ACCESS MICROBIOLOGY

RESEARCH ARTICLE

Blasdell *et al.*, *Access Microbiology* 2021;3:000223

DOI 10.1099/acmi.0.000223

MICROBIOLOGY SOCIETY

OPEN ACCESS

First detection of a novel 'unknown host' flavivirus in a Malaysian rodent

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Abstract

Current phylogenetic analysis of the flavivirus genus has identified a group of mosquito-borne viruses for which the vertebrate hosts are currently unknown. Here we report the identification of a novel member of this group from a peridomestic rodent species (*Sundamys muelleri*) collected in Sarawak, Malaysia in 2016. We propose to name this novel flavivirus Batu Kawa virus after the location in which it was identified, with the abbreviation BKWV. Characterization of the BKWV genome allowed identification of putative mature peptides, potential enzyme motifs and conserved structural elements. Phylogenetic analysis found BKWV to be most closely related to Nhumirim virus (from Brazil) and Barkedji virus (from Senegal and Israel). Both of these viruses have been identified in *Culex* mosquitoes and belong to a group of viruses with unknown vertebrate hosts. This is the first known report of a member of this group of viruses from a potential mammalian host.

INTRODUCTION

The genus *Flavivirus* within the family *Flaviviridae*, contains positive-sense, single stranded, enveloped RNA viruses, many of which are confirmed arboviruses that circulate between haematophagous arthropods and vertebrate hosts. Several well-known members of this genus are important human or veterinary pathogens, including dengue virus (DENV) [1], Zika virus (ZIKV) [2], yellow fever virus (YFV) [3], Japanese-encephalitis virus (JEV) [4], West Nile virus (WNV) [5] and tick-borne encephalitis virus (TBEV) [6]. However, not all *Flavivirus* spp. are arboviruses and the majority have not been associated with clinical disease. Several species have no known vector and are believed to circulate solely within their vertebrate hosts (often rodents or bats) [7] whilst others are insect specific [8].

In recent years numerous flaviviruses have been discovered, including some from aquatic systems and some which may circulate between marine arthropods and vertebrates [9, 10]. However, the majority of recognized flaviviruses are associated with terrestrial arthropods and/or terrestrial vertebrate hosts. Phylogenetic analysis of terrestrial members of the *Flavivirus* genus places most viruses into four strongly supported clades, comprising (1) mosquito-borne viruses (MBFVs), (2) tick-borne viruses (TBFVs), (3) no-known vector viruses (NKVs) and (4) insect-specific viruses (ISFVs) [11, 12]. The MBFV clade is further separated into two sub-clades, one containing *Aedes*-associated viruses and the other *Culex*-associated viruses. However, several viruses that fall within the *Aedes*-associated sub-clade currently have no recognized vertebrate host, leading to the suggestion that they may represent ISFV-like viruses that, like the true ISFVs, do not require vertebrate hosts to circulate [12].

With its tropical climate and position within the Sundaland hotspot of biodiversity [13], it is perhaps unsurprising that many *Flavivirus* spp. have been detected in Malaysia. At least four human pathogens are known to be present, including DENV, JEV, ZIKV and WNV, although the latter has not been associated with human disease in Malaysia [14]. In addition, seven other flavivirus species have been detected in either

Received 16 September 2020; Accepted 22 March 2021; Published 15 April 2021

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Keywords: Barkedji virus; flavivirus; Malaysia; mosquitoes; *Sundamys muelleri*.

Abbreviations: BJV, Barkedji virus; BKWV, Batu Kawa virus; DENV, dengue virus; DTMUV, duck Tembusu virus; ISFV, insect specific flavivirus; JEV, Japanese encephalitis virus; MBFV, mosquito-borne flavivirus; NHUV, Nhumirim virus; NKV, no-known vector flavivirus; ORF, open reading frame; TBEV, tick-borne encephalitis virus; TBFV, tick-borne flavivirus; TMUV, Tembusu virus; UTR, untranslated region; YFV, yellow fever virus; ZIKV, Zika virus.

GenBank accession: MT762108.

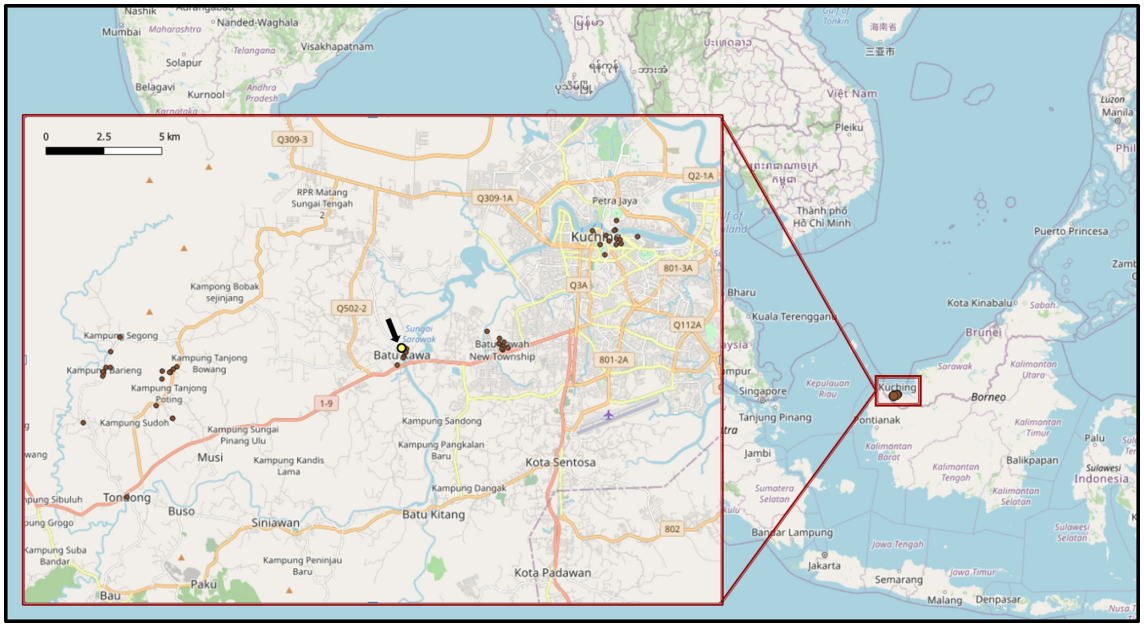

Fig. 1. Map of Borneo with inset map of Kuching and environs, showing rodent sampling sites (maroon circles), with the site where the BKWV was detected highlighted (black arrow pointing to yellow circle).

putative arthropod vectors or vertebrate hosts, including several of veterinary significance, namely Tembusu virus (TMUV), duck Tembusu virus (DTMUV) and Sitiawan virus (SV) [14]. Despite this, no novel flaviviruses have been detected in Malaysian wildlife in recent years. Here we report the identification of a novel flavivirus from a Muller's giant Sunda rat (*Sundamys muelleri*) detected during a study investigating the impact of urbanization on rodent-borne diseases. We described the genome of this novel virus, its relationship to other known flaviviruses and discuss its potential ecology.

METHODS

Collection of rodents

For a detailed description of rodent collection and processing, see [15]. In brief, rodents were collected from the city and environs of Kuching, Sarawak, Malaysia, between September 2015 and April 2016. Sampling was conducted at various locations along a ~25 km long transect from Kuching city centre to the Mount Singai region (Fig. 1). A total of 316 individuals primarily comprised of two species were collected: *Rattus rattus* R3 (165 individuals), *Sundamys muelleri* (100 individuals). Rodents were humanely euthanized using isoflurane, and blood and organ samples were collected and stored at −70 °C.

Sample preparation and Illumina sequencing

Serum samples were clarified prior to nuclease treatment with RNase A (Thermofisher), Turbo DNA-free (Thermofisher) and Benzonase (Sigma), followed by RNA extraction using the QIAmp viral RNA mini kit (Qiagen). Reverse transcription was performed using Superscript III (Thermofisher) and K-8N primer (GACCATCTAGCG ACCTCCACNNNNNNNN) [16], followed by treatment with RNase H (NEB). Serum was then pooled, with each pool containing samples from three to six individuals. Second-strand cDNA synthesis was performed on the pools using Klenow DNA polymerase (NEB). This was followed by a low cycle PCR using Platinum Taq polymerase (Thermofisher) and K primer (GACCATCTAGCGACCTCCAC) [16]. A detailed protocol is available on request. Following DNA quantification, pools were prepared for Illumina sequencing using the Nextera XT library preparation kit (Illumina) and sequenced on an Illumina MiSeq using a MiSeq reagent kit V3 600 cycle cartridge (Illumina).

Sequence analysis

Assembly of the novel flavivirus genome was performed by first trimming raw reads using a sliding window approach (window size of 10 and quality of above 15), and an eight base pair hard trim from the leading and trailing bases using Trimmomatic [17]. Only reads greater than 30 bp were retained for assembly. Metagenomic assembly was performed using quality trimmed reads with the assembler Megahit [18]. Assembled contigs were annotated using Diamond against the non-redundant database [19]. A single contig of 10867 nucleotides with sequence homology to flavivirus genomes was identified, and the 3′ and 5′ ends of the contig manually inspected/trimmed.

Open reading frames (ORFs) were identified using the genome browser tool Artemis [20]. Putative peptides, cleavage sites, cysteine residues and other significant regions were detected manually by comparison to the genomes of

closely related flaviviruses, with particular reference to Barkedji virus (BJV) [21]. Potential N-glycosylation sites were calculated using the program NETNGLYC 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Phylogenetic analysis

A multiple sequence alignment comprising the complete polyprotein sequence of BKWV and representative members of the *Flavivirus* genus was created using MAFFT v7.388 [22] (*N*=71). Ambiguously aligned residues were removed using GBLOCKS [23], resulting in an alignment 2297 amino acids in length. A maximum likelihood phylogenetic tree was estimated using IQ-TREE [24] and the Le and Gascuel model of amino acid substitution with six categories of rate variation (LG+R6), as determined by ModelFinder [25]. Nodal support was calculated using 1000 ultrafast bootstrap replicates and UFBoot2 [26], implemented in IQ-TREE. The resultant tree was visualized using FigTree v1.4.4 [27] and rooted using Tamana bat virus (AF285080) as an outgroup.

Molecular screening for novel flavivirus

The spleens and serum from all captured rodents (*N*=316) were screened for novel flavivirus RNA. RNA was extracted from each sample using the RNeasy mini kit (Qiagen) and reverse transcribed using random hexamers and Superscript III (Thermofisher). Primers targeted specifically to a 603nt portion of the NS22B/NS3 region of the novel flavivirus were designed using the sequence obtained from the Illumina data (OBK_4571F: CTAGAAGTTGGCAACGGTCAG; OBK_5173R: GATGGTGGTCTAATACAGTG). PCRs were conducting using AmpliTaq Gold 360 (Thermofisher) and the following protocol: 95 °C for 2 mins; 40 cycles of 95 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min; 72 °C for 7 mins.

RESULTS

Identification and genome characterization of Batu Kawa virus

During metagenomic analysis of pooled rodent sera, one long contig (>10 kb) was found to have significant sequence identity to several flaviviruses, which was confirmed to be a novel virus following interrogation of the complete GenBank database using BLAST (completed 01 July 2020). Further processing determined the near complete genome to be 10867, which includes the complete coding region (10392 nt) but lacks the extreme 5′ and 3′ untranslated regions (UTRs) (GenBank accession no. MT762108). The deduced complete polyprotein of the novel virus is 3464 aa in length and is comprised of the three structural (C, prM/M, E) and eight non-structural (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, NS5) flavivirus proteins. Putative cleavage site analysis suggests that the viral serine protease is most likely involved in the cleavage of C/anchored capsid (anchC), prM/M, NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/2K and NS4B/NS5, whilst anchC/prM, M/E, E/NS1, NS1/NS2A and 2K/NS4B are most likely cleaved by host proteases. Each of the putative proteins are comparative in size to those from related flaviviruses (Table 1).

Several regions conserved within the flaviviruses were identified in the genome and putative protein sequences of the novel virus. A conserved region of nine peptides in the prM protein, critical for flavivirus particle assembly, was present in the form ERDDIDCWC, identical to that found in BJV [21, 28, 29], whilst in Nhumirim virus (NHUV), this motif exists as DRDDIDCWC. A highly conserved sequence identical to that usually found in mosquito-borne flaviviruses (DRGWGNGCGLFGK) and thought to be homologous to the putative fusion peptide, was also detected in the E protein at positions 98–110. However, the integrin-binding motif, which is present in the E protein of several members of the JEV serogroup, was not present and instead was replaced with MGE at aa positions 390–392. In the NS1 protein of the novel virus, the corresponding site to Proline-250, which is implicated in neuroinvasiveness in JEV serogroup members [30], could be determined at position P-248, as also found in BJV (Fig. 2). Evidence of ribosomal frameshifting in the form of slippery heptanucleotides, as found in the NS2A/NS2B region for other flaviviruses (i.e. 'YCCUUUU' in JEV serogroup viruses; 'GGAUUUY' in insect-specific viruses) was not identified in the novel virus [31, 32]. Several conserved residues within the putative viral serine protease region [33] were identified in the NS3 protein, including the proposed catalytic triade (H-47, D-75, S-135), three of the five putative substrate-binding residues (Y-150, N-152, G-153) and the ultra-conserved residues of serine proteases (G-133, S-135, G-136, G-148, L-149, G-153). The RNA helicase motif DEAH at positions 285–288 was also conserved in the NS3. In the NS4A protein, the motif required for successful cleavage of protein 2K [34], is represented by the motif PDAE (positions 120–123) and the flavivirus-typical RNA-dependent RNA polymerase motif (residues G-666, D-667, D-668 [35];) is present in the NS5 protein.

Cysteine residues typical for flaviviruses were found in several proteins, including prM (six at positions 124, 135, 143, 156, 158, 170), E (12 at positions 3, 30, 60, 74, 92, 105, 116, 121, 190, 294, 311, 342) and NS1 (12 at positions 4, 15, 55, 141, 177, 221, 278, 289, 310, 311, 314, 327) proteins (Fig. 2). Cysteine residues were also identified in other proteins, including NS3 (nine, including six conserved across three or more viruses) and NS5 (16, including 12 conserved across four or more viruses). Potential N-glycosylation sites were also found in several proteins, including the prM (two, at positions 31 and 85), NS1 (three likely at positions 129, 205 and 282 and two possible at positions 94 and 288), NS2A (two at positions 48 and 167), NS3 (two at positions 66 and 499), NS4A (three likely at positions 25, 65 and 218 and one possible at position 21) and NS5 (five likely at positions 215, 235, 341, 654 and 856 and one possible at position 894). Two of the N-glycosylation sites predicted in the NS1 of the novel flavivirus (at positions 129 and 205) correspond to two of the three N-glycosylation sites (N-130, N-175 and N-207) that have been linked to neuroinvasiveness in the

Table 1. Comparison of BKWV putative protein amino acid lengths and cleavage sites with those in other MBFVs

Virus	Polyprotein total length (aa)	C/anchC	anchC/prM	anchC (aa)	prM/M	prM (aa)	M/E	E (aa)	E/NS1	NS1 (aa)	Ns1/NS2A	NS2A (aa)
BKWV	3464	QKKRR/ STGTG	ATTLC/ FTLSF	122	RRSRR/ SVAVA	170	APAYS/ FKCAS	504	TVVAG/ DSGCA	350	SWVTA/ GSTTG	256
BJV	3420	KTSKR/ GLQQS	TMAAC/ ATLGM	108	RRSKR/ SVAIA	167	APAYS/ LHCSR	501/503	TTVAG/ DVGCN	350	SWTTA/ GNATG	233
NHUV	3445	RRARR/ GMGIP	TMVAC/ VTVGT	111	RRSRR/ SVALS	187	APAYS/ THCVR	503	TSAHA/ EVGVS	350	SWVTA/ GQMTG	233
WNV	3433	KQKKR/ GGKTG	ASVGA/ VTLSN	123	RRSRR/ SLTVQ	167	APAYS/ FNCLG	501	VNVHA/ DTGCA	352	SQVNA/ YNADM	231
JEV	3432	KQNKR/ GGNEG	AYAGA/ MKLSN	127	KRSRR/ SVSVQ	167	APAYS/ FNCLG	500	TNVHA/ DTGCA	352	SQVDA/ FNGEM	227
Virus	NS2A/NS2B	NS2B (aa)	NS2B/NS3	NS3 (aa)	NS3/NS4A	NS4A (aa)	NS4A/2K	2K (aa)	2K/NS4B	NS4B (aa)	NS4B/NS5	NS5 (aa)
BKWV	VNGQR/ SLPMG	131	RSWQR/ SGALW	621	AAGRR/ SGLDV	126	AEKQR/ SALDN	23	GMVAA/ NEYGF	255	KAARR/ GRPAG	906
BJV	GSGKR/ SVSMG	129	KGTQK/ AGAMW	621	AEGRR/ GASDI	126	AEKQR/ SAIDN	23	LAVTA/ NEKGL	255	KSARK/ GTPGG	905
NHUV	KSGKR/ SVSMG	130	SATQR/ AGAMW	622	AEGRR/ GAMDL	126	AEKQR/ SALDN	23	LMIAA/ NEKGL	255	KSARK/ GTPGG	906
WNV	PNRKR/ GWPAT	131	QYTKR/ GGVLW	619	ASGKR/ SQIGL	126	PEKQR/ SQTDN	23	SAVAA/ NEMGW	255	PGLKR/ GGAKG	905
JEV	PNKKR/ GWPAT	131	KTTKR/ GGVFW	619	AAKGR/ SAISF	126	PEKQR/ SQTDN	23	GVVAA/ NEYGM	255	PSLKR/ GRPGG	905

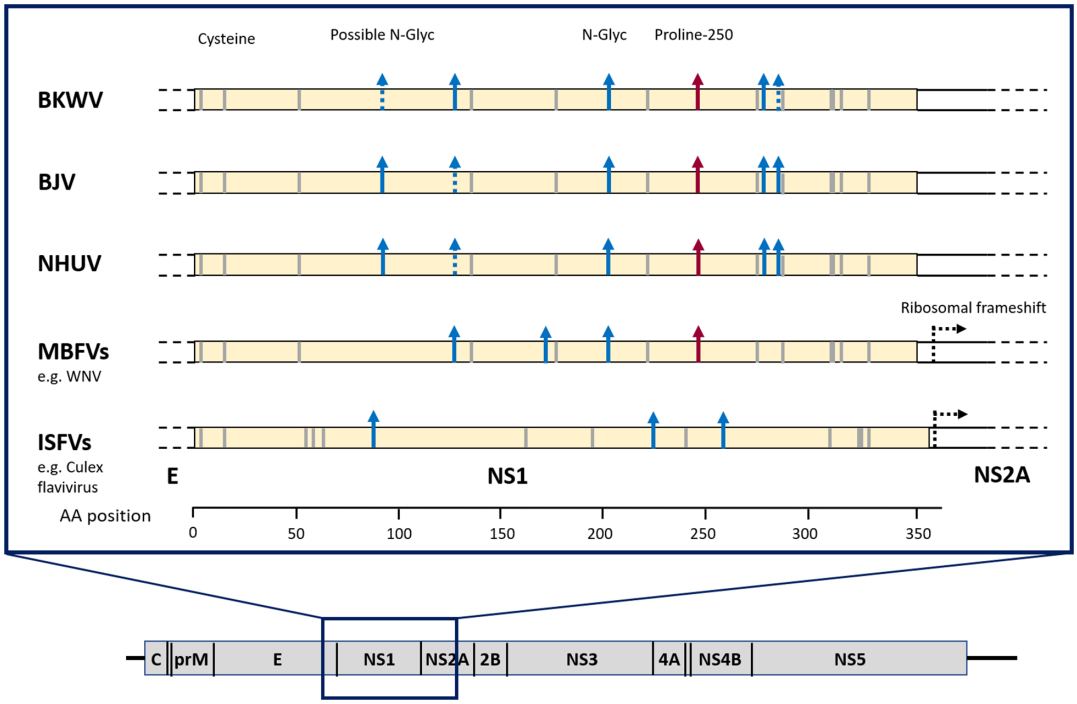

Fig. 2. Comparison of the NS1 region of BKWV, BJV and NHUV to representatives of the MBFVs (WNV) and ISFVs (*Culex flavivirus*). Conserved cysteine residues are shown as grey lines, probable N-glycosylation sites as solid blue arrows, possible N-glycosylation sites as dashed blue arrows, proline-250 as a solid red arrow, and possible ribosomal frameshift locations as dashed black arrows.

JEV serogroup of viruses [36]. In comparison, only one of these sites (at position 205, corresponding to N-207) was predicted to be glycosylated in both BJV and NHUV [21] (Fig. 2). No N-glycosylation sites were identified in the E protein, with the highly conserved N-glycosylation site NYS (aa 154–156) of the JEV serogroup, replaced by DTS.

Although the extreme termini of the 5′ and 3′ UTRs of BKWV could not be resolved, 400 nucleotides of the 3′ UTR were determined. Within this region the highly conserved flavivirus sequences CS1 and CS2 [37, 38] were identified in the forms AGCATATTGACACCAGGGAAAGAC and GGACTAGTGGTTAGAGGAGACCC at 341 and 294 nucleotides from the 3′ end of the polyprotein sequence, respectively.

Phylogenetic and sequence analysis of Batu Kawa virus

Pairwise analysis of the complete polyprotein found that the novel virus is most similar to NHUV (62.6 and 62.5%) and BJV (62.2 and 62.4%), at both the nucleotide and amino acid levels, respectively. The novel virus also clusters closely with these two viruses in our phylogenetic tree and falls more broadly within the larger group of 'insect specific-like' flaviviruses, that have not yet been associated with a vertebrate host (Fig. 3). Based on the ICTV demarcation criteria for flaviviruses (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/360/genus-flavivirus) [39], these sequence identities, geographic location, and species in which the novel virus was detected, together indicate that this virus should be considered a novel species with the proposed name *Batu Kawa virus*, and abbreviation BKWV.

Molecular survey of Malaysian rodents for infection with Batu Kawa virus

Only a single animal (MYR-097) was found to be positive by BKWV-specific PCR for both spleen and serum. Subsequent testing of other organs from this animal also found the kidney to be positive, which was confirmed by sequencing. The positive animal belonged to the species *Sundamys muelleri* and was collected in a green space on the edge of a village in a suburban area (1.513° N, 110.270° E). Attempts to isolate BKWV in *Aedes albopictus* C636, Vero and BHK-BSR cells from infected serum and tissues were unsuccessful.

DISCUSSION

Several flaviviruses, many of which are of medical and/or veterinary importance, have been recorded from Malaysia [14, 40], but this is the first report of a flavivirus detected in wild rodents. As BKWV was only detected in a single Muller's giant Sunda rat, it is unknown if this species represents the natural host of this virus. However, detection of the virus in multiple organs as well as serum does suggest a genuine infection in this animal. Muller's giant Sunda rat is found across parts of Peninsular and Island Southeast Asia [41], where it

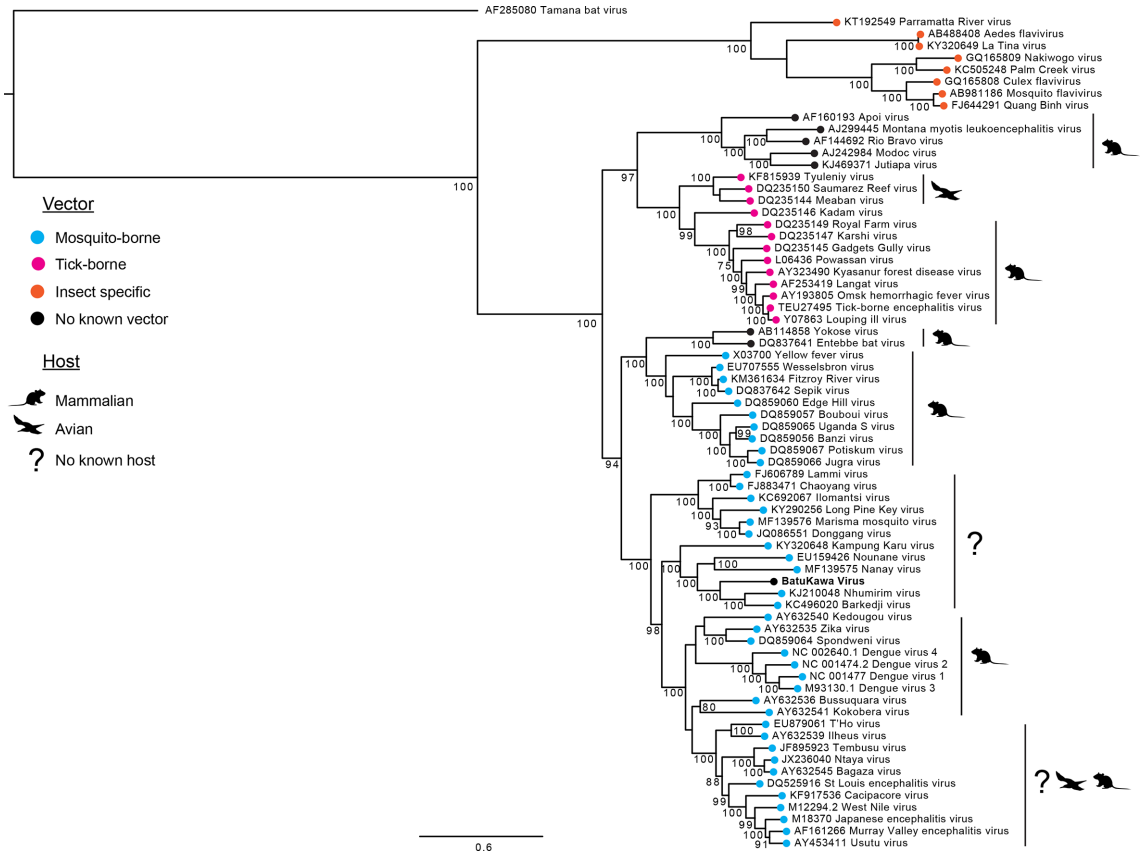

Fig. 3. Phylogenetic relationships of BKWV and representative flaviviruses (*N*=71) based on a trimmed alignment of the complete polyprotein (2297 aa). Bootstrap values are indicated for nodes with ≥75% support. The position of the mosquito-borne viruses (MBFVs), tick-borne viruses (TBFVs), no-known vector viruses (NKVs) and insect-specific viruses (ISFVs) are indicated.

shows some degree of adaption to urban areas [42, 43]. In this study it was found across the transect, including in remnant green patches within the urban centre of the city of Kuching [15]. If this rodent species is a natural host of BKWV, then the opportunity exists for BKWV to co-circulate in regional urban areas alongside dengue virus [44]. Several other flavivirus species have also been associated with rodents, but none are closely related to BKWV and most belong either to the TBFV or NKV clades [7, 45]. As BKWV falls within a subclade containing *Flavivirus* spp. with unknown vertebrate hosts, no further conclusions can yet be drawn about this relationship. However, if this is the true vertebrate host of BKWV it is possible that other species in this 'ISFV-like' subclade also circulate in vertebrates and are not mosquito-specific as has been proposed [12].

By looking at the closest relatives of BKWV, BJV and NHUV, it may be possible to infer some information about its vector. The former is named for the region where it was originally identified in Senegal and appears to have a wide geographic range, having since been identified in *Culex perexiguus* mosquitoes in Israel and the United Arab Emirates [21, 46], and (based on sequences available in GenBank) in *C. quinquefasciatus* mosquitoes in Oman and Zambia. In contrast, NHUV has only been detected once in *C. chidesteri* from Brazil [47]. This suggests that BKWV most likely utilizes a *Culex* sp. mosquito vector. *Culex quinquefasciatus* mosquitoes were detected at the same site where the rodent infected with BKWV was collected (data not published), but further studies are needed to establish if this species is the vector of BKWV.

Batu Kawa virus contains numerous conserved sequence elements that are common to viruses within the genus *Flavivirus* and to the MBFVs in particular (Fig. 2). These include three putative markers in the NS1 protein that have been related to neuroinvasiveness in JEV serogroup viruses, namely Proline-250 (at position 248 in BKWV) and the N-glycosylation sites N-130 and N-207 (at positions 129 and 205 respectively in BKWV) [30, 36]. In comparison, position 129 was not predicted to be N-glycosylated in the closest relatives of BKWV, BJV and NHUV, due to the surrounding residues. However, the ribosomal frameshift-stimulating motif responsible for the generation of the NS1' protein, which has been experimentally associated with neuroinvasiveness [48], was not present in either BKWV, BJV or NHUV. This suggests that BKWV is unlikely to pose a public health threat.

Although BKWV clusters with BJV and NHUV in a well-supported clade, it is clearly genetically distinct with both nucleotide and amino acid sequence identities of <63% to both viruses. Unfortunately, as attempts to culture this virus in several cell lines were unsuccessful, further characterization, including an assessment of its antigenic relationships, could not be performed. However, as this is the first flavivirus associated with rodents in Malaysia, and the first one to be isolated from Muller's giant Sunda rat, we propose that BKWV fulfils the ICTV criteria needed to designate it as a novel species within the *Flavivirus* genus [39].

As BKWV does not group closely with flavivirus species known to be pathogenic for humans, it is unlikely that this virus represents a zoonotic threat. However, it does contain some markers that have been linked to neuroinvasiveness and is potentially hosted by a locally common rodent species that is resident in urban areas. Therefore, further studies on this virus, including additional isolation attempts in other cell lines, would be warranted to fully ascertain its biology, ecology and potential risk to human and/or animal health.

Funding information
This study was supported by a Discovery Early Career Researcher Award to CF from the Australian Research Council (DE150101259). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
The authors thank Andrew Alek Tuen (Universiti Malaysia Sarawak), Andrew Joris Noyen (Padawan Municipal Council), Basheer Ahmed (Kuching North Municipal Council), Danielle Levesque (Universiti Malaysia Sarawak), Dilop Jina (Padawan Municipal Council), Jean-Bernard Duchemin (CSIRO), Lee Trinidad (CSIRO), Nurshilawati Abdul Latip (Universiti Malaysia Sarawak), Patrick Lai Ganum (Kuching South Municipal Council), Rachel Amos-Ritchie (CSIRO), and Samuel Wong (Universiti Malaysia Sarawak).

Author contributions
K. R. B., was involved in the conceptualisation of the project, development and design of the methodology, conducting the experiments, interpretation of results and writing the manuscript draft. J. W. W., was involved in the analysis of the data and writing the manuscript draft. D. P., was involved in writing the manuscript draft. C. F., was involved in the conceptualisation of the project, securing of funding, development and design of the methodology, conducting the experiments, interpretation of results and writing the manuscript draft.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All animal sampling was conducted under animal ethics project protocol number 1750, authorized through the Australian Animal Health Laboratory, and the Sarawak Forests Department (Permit: NCCD.907.4.4 (JLD.12)–131).

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