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A Novel Mosquito-Borne Orbivirus Species found in Southeast Asia.

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ABSTRACT

The *Orbivirus* genus of the family *Reoviridae* includes a genetically diverse group of dsRNA arthropod-borne viruses that infect a wide variety of animal species. Here we report the complete genome and phylogenetic analysis of a novel *orbivirus* (IAn-66411 or Sathuvachari virus, SVIV) isolated in 1963 from starlings (*Brahminy myna*) collected in Vellore, Tamil Nadu, India. Comparative genetic analysis of the SVIV polymerase (VP1 protein), core protein (VP3) and outer core protein (VP7) confirmed that SVIV is most closely related to the mosquito-borne orbiviruses, but that it is equally divergent from all known species. Therefore, SVIV should be tentatively considered as the prototype of a new mosquito-associated *Orbivirus* species. These findings will aid in the development of molecular reagents that can identify genetically similar orbiviruses and help elucidate their geographical distribution, epidemiology, species tropism and possible disease association.

TEXT

The genus *Orbivirus* of the family *Reoviridae* includes 22 virus species as well as 10 unclassified or unassigned viruses (Attoui *et al.*, 2011). Orbiviruses are icosahedral, non-enveloped viruses with double-stranded RNA (dsRNA) genomes that encode seven distinct structural proteins (virion proteins, VP1-VP7) and four distinct non-structural proteins (NS1-NS4) (Attoui *et al.*, 2011; Ratinier *et al.*, 2011). Orbiviruses are global in distribution and infect a wide variety of vertebrate hosts, including wild and domestic ruminants and equids, rodents, bats, marsupials, birds, sloths and primates, including humans. Orbiviruses also replicate in and are transmitted by a variety of hematophagous arthropods (mosquitoes, ticks, phlebotomine sandflies and biting midges, depending on the virus).

Genetic characterization is crucial for the appropriate classification of unidentified viruses (Kapoor *et al.*, 2008a; Kapoor *et al.*, 2008b). In turn, genomic sequence data can be used to develop molecular reagents for assessing the epidemiology and disease associations of novel viruses and their genetic relatives (Burbelo *et al.*, 2012). We report here the complete genomic sequence of a novel orbivirus that was initially isolated in 1963 from a starling (*Brahminy myna*) collected in Vellore (Tamil Nadu, India). Based on comparative genetic analysis of all 10 genomic segments (GenBank accession no. KC432629-KC432638), IAn-66411 isolate (Sathuvachari virus, SVIV) is highly divergent from other known *orbivirus* species, but is most closely related to the mosquito-borne virus species. A homology-based search against the NCBI non-redundant nucleotide sequence database yielded a partially characterized/unclassified orbivirus, JKT-8132, as the nearest genetic relative of SVIV. JKT-8132 (Tagtag virus, TGV) was

isolated at the U.S. Naval Medical Research Unit 2 (NAMRU-2), Jakarta (Indonesia), from a pool of *Culex vishnui* mosquitoes collected in Tag-tag, Bali, Indonesia in 1980. On the basis of the phylogenetic analysis presented herein, we propose that SVIV and TGV viruses define a novel mosquito-transmitted species within the genus *orbivirus*.

SVIV was initially isolated by intracerebral inoculation of newborn mice at the Virus Research Centre, Christian Medical College, Vellore, India (Carey *et al.*, 1968a; Carey *et al.*, 1968b). TGV was first isolated in baby hamster kidney (BHK) and *Aedes pseudoscutellaris* (MOS-61) cells at NAMRU-2 (Converse, J.D., NAMRU-2, personal communication, 1982). Both viruses were subsequently sent to the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch for further characterization. Our initial attempts to culture SVIV from old lyophilized stocks by inoculation of newborn mice and culture in BHK and Vero cells were unsuccessful. However, subsequent attempts to grow the virus in C6/36 (*Aedes albopictus*) cells were successful. In contrast, TGV was viable and produced moderate cytopathic effect (CPE) in BHK and Vero E6 cells after 5 days of incubation at 37°C. It also produced CPE in C6/36 cells after 4 days of incubation at 28°C. Newborn mice inoculated intracerebrally with TGV became ill on the fourth and fifth days post-infection with symptoms of spasticity and incoordination. Antisera for serologic tests were prepared in adult mice, using 10% crude homogenates of TGV-infected newborn mouse brain in phosphate-buffered saline as the immunogens. The immunization schedule consisted of four intraperitoneal injections of antigen mixed with Freund's adjuvant, given at weekly intervals (Beaty *et al.*, 1989). After the final immunization, mice were inoculated with sarcoma 180 cells, and the resulting immune

ascitic fluids were collected. Complement fixation (CF) tests were performed by the microtiter technique, using 2 U of guinea pig complement and overnight incubation of the antigen and antibody at 4°C (Knudson *et al.*, 1984; Tesh *et al.*, 1986). Antigens used in the CF tests were prepared from infected newborn mouse brain by the sucrose acetone extraction method and were inactivated with 0.05% β-propiolactone (Sigma, St. Louis, MO). No antigenic relationship could be shown between TGV and SVIV and other known orbiviruses in CF tests; however transmission electron microscopy confirmed both viruses to have characteristic reovirus morphology (Figure-1).

For genetic characterization of SVIV, mouse brain suspensions, prepared by reconstituting old lyophilized virus stocks prepared in 1968. Virus nucleic acids were extracted from the filtrate and subjected to unbiased high-throughput sequencing (454 Roche) and applicable bioinformatics approaches (Kapoor *et al.*, 2011; Victoria *et al.*, 2009). Initial bioinformatics analysis indicated SVIV as a highly divergent orbivirus. Sequencing results were analyzed further to acquire partial genomic sequences of all 10 segments of the virus. Assembled contigs (batch of sequences showing >95% nt identity over >40 nt length) from the shotgun 454 sequencing reads were amplified by reverse transcription-PCR (RT-PCR), which was followed by Sanger sequencing. Gaps between contigs were closed by designing PCR primers from the existing contigs spanning each gap. When needed, PCR products were cloned into pGEMT-easy vector and sequenced. 5' rapid amplification of cDNA ends (RACE) and 3'RACE were used to acquire the terminal sequence of all 10 genomic segments (Kapoor *et al.*, 2011).

The complete genome of SVIV is comprised of 18,834 nt pairs. SVIV segments 1 to 10 are 4015, 2860, 2393, 1997, 1789, 1644, 1205, 1188, 933 and 810 nt long and encodes VP1, VP3 (T2), VP2, VP4, NS1, VP5, NS2, VP7, VP6 and NS3 viral proteins, respectively. All these sequences were submitted to GenBank under the accession numbers: KC432629-KC432638. Genomic features and genetic relatedness of all 10 segments of SVIV is described in supplementary table-1. The orbiviruses share hexanucleotide termini that are partially conserved between the genomic segments of viruses within the same species and, to a lesser extent, between viruses of different species (Belaganahalli *et al.*, 2011). The genomic segments of SVIV share six completely conserved nucleotides at their 5' ends as well as 4 conserved nucleotides at their 3' ends (5'-GGUU$^{U}/_{A}$A- virus gene- TACC-3'). Moreover, the first and last pair of nucleotides for each genome segment are inverted complements and identical to those reported for other known orbiviruses (Attoui *et al.*, 2009; Attoui *et al.*, 2005; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011). Previous studies have observed that the genomes of orbiviruses contain 5.03% to 5.695% of non coding region (NCR) in the mosquito-borne group, 4.47 – 4.9% of NCR in the tick-borne group, and 3.5 – 4.1% NCR in *Culicoides* borne viruses (Belaganahalli *et al.*, 2011). Analysis of the SVIV genome revealed 4.874% NCR in its entire genome. It is noteworthy that SVIV has a lower percentage of NCRs relative to previously characterized mosquito-borne orbiviruses. The biological significance of lower percentage of NCR observed in SVIV is unknown. In this respect, SVIV more closely resembles the tick-borne agent Great Island virus (4.978% NCR). Moreover, the T2- subcore protein (VP3) of SVIV also possesses more sequence similarity (46%) with Great Island virus(Belhouchet *et al.*, 2010) than with the mosquito-borne viruses (45% NCR). In addition, SVIV shares <36% identity in its VP5 protein

154 with Great island virus, the same percentage observed with other mosquito-borne orbiviruses.
155 The G+C content of SVIV is 42.3%. Previous studies of other mosquito-borne viruses found a
156 G+C content of36.72% in Peruvian horse sickness virus, 41.53% in Umatilla virus and 41.55% in
157 Yunnan virus (Attoui et al., 2009). For tick-borne viruses, G+C content is between 51.93% (St.
158 Croix River virus) and 57.29% (Great Island virus), while the midge-borne orbiviruses have an
159 intermediate G+C content: 39.89% in Chuzan virus to 45.89% in equine encephalosis virus
160 (Belaganahalli *et al.*, 2011). Our analysis demonstrates that SVIV has a slightly higher G+C
161 content than previously characterized mosquito viruses, and that this percentage is more
162 similar to midge-borne viruses. In most orbivirus genome segments, the 5' NCRs are shorter
163 than the 3' NCRs. In case of SVIV, all of the segments have shorter 5'NCRs than 3' NCRs, except
164 for segment 7 (NS2) that has a 5'NCR that is 57bp and a3'NCR that is 47bp. An earlier report
165 found that segment 7 (NS2) and segment 9 (VP6) from Umatilla virus express 5'NCRs longer
166 than their 3'NCRs. Also, segment 6 (VP5) of Yunnan orbivirus and segment 9 of Great island
167 virus contains longer 5'NCRs relative to their 3'NCRs (Belaganahalli *et al.*, 2011). A unique
168 feature of the orbivirus genome is the coding assignment of its different segments; i.e., each
169 segment encodes protein(s) with their own putative function. The coding assignment varies
170 between the strains / species, and the pattern can indicate which arthropod vector is preferred
171 for the individual viruses (Attoui *et al.*, 2005; Belaganahalli *et al.*, 2011). Therefore, each
172 segment of SVIV was analyzed by BLASTx and/or conserved domain search (NCBI) to determine
173 the type of protein that it encodes and its putative function. The SVIV coding assignments were
174 compared to assignments previously published for other orbiviruses. The analysis revealed that
175 segment 1, 9 and 10 of SVIV putatively encode polymerase, helicase and the viral release

protein, respectively. SVIV segment 2 encodes VP3 (T2) and segment 3 is VP2 (OCP1), which is distinct from previously investigated orbiviruses.

To determine the genetic relationship of SVIV with other viruses in the *orbivirus* genus, we conducted phylogenetic analysis on all 10 virus genome segments (Fig. 2 and 3). The type member for each *orbivirus* species was included in the phylogenetic trees. The deduced amino acid sequences of SVIV virus were aligned with the homologous protein sequences of well-characterized orbiviruses using ClustalW default parameters and BLOSUM protein weight matrix, as implemented in MEGA5 (Tamura *et al.*, 2011). Protein alignments were used to calculate the Bayesian information criterion (BIC) for 48 unique protein substitution models, and the maximum likelihood amino acid substitution model with the lowest BIC score was used to construct the phylogenetic tree (Tamura *et al.*, 2011). The RNA-dependent RNA polymerase VP1 protein is the most evolutionarily conserved of all orbivirus proteins and has been used to classify new orbiviruses into taxonomic groups (Attoui *et al.*, 2009; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011). Our phylogenetic analysis of VP1 protein from SVIV and TGV suggest their close relationship with other mosquito-borne orbiviruses; thus, suggestive of their vector origin. However, the sequences from both new viruses formed a separate branch within the group (Fig. 2). Confidence in phylogenetic analyses was accessed using bootstrap method. We observed that the trees recapitulated previously reported classification of all well-characterized orbiviruses and that there was genetic clustering of viruses transmitted by a common arthropod vector (shown as different color shades in Fig. 2) (Attoui *et al.*, 2009; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011). Almost the same tree topology was observed in phylogenetic analyses for all remaining 9 segments (Fig. 3). Statistically, the VP1 protein of SVIV

showed 10% more amino acid identity with mosquito-borne viruses than with the tick- or midge-borne orbiviruses. The comparison of deduced amino acid sequences of all the segments of SVIV revealed that it is clearly distinct from other orbivirus species investigated. SVIV showed <55 % identity in the polymerase protein, <46% in T2 subcore protein, <18% in outer capsid protein 1 [VP2] and <36% in outer capsid protein 2 [VP5] with known orbiviruses (data not shown). We sequenced partial VP1, VP5, VP6, VP7, NS1 and NS2 genes of Tagtag virus (GenBank accession no. KC439154-KC439159) using the same primers that used for SVIV. The analysis showed that TGV had maximum of 99% (range-94-99%) identity with SVIV at amino acid level. Our analysis confirms that SVIV and TGV viruses are genetically related variants of the same orbivirus species.

Orbiviruses are known to infect multiple animal species (Attoui *et al.*, 2011). Traditionally orbivirus isolates were classified based on their serological properties (Attoui *et al.*, 2005; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Palacios *et al.*, 2011). The development of next generation sequencing technologies have repeatedly demonstrated their utility in identifying non cultivable viruses and also in characterizing existing virus isolates that cannot be identified by more traditional laboratory methods (Victoria *et al.*, 2008). Many studies that included all known orbiviruses have demonstrated that phylogenetic analysis and genetic relatedness can be used to classify uncharacterized orbiviruses (Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Palacios *et al.*, 2011). Moreover, the orbiviruses transmitted by a common arthropod vector (i.e. mosquito, tick, midge) also show common ancestry or closer genetic relatedness. In the present study, these concepts and methods were adopted to

completely characterize the genome of SVIV, which was isolated almost 50 years ago. Both SVIV and TGV were deposited in the WRCEVA collection as unknowns. However, their full characterization was not possible until full genome sequencing was done. Moreover, the sequence data generated for SVIV helped us to characterize another previously unclassified orbivirus, TGV http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=10892. The genetic information presented in this study should allow development of molecular reagents (PCR primers, serological assays etc.) that can now be used to define the prevalence, epidemiology, host range and possible disease association of SVIV and TGV.

Nonetheless, this is also an example of the continuing importance of primary virus isolation and deposition of unknown or novel viruses in permanent virus collections or repositories, so that such agents are available for study as new techniques become available or new pathogens appear (Arrigo *et al.*, 2012). To date, nothing is known about the potential public health or veterinary importance of SVIV and TGV viruses, but their characterization now makes such studies feasible. Wider geographic sampling of vectors, animals and humans will provide better description of the genetic diversity of this proposed new *Orbivirus* species. Serological assays will be needed to determine whether these viruses infect animals, including humans. (Burbelo *et al.*, 2011; Burbelo *et al.*, 2012). The genetic characterization of a second novel virus (TGV) with a genetically divergent VP1 and other genes indicates that wider geographic sampling for related viruses will likely reveal other novel variants. The genetic diversity within this proposed species may also reflect a range of disease phenotypes upon their host. In conclusion, the

242 sequence data of SVIV should provide sufficient information to develop specific molecular
243 diagnostic assays that will allow confirmation of future outbreaks or cases of orbivirus infection
244 and retrospective analysis of previously unconfirmed case; and it will also facilitate
245 epidemiological studies.
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FIGURE LEGENDS

Fig.1. Electron micrographs of viruses SVIV (IAn-66411) and Tagtag virus (JKT-8132). (A) and TGV in BHK cells (B). Virions are shown by pointed arrows. (A). JKT-8132. Ultrastructure of reovirus fibrillar aggregate in the cytoplasm of a BHK cell with virus particles and cores, distance bar = 100 nm. (B). IAn-66411 #5670. An aggregate of reovirus particles ~60 nm in diameter in the cytoplasm of a C6/36 cell, distance bar = 100 nm. (C). IAn-66411 #5672. A portion of a C6/36 cell infected with a reovirus IAn-66411 showing viral protein aggregates with forming cores and virus particles ~60 nm in diameter (thick arrows) and microtubules (thin arrow) inside a cistern of granular endoplasmic reticulum which is expanded at one end, distance bar = 100 nm.

Fig.2. Phylogenetic analyses of inferred amino acid sequences of the VP1 fragment of SVIV and TGV with other known orbiviruses; bootstrap values of >70% are shown. The strains used for comparison with SVIV were retrieved from GenBank (accession numbers are YP_052968, ACY02806, YP_003240108, BAD89093, AFH41509, AEE98368, YP_002925132, YP_460038, YP_443925, YP_003896058, YP_052966, YP_052935, ADM88609, ADM88603, ADM88606, ACJ06234, YP_052942).

Fig.3. Phylogenetic analyses of inferred amino acid sequences of the nine genomic segments of SVIV. A to I are the phylogenetic trees showing analysis of nine proteins encoded by segments VP2-7 and NS1-3, respectively. The GenBank accession numbers of strains that were used for this analysis are as follows; VP2: YP_052943, AFH41510, ADI79209, ACJ06245, AEY69029, YP_052931, CAN89166, YP_460040, ACJ06702, ADU57369; VP3: YP_002925133, YO_443926, YP_003896059, ADM88610, ADM88607, ADM88604, YP_052943, ACJ06236, AEE98369, AC053603, BAC67379, YP_052934, AEY69030, AAC40995, ACR58460,AFH41511; VP4: YP_460041, YP_443928, AEE98372,YP_052936, CAN89107, CAP04843, ACJ06237, YP_003896060, ADZ96231, ADZ96221, YP_052945, ACR58461, AFH41512, ACY02808; VP5: AEE98373, YP_003896063, YP_443930, ADZ96224, ADZ96234, ADM88605, YP_460042,

YP_052946, YP_052932, CAE52975, YP_003240113, ACJ06239, ACJ06704, BAA93693, AFH41514, YP_052963; VP6: YP_460043, ADZ96227, YP_003896066, AEE98376, ADZ96237, ACO53605, AFH41517, CAN89173, YP_052937, ACJ65038, ACJ06250, CAN89112, YP_052950, ACJ06707, ACJ06242; VP7: AEE98375, YP_460044, YP_003896064, YP_443932, ADZ96226, ADZ96236, YP_052933, CAP04847, ACJ06241, P18259, YP_052949, ACJ06705, AFH41515, CAN89110, BAC20279; NS1:YP_443929, YP_460045, AEE98371, ADZ96222, ADZ96233, YP_003896061, ACH92681, YP_052938, AFH41513, CA085724, AAA91963, YP_052947, ACJ06238, ACJ06703; NS2: YP_460046, YP_443931, YP_003896065, ADZ96225, ADZ96235, CAP04848, YP_003240115, CAP12633, ACJ06240, YP_052948, ACJ 06706, AFH41516, YP_052939, AEE98374, BAC22192; NS3: AAB03411, AFH41518, ABU48536, ADZ96228, BAF40427, YP_443934, ACO53602, YP_003240117, AEP95960, YP_052951, YP_003896068, AEE98377, ADZ96238, YP_052940, ACJ06708.

Figure-1

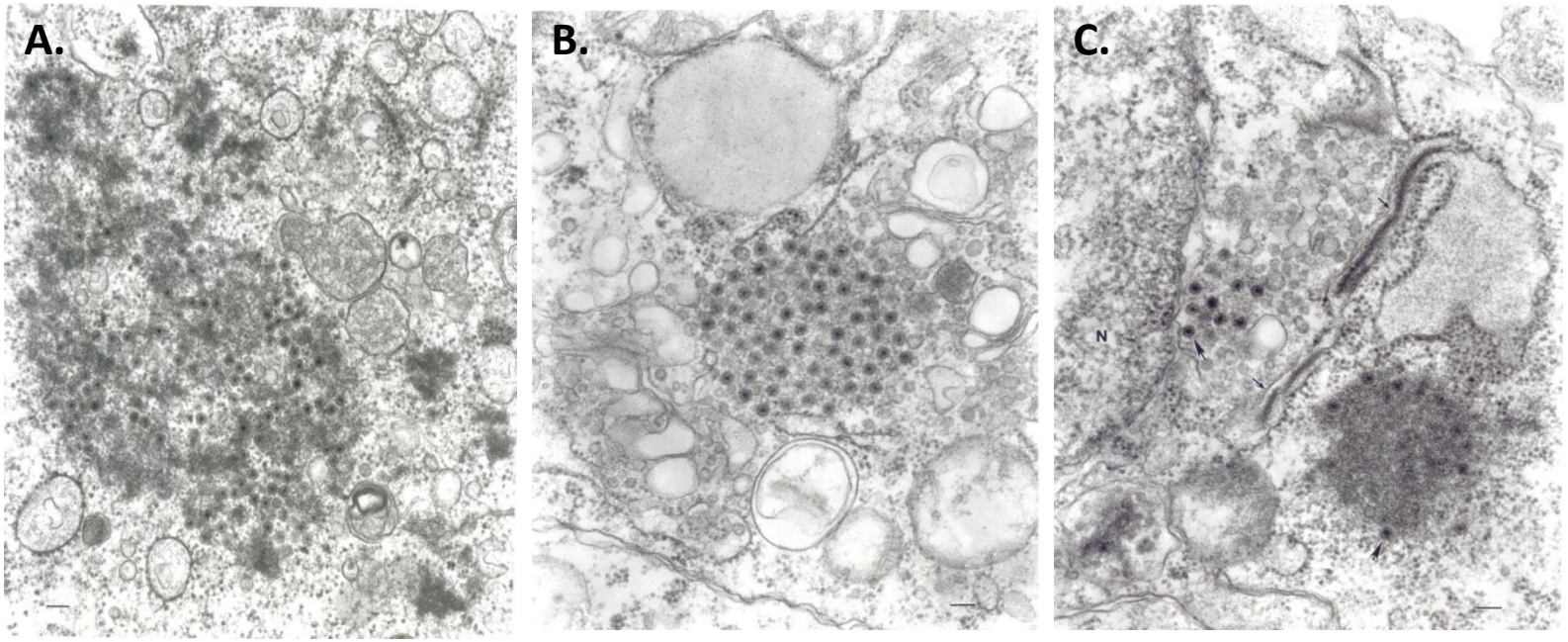
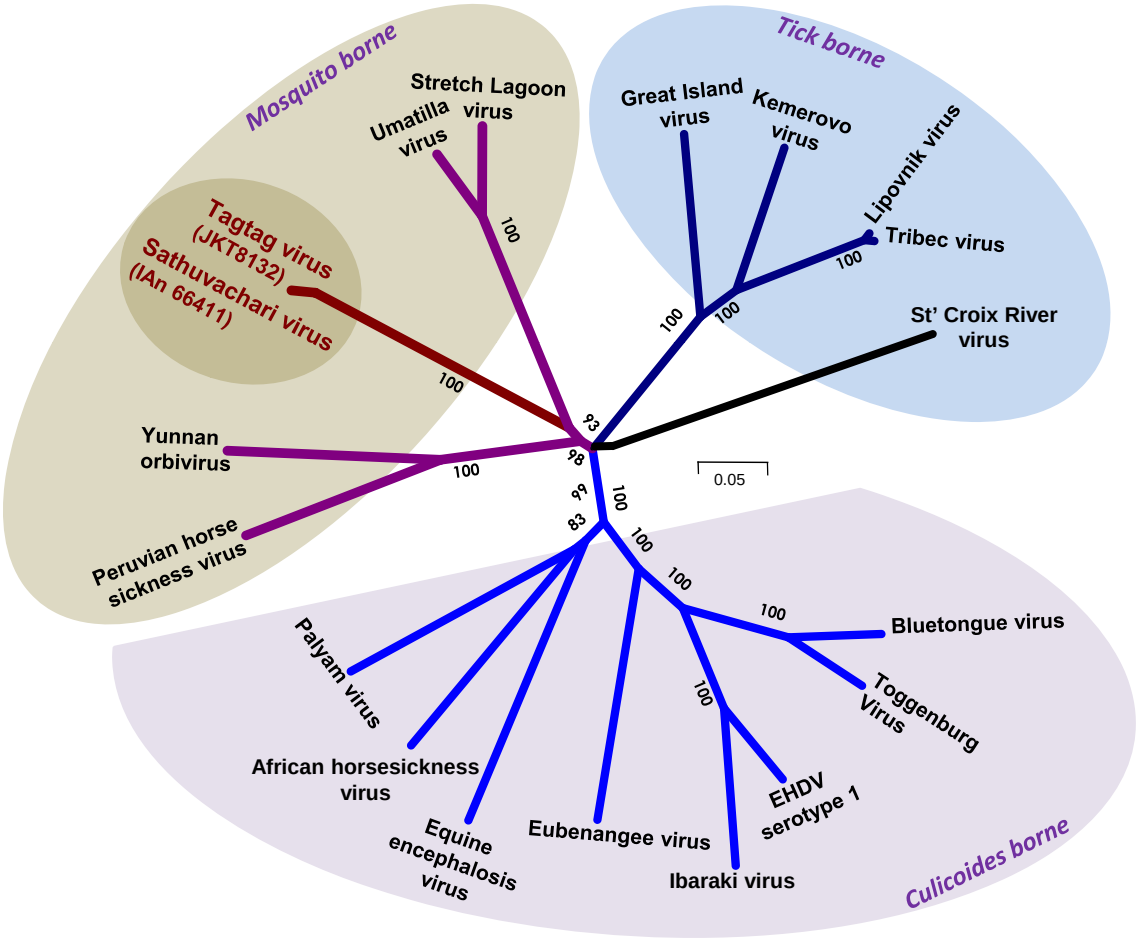
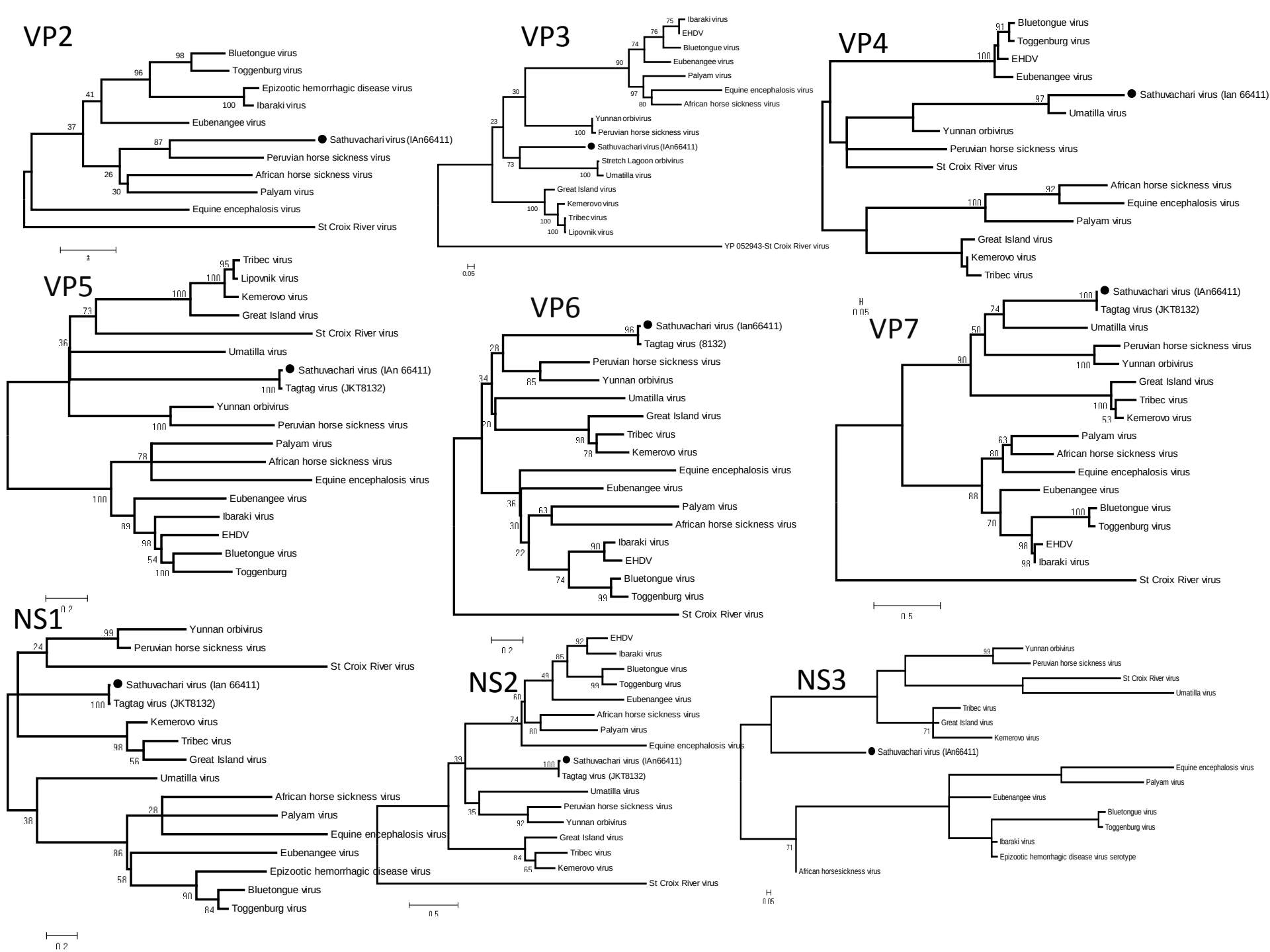


Figure-2


Mosquito borne
Tick borne
Culicoides borne
Stretch Lagoon virus
Umatilla virus
Tagtag virus (JKT8132)
Sathuvachari virus (IAn 66411)
Great Island virus
Kemerovo virus
Lipovnik virus
Tribec virus
St' Croix River virus
Yunnan orbivirus
Peruvian horse sickness virus
Palyam virus
African horsesickness virus
Equine encephalosis virus
Eubenangee virus
Ibaraki virus
EHDV serotype 1
Toggenburg Virus
Bluetongue virus
0.05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Bluetongue virus
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Ibaraki virus
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Sathuvachari virus (IAn66411)
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African horse sickness virus
Palyam virus
Equine encephalosis virus
St Croix River virus
VP3
Ibaraki virus
EHDV
Bluetongue virus
Eubenangee virus
Palyam virus
Equine encephalosis virus
African horse sickness virus
Yunnan orbivirus
Peruvian horse sickness virus
Sathuvachari virus (IAn66411)
Stretch Lagoon orbivirus
Umatilla virus
Great Island virus
Kemerovo virus
Tribec virus
Lipovnik virus
YP 052943-St Croix River virus
VP4
Bluetongue virus
Toggenburg virus
EHDV
Eubenangee virus
Sathuvachari virus (Ian 66411)
Umatilla virus
Yunnan orbivirus
Peruvian horse sickness virus
St Croix River virus
African horse sickness virus
Equine encephalosis virus
Palyam virus
Great Island virus
Kemerovo virus
Tribec virus
VP5
Tribec virus
Lipovnik virus
Kemerovo virus
Great Island virus
St Croix River virus
Umatilla virus
Sathuvachari virus (IAn 66411)
Tagtag virus (JKT8132)
Yunnan orbivirus
Peruvian horse sickness virus
Palyam virus
African horse sickness virus
Equine encephalosis virus
Eubenangee virus
Ibaraki virus
EHDV
Bluetongue virus
Toggenburg
VP6
Sathuvachari virus (Ian66411)
Tagtag virus (8132)
Peruvian horse sickness virus
Yunnan orbivirus
Umatilla virus
Great Island virus
Tribec virus
Kemerovo virus
Equine encephalosis virus
Eubenangee virus
Palyam virus
African horse sickness virus
Ibaraki virus
EHDV
Bluetongue virus
Toggenburg virus
St Croix River virus
VP7
Sathuvachari virus (IAn66411)
Tagtag virus (JKT8132)
Umatilla virus
Peruvian horse sickness virus
Yunnan orbivirus
Great Island virus
Tribec virus
Kemerovo virus
Palyam virus
African horse sickness virus
Equine encephalosis virus
Eubenangee virus
Bluetongue virus
Toggenburg virus
EHDV
Ibaraki virus
St Croix River virus
NS1
Yunnan orbivirus
Peruvian horse sickness virus
St Croix River virus
Sathuvachari virus (Ian 66411)
Tagtag virus (JKT8132)
Kemerovo virus
Tribec virus
Great Island virus
Umatilla virus
African horse sickness virus
Palyam virus
Equine encephalosis virus
Eubenangee virus
Epizootic hemorrhagic disease virus
Bluetongue virus
Toggenburg virus
NS2
EHDV
Ibaraki virus
Bluetongue virus
Toggenburg virus
Eubenangee virus
African horse sickness virus
Palyam virus
Equine encephalosis virus
Sathuvachari virus (IAn66411)
Tagtag virus (JKT8132)
Umatilla virus
Peruvian horse sickness virus
Yunnan orbivirus
Great Island virus
Tribec virus
Kemerovo virus
St Croix River virus
NS3
Yunnan orbivirus
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Equine encephalosis virus
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Eubenangee virus
Bluetongue virus
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Ibaraki virus
Epizootic hemorrhagic disease virus serotype
African horsesickness virus