8% to 79.5% and L protein aa identities vary from 46.7% to 92.2%, falling well within the range seen for other genera in the

TABLE 1
Full genome pairwise nucleotide sequence identities for all proposed ledanteviruses

	NKOV	NISHV	BARV	FUKAV	OITV	MEBV	FIKV	KOLEV	KCV	LEDV	KEUV
NKOV											
NISHV	59.2										
BARV	59.3	75.2									
FUKAV	59.8	76.2	79.5								
OITV	45.4	46.8	46.5	47.1							
MEBV	47.2	49	49.3	49.3	50						
FIKV	46.6	47.2	46.7	47	53	50.5					
KOLEV	46.7	47.8	47.4	47.8	53.3	50.8	62				
KCV	47.6	48.9	48.8	49.3	45.4	46.8	45.1	45.5			
LEDV	48.2	49.2	49.4	49.4	45.8	47.6	44.8	46.1	55.6		
KEUV	48.1	48.9	48.6	49.7	45.7	47.3	45.8	45.8	55	69.2	

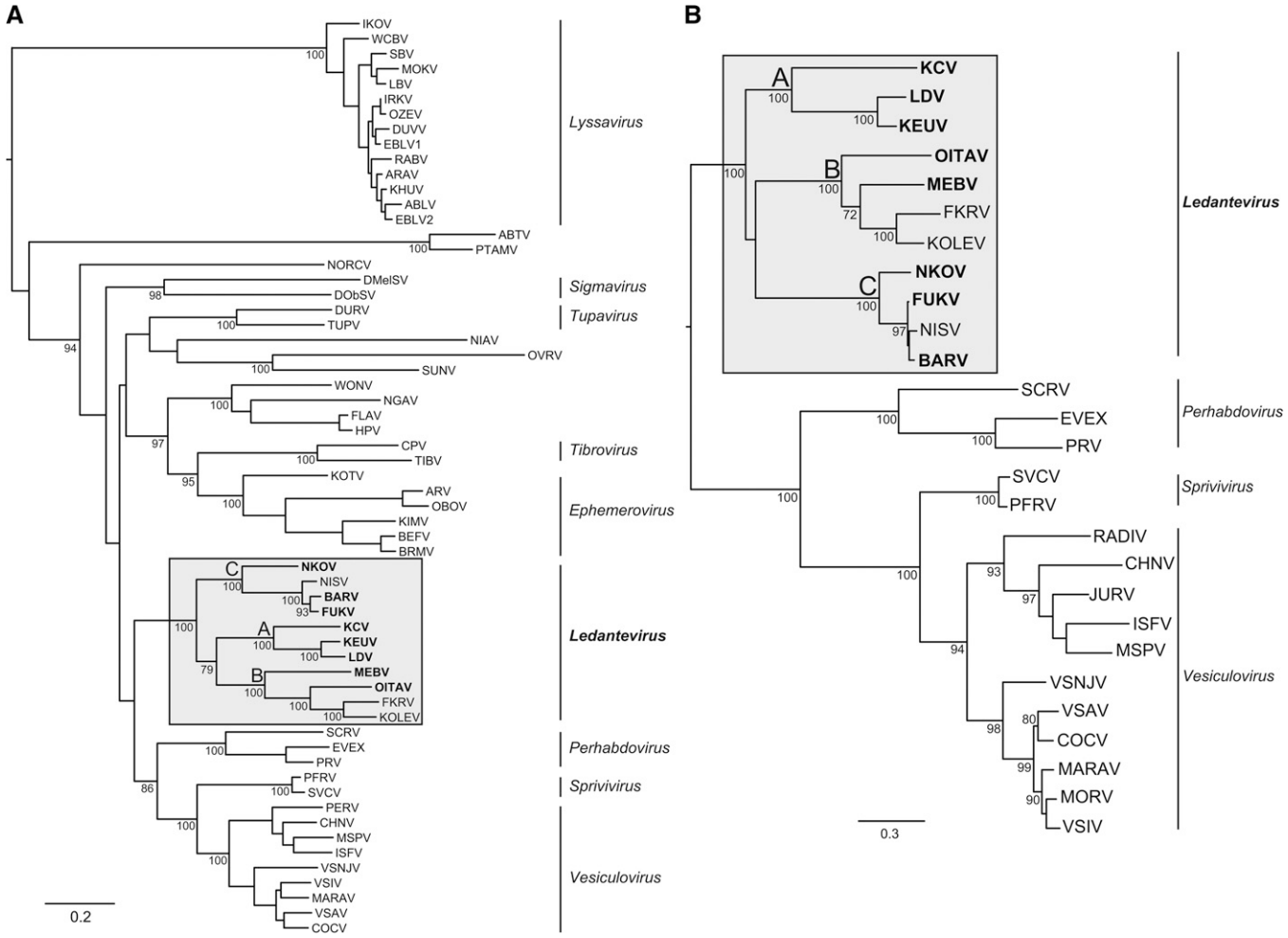

FIGURE 2. Maximum likelihood phylogenetic trees of the ledanteviruses (boxed) and select members of the *Rhabdoviridae* based on L (**a**) and N (**b**) protein alignments. Sequences generated in this study are shown in bold, and subgroups **A**, **B**, and **C** are indicated above the respective nodes. Bootstrap support values > 70% are indicated below each node; for clarity these values are given for major clades in the L tree only. Rhabdoviruses included in these analyses comprised: Ikoma virus (IKOV), West Caucasian bat virus (WCBV), Shimoni bat virus (SBV), Mokola virus (MOKV), Lagos bat virus (LBV), Ozernoe virus (OZEV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1) and 2 (EBLV-2), Rabies virus (RABV), Aravan virus (ARAV) Khujand virus (KHUV), Australian bat lyssavirus (ABLV), Arboretum virus (ABTV), Puerto Almendras virus (PTAMV), North Creek virus (NORCV), *Drosophila melanogaster* sigmavirus (DMelSV), *Drosophila obscura* sigmavirus (DObSV), Durham virus (DURV), Tupai virus (TUPV), Niakha virus (NIAV), Oak vale rhabdovirus (OVRV), Sunguru virus (SUNV), Wongabel virus (WONV), Ngaingan virus (NGAV), Flanders virus (FLAV), Hart Park virus (HPV), Coastal Plains virus (CPV), Tibrogargan virus (TIBV), kotonkon virus (KOTV), Adelaide River virus (ARV), Obodhiang virus (OBOV), Kimberley virus (KIMV), bovine ephemeral fever virus (BEFV), Berrimah virus (BRMV), Nkolbisson virus (NKOV), Nishimuro virus (NISV), Barur virus (BARV), Fukuoka virus (FUKV), Kern Canyon virus (KCV), Keuraliba virus (KEUV), Le Dantec virus (LDV), Mount Elgon bat virus (MEBV), Oita virus (OITAV), Fikirini virus (FKRV), Kolente virus (KOLEV), *Siniperca chuatsi* rhabdovirus (SCRV), Eel virus X (EVEX), Perch rhabdovirus (PRV), Pike fry rhabdovirus (PFRV), spring viremia of carp virus (SVCV), Perinet virus (PERV), Chandipura virus (CHNV), Malpais Spring virus (MSPV), Isfahan virus (ISFV), vesicular stomatitis virus New Jersey (VSNJV), Indiana (VSIV) and Alagoas (VSAV), Maraba virus (MARAV), Cocal virus (COCV), Radi virus (RADIV), Jurona virus (JURV), Morreton virus (MORV).

Rhabdoviridae.[29] The genome organizations of the viruses are also similar, comprising five ORFs encoding the structural proteins, concise intergenic regions and a small additional ORF between the G and L in only three of the viruses. This relatively simple genome organization is similar to that of vesiculoviruses.[30]

Based on the N and L phylogenies and full genome nt sequence pairwise comparisons, the ledanteviruses can be further subdivided into three subgroups. This is partially supported by the complement fixation tests reported previously, although strong serological cross-reactions were generally only observed between closely related viruses (BARV and FUKV; KEUV, and LEDV); NISV and FKRV were not included in this study.[20] Although this previous study also included Gossas virus, which had previously been identified as a rhabdovirus, subsequent sequence analysis of the source material indicated that the virus used was actually NKOV and that Gossas virus is not a rhabdovirus (data not shown). Some differences in genome organization were also observed between the three subgroups, with an additional ORF between the G and L ORFs in all subgroup A viruses, whereas subgroup B viruses contained a considerably longer P ORF than that found in the other subgroups. Based on the isolation data available for each virus, there are indications that there may also be differences in the ecology of viruses associated with each of these subgroups.

Species demarcation criteria for the *Rhabdoviridae* vary among different genera, complicated by differences in inter-species genetic diversities that are likely to be associated with different rates of evolution and/or periods of speciation. However, intra-species sequence diversities have been reported previously for several rhabdoviruses. Diversity analysis of the P proteins of 77 isolates of rabies virus from China identified a minimum aa identity of 85%, whereas intra-species (intra-genotype) P protein variation among 128 lyssavirus isolates identified aa identities of ≥ 73.5% and inter-species identities of ≤ 65.9%.[31,32] Full genome analysis of nine virus isolates of vesicular stomatitis New Jersey virus from throughout the known geographic range of the virus identified minimum aa identities of ≥ 91.5% for all proteins except P for which the minimum identity was 82.8%.[33] Analysis of the ephemeroviruses bovine ephemeral fever virus (BEFV) and Kimberley virus showed minimum intra-species aa sequence identities of 79.1% for the partial P protein and 94.9% for the partial G protein, whereas maximum inter-species identities between BEFV and the closely related Berrimah virus were 44.9% and 90.8% for the partial P and G proteins, respectively.[34] In the current study, aa sequence identities were < 81% for L and N, < 70.0% for M and G, and ≤ 57% for all viruses except BARV, FUKV, and NISV. Amino acid sequence identities between BARV and FUKV were > 90% for the N, M, and L proteins and > 80% when compared with NISV. Amino acid sequence identities of P and G proteins were lower but were still 81.1% and 84.4%, respectively, between BARV and FUKV, and > 70% for NISV. Although identities for most proteins are similar to those observed previously for intra-species diversity of other rhabdovirus species, sequence identity of the G protein is much lower. Sequences analysis of further isolates would be required to ascertain the intra-species sequence diversity for the ledanteviruses and to establish if BARV, FUKV, and NISV should be considered as separate species or genotypes of a single species.

Several of the genera within the *Rhabdoviridae* show associations with a dominant group of vertebrate hosts. Ephemeroviruses and tibroviruses appear to be hosted by cattle, with many members of each genus isolated either from cattle and/or from mosquitoes or biting midges that feed on cattle.[35,36] Lyssaviruses circulate in bats, whereas novirhabdoviruses, spriviviruses, and perhabdoviruses are associated with fish.[37–39] There is a strong ecological association of the proposed genus *Ledantevirus* with infection of bats, including all proposed species in subgroup B (FKRV, KOLEV, MEBV, and OITAV) and two of the three viruses in subgroup A (KCV and LDV). There is also some suggestion that the viruses in these two subgroups are each associated with a different subgroup of microchiropteran bats. Subgroup A viruses have been detected in bats from the family Vespertilionidae within the Yangochiroptera subgroup of bats, whereas subgroup B viruses have been found in the sister families Rhinolophidae and Hipposideridae in the other sub-group of bats, the Rhinolophoidea, with the two viruses isolated from *Hipposideros* bat species (FKRV and KOLEV) showing the greatest identities.[40] The detection of various other ledanteviruses in ungulates, rodents and humans may suggest broader natural host specificity or may have been caused by spillover events from a natural reservoir. Possible host switching events have previously been identified in at least two genera of rhabdoviruses.[41,42] However, sampling of more viruses from each of these subgroups is required before firm conclusions on natural host range can be drawn. Furthermore, although some ledanteviruses have been isolated from arthropods and the clade sits within the dimarhabdovirus supergroup, it is not clear at this time if vector-borne transmission is a common characteristic of all viruses in the proposed new genus.[6,7,10,11,13]

Collectively, the data show that the 11 viruses analyzed here form a monophyletic group within the *Rhabdoviridae*, which we propose should be assigned as the novel genus *Ledantevirus*. Although little is known about the ecology of most of these viruses, two subgroups appear to be hosted predominantly by bats, whereas a third subgroup contains several viruses suspected of being vectored by arthropods. Further studies are needed to assess the genetic diversity, ecology, and pathogenicity of these viruses and to better define the risks they may pose to human and animal health.

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