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# Discovery and characterisation of a new insect-specific *bunyavirus* from *Culex* mosquitoes captured in northern Australia ☆

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ABSTRACT

Insect-specific viruses belonging to significant arboviral families have recently been discovered. These viruses appear to be maintained within the insect population without the requirement for replication in a vertebrate host. Mosquitoes collected from Badu Island in the Torres Strait in 2003 were analysed for insect-specific viruses. A novel bunyavirus was isolated in high prevalence from *Culex spp.* The new virus, provisionally called Badu virus (BADUV), replicated in mosquito cells of both *Culex* and *Aedes* origin, but failed to replicate in vertebrate cells. Genomic sequencing revealed that the virus was distinct from sequenced bunyavirus isolates reported to date, but phylogenetically clustered most closely with recently discovered mosquito-borne, insect-specific *bunyaviruses* in the newly proposed *Goukovirus* genus. The detection of a functional furin cleavage motif upstream of the two glycoproteins in the M segment-encoded polyprotein suggests that BADUV may employ a unique strategy to process the virion glycoproteins.


## Introduction

The family *Bunyaviridae* comprises over 350 antigenically distinct RNA viruses that are characterised into five genera: *Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus* and *Tospovirus* (King et al., 2012). Bunyaviruses have a negative-sense (or ambi-sense), single stranded RNA, tripartite genome encoding the RNA dependent RNA polymerase (RdRp; L segment), two glycoproteins (Gn and Gc; M segment) and the Nucleocapsid protein (N; S segment). Some viruses of the *Orthobunyavirus, Phlebovirus* and *Tospovirus* genera also encode nonstructural proteins NSs and NSm using an overlapping open reading frame or ambi-sense coding.

Members of the *Phlebovirus* genus are predominantly transmitted by phlebotomine sandflies, although ticks and mosquitoes are also associated with the transmission of significant viruses of this genus including Uukuniemi (UUKV) and Rift Valley fever viruses (RVFV), respectively. Arthropod-borne bunyaviruses of medical relevance, considered to be new species of the *Phlebovirus* genus have recently been reported. The closely related viruses, Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) and Heartland virus, both associated with ticks, have been linked to severe febrile illness and thrombocytopenia in patients in China and North America, respectively, since 2009 (McMullan et al., 2012; Pastula et al., 2014; Yu et al., 2011; Zhang et al., 2012). These viruses, along with the recent emergence of the agriculturally significant orthobunyavirus, Schmallenberg virus (Hoffmann et al.,

☆The Genbank accession numbers for the Badu virus L, M and S segments are KT693187, KT693188 and KT693189 respectively

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2012), highlight the continued significance of bunyaviruses to human and animal health.

With the application of enhanced technologies, such as next generation sequencing, additional viruses which have not been associated with disease, have been detected in or isolated from a number of arthropod species, including mosquitoes and ticks (Coffey et al., 2014; Cook et al., 2013; Warrilow et al., 2014; Zirkel et al., 2011). Of particular interest are four unusual and divergent groups of bunyaviruses recently identified in mosquitoes that do not appear to replicate in vertebrate cells (Marklewitz et al., 2011, 2015, 2013). Gouleako virus (GOLV) was isolated from mosquitoes collected in Cote d'Ivoire and found to be evolutionarily related to the phleboviruses (Marklewitz et al., 2011). However, due to multiple characteristics making it distinct from the phleboviruses, a new genus was proposed and tentatively named "*Goukovirus*" (Auguste et al., 2014; Marklewitz et al., 2011, 2015). The most recent relative of GOLV discovered is Cumuto virus (CUMV) (Auguste et al., 2014) which was isolated from mosquitoes collected in Trinidad. While CUMV was unable to cause cytopathic effect (CPE) in vertebrate cell lines, nor cause disease in two day old mice, extensive assays are yet to be performed to confirm that the tropism of this virus is restricted to insect cells (Auguste et al., 2014). Similarly, the second, third and fourth unique groups of viruses, also initially identified from the cohort of mosquitoes collected in Cote d'Ivoire, do not replicate in vertebrate cells and phylogenetically could not be assigned to an established bunyavirus genus (Marklewitz et al., 2015, 2013).

Viruses that appear only to replicate in a mosquito vector, without the requirement for vertebrates as part of their transmission cycle, have also been identified in other arbovirus families (Cook et al., 2006; Crabtree et al., 2003; Hoshino et al., 2007; Nasar et al., 2012). Recent analysis of these viruses is providing valuable information on the evolution and genetic divergence of individual viral species, and molecular determinants of transmissibility and pathogenesis. Moreover, there are indications that persistent infection with insect-specific viruses may interfere with the ability of mosquitoes to transmit viral pathogens of vertebrates (Bolling et al., 2012; Kent et al., 2010; Newman et al., 2011). Thus, the discovery and characterisation of new insect-specific viruses will not only enhance our understanding of the mosquito virome, it will also enhance our understanding of virus transmission.

After Japanese encephalitis virus (JEV) emerged in 1995 in the Torres Strait, the region that separates mainland Australia from Papua New Guinea (PNG), a surveillance programme was established on Badu Island in 2003 (Hanna et al., 1999, 1996). Mosquitoes were trapped and tested for the presence of JEV using virus-specific real-time RT-PCR assays (van-den-Hurk et al., 2008), but the presence of other arboviruses in the mosquito pools collected during the 2003 trapping period was not determined. In the current paper we report the isolation, and genetic and phenotypic characterisation of a novel bunyavirus from *Culex* mosquitoes collected from Badu Island. This proposed new virus fails to replicate in cultured mammalian and avian cell lines, and is likely to represent a distinct genetic lineage within the newly proposed insect-specific genus, *Goukovirus*, of the *Bunyaviridae* family.

## Results

### *Detection, isolation and culture of the prototype BADUV isolate from Badu Island*

During surveillance for Japanese encephalitis virus (JEV) in the Australasian region in 2003, mosquitoes were trapped at various sites on Badu Island (van-den-Hurk et al., 2008). A subset of mosquito pools ($n=100$) that were not positive for JEV, nor caused cytopathic effect (CPE) in vertebrate cells, were further examined for the presence of insect-specific viruses. One pool of *Culex sitiens* (pool TS6347) caused clear morphological changes to the C6/36 cell monolayer after 6 days that was characterised by the fusion of the cells and syncytia. Reverse transcription PCR (RT-PCR) of extracted RNA using primer sets targeted to common viruses present in Australian mosquitoes revealed that the virus was not a flavivirus, mesonivirus, nor Liao ning virus – a recently discovered reovirus that is highly prevalent in Australian mosquito populations (data not shown, (Coffey et al., 2014)). Sequencing of extracted RNA by deep sequencing revealed that at the time of sequencing, the unknown virus was a bunyavirus (based on partial L segment sequence) that was distantly related to the Uukuniemi serocomplex of the *Phlebovirus* genus. Consistent with this identification, partial sequence for all 3 segments of the negative sense genome were obtained. This virus was tentatively named Badu virus (BADUV).

### *Detection of BADUV in additional mosquito pools from Badu Island*

Testing of additional pools of *Culex spp*. mosquitoes collected from a number of different trapping sites on Badu Island by RT-PCR and primers designed to the prototype sequence, revealed a high prevalence of BADUV in pools of both *C. sitiens* and *C. annulirostris* (100% (6/6) and 39% (7/18) respectively) (Table 1). Furthermore, sequencing of a 532 nt region of the M segment of each isolate gave $\geq$97% nucleotide identity to the prototype (pool TS6347), suggesting that these are likely to be strains of the same virus species.

### *BADUV virions display typical bunyavirus morphology*

Electron microscopy of negative-stained BADUV virions revealed spherical, enveloped particles of uniform size and appearance, consistent with the typical morphology of a bunyavirus (Fig. 1). The images of 113 intact virions were selected and the mean $\pm$ SD diameter was determined to be 131.9 $\pm$ 4.9 nm. Damaged virions were also observed within the electron micrographs (black arrows), revealing the presence of presumed ribonucleocapsid proteins (RNPs), which have a thread-like appearance typical of *Bunyaviridae* RNPs (Fig. 1b); (Li et al., 2013; Raymond et al., 2010).

### *BADUV growth is restricted to mosquito cells*

Permissiveness for BADUV to replicate in four different vertebrate cell lines and three mosquito cell lines was assessed by

**Table 1**
Summary of mosquito pools that were positive for BADUV by RT-PCR collected from Badu Island.

| Collection location | Collection year | Mosquito species | No. of pools positive/number tested by RT-PCR | % pairwise identity to isolate TS6347[a] nt/**aa** (number of pools assessed for sequencing) |
|---|---|---|---|---|
| Badu Island | 2003 | *Cx. annulirostris* | 7/18 | 97–99/**98–99** (4) |
| Badu Island | 2003 | *Cx. sitiens* | 6/6 | 97–99/**98–99** (6) |

[a] Nucleotide identity over a 532 nt fragment of M segment.

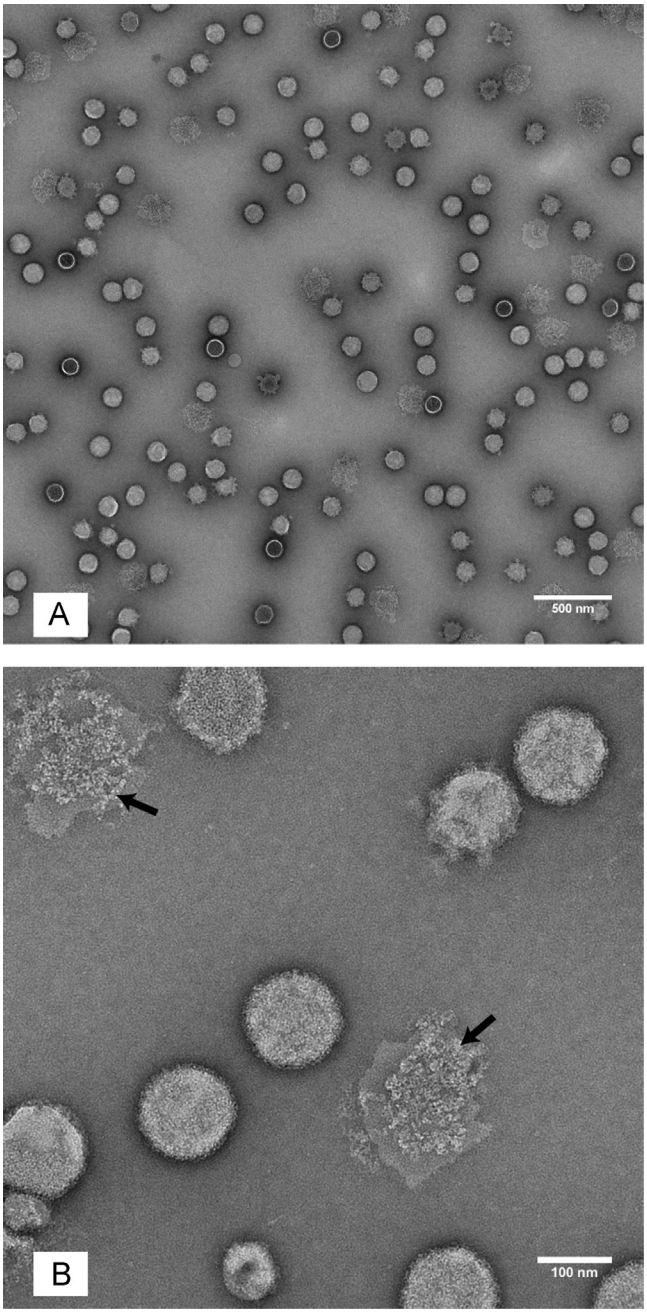


**Fig. 1.** BADUV Morphology. (a) and (b) Negative-staining electron micrograph of BADUV particles following potassium tartrate gradient purification and staining with 1% uranyl acetate. Presumed RNPs are indicated with an arrow (b).

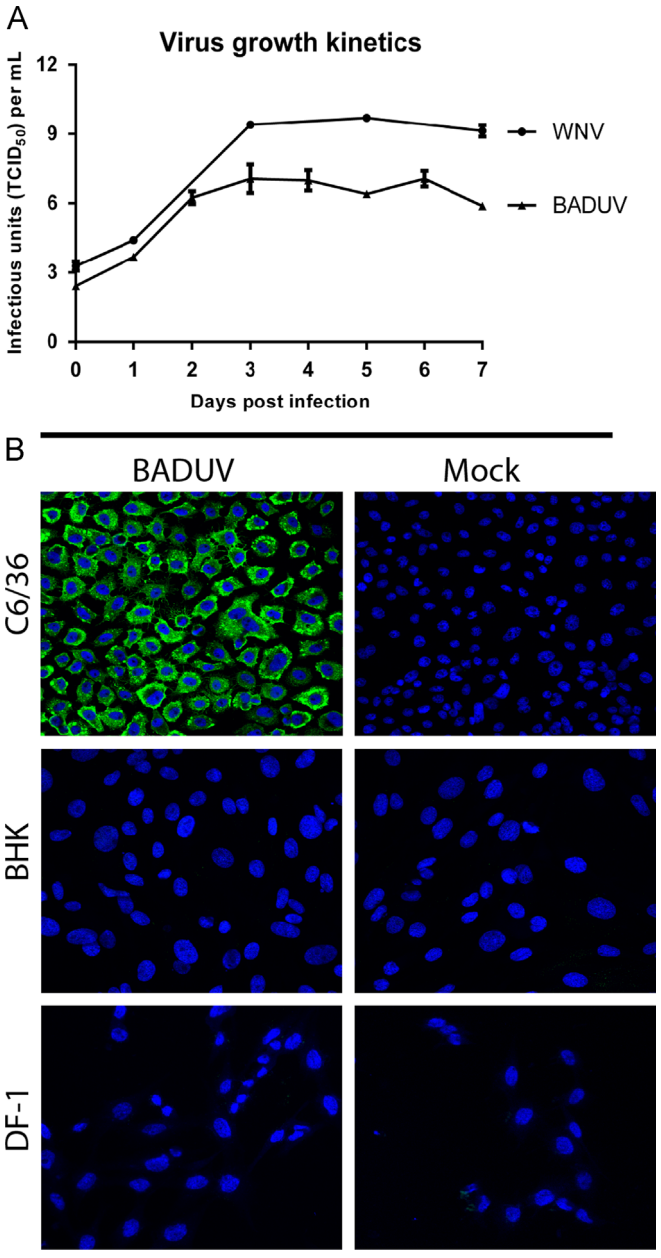


**Fig. 2.** Growth of BADUV in various cell lines. (a) C6/36 cells were inoculated with BADUV or WNV at an MOI of 0.1. Levels of infectious virus were determined by $TCID_{50}$ over 7 days, (b) vertebrate (BHK, DF-1) and mosquito cell monolayers (C6/36) were inoculated with BADUV at an MOI of 10, or mock inoculated and fixed with acetone after 2 days. IFA was perfomed by probing with BADUV-immune mouse serum. The nucleus of each cell was stained with Hoechst. Images were taken using a x40 objective lens.

monitoring the inoculated monolayers for evidence of CPE and by assessing the cell culture supernatant for the presence of viral RNA by RT-PCR. There was no evidence of CPE in any of the BADUV-inoculated cell monolayers (Table 2). BADUV replicated in the two *Culex* cell lines (Chao Ball and HSU), in addition to the C6/36 cells, replicating to a maximum infectious titre of $10^{7.07}$ infectious units per mL after three days in the C6/36 cells (Fig. 2a). In contrast, WNV, which was assayed in parallel as a control, replicated to titres above $10^9$ infectious units per mL. No replication of BADUV was detected in Vero, BHK-21 and SW13 cell supernatants following three passages (Table 2), although a trace RT-PCR product was detected following analysis of the third passage of BADUV on DF-1 cells, which may be attributable to carry-over RNA or extremely low levels of replication. The ability of each of the vertebrate cell lines to support the replication of another bunyavirus was confirmed by the inoculation and subsequent detection by RT-PCR of Catch-Me-Cave virus (CMCV) RNA and the detection of clear CPE induced in the vertebrate cell lines by representative members of three bunyavirus genera (Akabane virus, AKAV (Orthobunyavirus); Finch Creek virus (FCV, Nairovirus); CMCV (Phlebovirus)) (Table 2).

In further support of the inability of BADUV to replicate in vertebrate cells, IFA analysis showed no specific binding of BADUV-reactive mouse serum to BADUV-inoculated vertebrate cells, in contrast to the strong staining of similarly inoculated C6/36 cells at both a high MOI (10) and a low MOI (0.01) (Fig. 2b, Fig. S1).

**Table 2**
Replication of viral isolates in various cell lines.

| | | BADUV | | CMCV (Phlebovirus) | | FCV (Nairovirus) | AKAV (Orthobunyavirus) | mock |
|---|---|---|---|---|---|---|---|---|
| | | CPE[a] | RT-PCR P3 supernatant | CPE | RT-PCR P3 supernatant | CPE | CPE | CPE |
| **C6/36** | *Aedes albopictus* | − | + | − | − | − | − | − |
| **Chao Ball** | *Culex tarsalis* | − | + | − | #+/− | ND | ND | − |
| **HSU** | *Culex quinquefasciatus* | − | + | − | #+/− | ND | ND | − |
| **Vero** | Monkey | − | − | + | + | + | + | − |
| **BHK** | Hamster | − | − | + | + | + | + | − |
| **SW13** | Human | − | − | + | + | + | + | − |
| **DF-1** | Chicken | − | #+/− | + | + | − | + | − |

BADUV – Badu virus, CMCV – Catch-me-cave virus, FCV – Finch Creek virus and AKAV – Akabane virus.
# trace PCR product detected.
[a] CPE P1.

**Table 3**
Genome and segment sizes and comparison of consensus terminal nucleotide sequences of Badu virus with other bunyaviruses.

| Genus/virus | Consensus terminal nucleotides | Segment length | |
|---|---|---|---|
| | | S | L |
| *Phlebovirus*/Rift Valley fever virus | 3'-**UGUGUUUC**<br>5'-**ACACAAAG** | 1690 (X53771) | (X56464) |
| Unassigned/Gouleako virus | S and M segments<br>3'-**UGUGU**UUC<br>5'-**ACACA**GUG<br>L segment<br>3'-**UGUGUUUC**<br>5'-**ACACAAAG** | 1087 (HQ541736) | 6358 (HQ541738) |
| Unassigned/Badu virus | S segment<br>3'-**UGUGUCUCU**<br>5'-**ACACA**AAGA<br>L segment<br>3'-**UGUGUUUCUG**<br>5'-**ACACAAAGAC** | 1513 (KT693189) | 6863 (KT693187) |

*Bold face type indicates complementary conserved terminal nucleotides.

Together, these data indicated that the replication of BADUV is likely to be restricted to insect cells.

### *Genome sequence, organisation and phylogenetics*

The genome of BADUV isolate TS6347 was sequenced using a combination of deep sequencing and Sanger sequencing (Genbank accession numbers: L segment KT693187; M segment KT693188; S Segment KT693189). The termini for the S and L segments were determined by 5' and 3' rapid amplification of cDNA ends-PCR (RACE-PCR, Table 3). The termini of the M segment could not be determined despite multiple attempts. The BADUV L segment termini were similar to those of the genus *Phlebovirus*, but with 10 complementary nucleotides instead of 8 (Table 3). The S segment termini were shorter, with a length of 5 complementary nucleotides, which is attributable to a single mismatch at position 6 in the 3' terminal region.

Each segment had a single predicted open reading frame. The L segment was 6863 nt with a predicted ORF of 2221 aa in cRNA, encoding the putative 256 kDa RdRp. The amino acid sequences of the RNA polymerase of BADUV, representative members of the phleboviruses, nairoviruses, orthobunyaviruses, two members of the newly proposed *goukoviruses* (Auguste et al., 2014; Marklewitz et al., 2011), and a group of mosquito-associated viruses distantly related to the orthobunyaviruses (Marklewitz et al., 2013) were aligned. The alignment was used to generate a phylogenetic tree using a maximum likelihood model (Fig. 3). BADUV clustered with reasonable support with the newly proposed *goukoviruses*, branching deeply within the clade, along with the Phasi Charoen-like (PCLV) sequence obtained following deep sequencing of total RNA from *Ae. aegypti* mosquitoes (Chandler et al., 2014). Pairwise alignment of the predicted BADUV RdRp with that of PCLV, GOLV, CUMV and selected phleboviruses revealed identities of 55.8% for PCLV and between 24.6% and 26.8% for the remainder (Table 4).

A near complete sequence for the M segment was resolved, containing the entire ORF and partial 5' and 3' UTRs. A single ORF encodes a 1239 aa polyprotein. Upon pairwise alignment of select Glycoprotein C (Gc) sequences of the gouko- and phlebo-viruses, the predicted Gc encoded at the terminus of the BADUV polyprotein showed highest homology to PCLV (45.3%) and sequence identities between 22.1% and 25.6% for the remainder analysed (Table 4).

The BADUV S segment of 1513 nt is considerably longer than the 1087 nt of GOLV and 1176 nt of CUMV and is more similar in length to those of the phleboviruses (albeit shorter e.g. UUKV 1720 nt, RVFV ~1690 nt) and of PCLV (1389 nt). However, the complete sequence of the S segment of PCLV has not been ascertained and is likely to be longer. Unlike the viruses in the *Phlebovirus* genus, the BADUV S segment does not appear to encode a NSs protein. Analysis for potential ORFs in the reverse orientation

**Fig. 3.** Phylogenetic tree showing the relationship between BADUV and other bunyaviruses across the RdRp protein. The mid-point rooted tree was based on an MAFFT amino acid sequence alignment of the RdRp (L protein) and was generated by a maximum likelihood model using MEGA 5.03. Representative members of the bunyavirus genera *Phlebovirus* (various serocomplexes), *Orthobunyavirus* and *Nairovirus* are shown. BADUV (shown in bold) branches basally within the *goukovirus* clade. Bootstrap values (1000 replicates) are given as a percentage. Possible new genera are indicated (*). Accession numbers: Aguacate virus YP_004414703, Alenquer virus AEA30054, Arbia virus AGA82737, Ariquemes virus AEA30056, Arumowot virus AEF30501, Bunyamwera virus NP_047211, Candiru virus YP_004347993, Chagres virus AEL29642, Chize virus AFH08728, CoAr 170255 AEL29646, CoAr 171616 AEL29649, Cumuto virus AHH60917, Echarate virus AEA30058, EgAN1825-61 virus AEL29654, Erve virus AFH89032, FinV707 virus AFN73038, Forecariah virus AGC60103, GGP-2011a AEL29685, Gouleako virus AEJ38175, Granada virus ADO17679, Grand=Grand Arbaud virus AFH08732, Heartland virus AFP33396, Herbert virus AGX32059, Itaituba virus AEA30059, Jacunda virus AEA29965, Kibale virus AGX32058, La Crosse virus AAM94387, Maldonado virus AEA30055, Manawa virus AFN73042, Massilia virus V2 ABG56143, Morumbi virus AEA30061, Mucura virus AEA30060, Munguba virus AEL29657, Murre virus AFH08736, Murrumbidgee virus YP_008709776, Nairobi sheep disease virus AED88229, Naples-like=sand fever Naples-like virus AEL29683, Nique virus AEA30062, Oriximina virus AEA30065, Oropouche virus NP_982304, Palma virus AFO66276, Phasi Charoen-Like virus AIF71030, Precarious point virus AEL29680, Rift=Rift Valley fever virus AEB20483, RML-105355 AFH08740, Salehabad virus AGA82741, Salt Ash virus AGX00984, Serra=Serra Norte virus AEA30063, SFTS=Severe fever with thrombocytopenia syndrome virus, AGT98505, Toscana virus AAB25907, Tai virus AGX32057, Turkey=Sandfly fever Turkey virus ACZ55880, Turuna virus AEA30064, Uriurana virus AEL29690, Uukuniemi virus NP_941973, Wyeomyia virus AEZ35273, Zaliv Terpenia virus AEL29693.

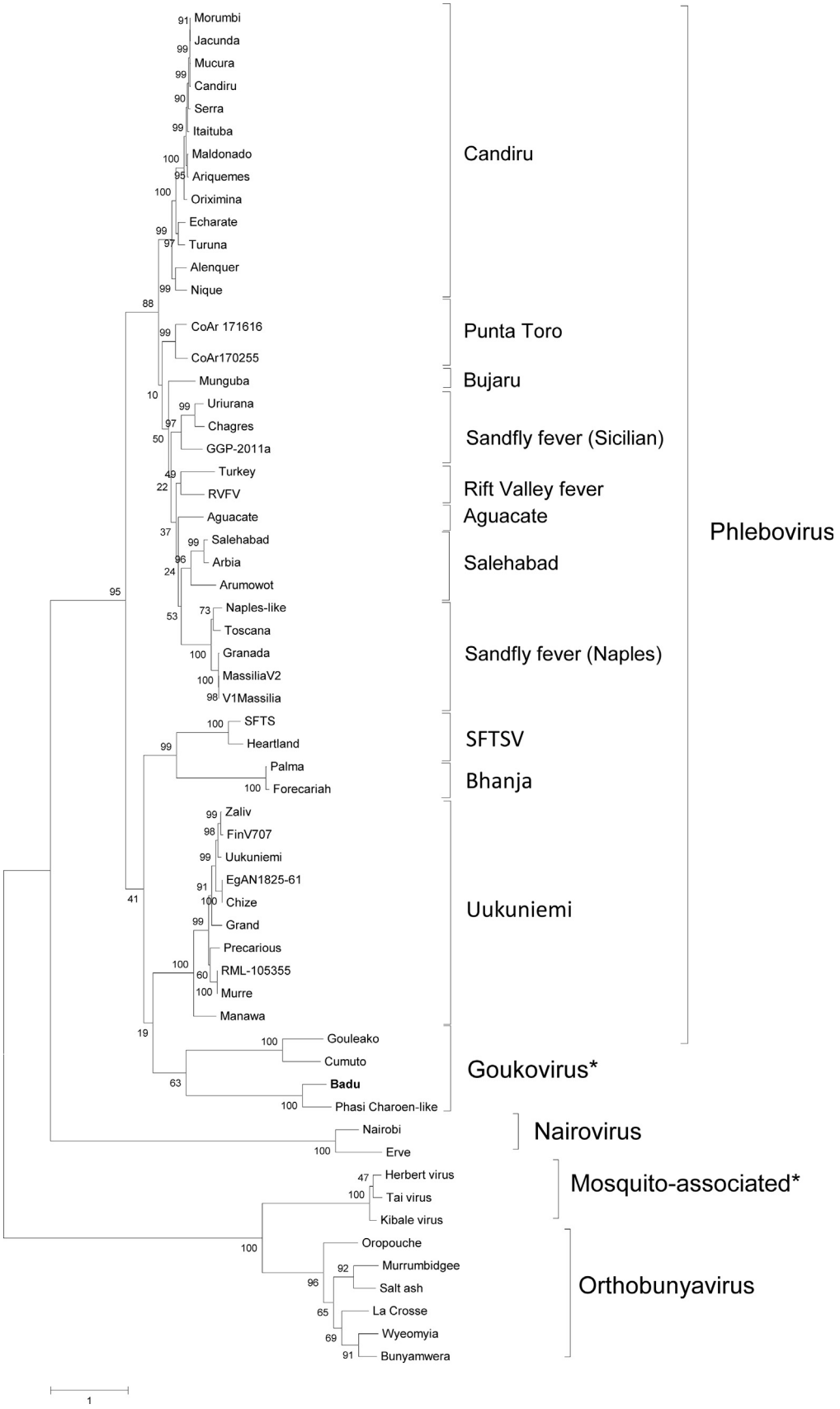
Morumbi
Jacunda
Mucura
Candiru
Serra
Itaituba
Maldonado
Ariquemes
Oriximina
Echarate
Turuna
Alenquer
Nique
CoAr 171616
CoAr170255
Munguba
Uriurana
Chagres
GGP-2011a
Turkey
RVFV
Aguacate
Salehabad
Arbia
Arumowot
Naples-like
Toscana
Granada
MassiliaV2
V1Massilia
SFTS
Heartland
Palma
Forecariah
Zaliv
FinV707
Uukuniemi
EgAN1825-61
Chize
Grand
Precarious
RML-105355
Murre
Manawa
Gouleako
Cumuto
Badu
Phasi Charoen-like
Nairobi
Erve
Herbert virus
Tai virus
Kibale virus
Oropouche
Murrumbidgee
Salt ash
La Crosse
Wyeomyia
Bunyamwera
Candiru
Punta Toro
Bujaru
Sandfly fever (Sicilian)
Rift Valley fever
Aguacate
Salehabad
Sandfly fever (Naples)
SFTSV
Bhanja
Uukuniemi
Goukovirus*
Nairovirus
Mosquito-associated*
Orthobunyavirus
Phlebovirus
1

**Table 4**
Amino Acid pairwise identity values for BADUV and selected members of the Phlebovirus and *Goukovirus* groups.

| Protein | Virus | % amino acid sequence identity | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | BADUV | PCLV | GOLV | CUMV | UUKV | Candiru | Munguba |
| RdRp | | | | | | | | |
| | PCLV | 55.8 | | | | | | |
| | GOLV | 24.6 | 24.3 | | | | | |
| | CUMV | 25.6 | 25.1 | 47.4 | | | | |
| | UUKV | 26.8 | 26.5 | 27.0 | 26.8 | | | |
| | Candiru | 26.7 | 25.9 | 26.8 | 27.5 | 37.1 | | |
| | Munguba | 26.4 | 25.0 | 26.3 | 26.1 | 36.0 | 36.6 | |
| | Toscana | 25.9 | 25.7 | 26.5 | 26.2 | 36.4 | 37.1 | 36.4 |
| Gc | PCLV | 45.3 | | | | | | |
| | GOLV | 22.1 | 22.7 | | | | | |
| | CUMV | 22.3 | 24.0 | 44.2 | | | | |
| | UUKV | 25.6 | 25.7 | 22.9 | 22.0 | | | |
| | Candiru | 23.1 | 22.1 | 22.6 | 23.1 | 29.5 | | |
| | Munguba | 23 | 21.1 | 20.6 | 22.3 | 23.9 | 47.8 | |
| | Toscana | 23.7 | 22.8 | 20.9 | 21.2 | 28.5 | 46.5 | 41.7 |
| N | PCLV | 44.3 | | | | | | |
| | GOLV | 19.9 | 17.8 | | | | | |
| | CUMV | 20.1 | 21.6 | 39.6 | | | | |
| | UUKV | 20.8 | 25.1 | 23.2 | 22.1 | | | |
| | Candiru | 21.5 | 22.4 | 23.3 | 21.8 | 35.8 | | |
| | Munguba | 23.5 | 27.3 | 25.2 | 26.1 | 38.2 | 54.9 | |
| | Toscana | 25.1 | 26.1 | 23.9 | 22.9 | 29.8 | 48.2 | 47.8 |

RdRp – RNA dependent RNA polymerase, Gc – Glycoprotein C, N-Nucleocapsid
Accession numbers RdRp, glycoprotein, nucleocapsid: PCLV (Phasi Charoen-like virus – AIF71030, AIF71031, AIF71032); GOLV (Gouleako virus-AEJ38175, AEJ38174, AEJ38173), CUMV (AHH60917, AHH60918, AHH60919), UUKV (Uukuniemi virus-NP_941973, NP_941979, AAA47958), Candiru virus (YP_004347993, YP_004347992, YP_004347994), Munguba virus (AEL29657, AEL29658, AEL29659), Toscana virus (ABZ85666, ABZ85665, ABZ85663).

revealed only small putative ORFs, the largest being 85 aa with a predicted molecular mass of 9 kDa, and no matches to sequences in Genbank. In the absence of a NSs ORF, it appears that the BADUV mRNA has an uncharacteristically long 5' non coding region (463 nt) prior to the predicted nucleocapsid ORF (269 aa; predicted mass of 29.9 kDa).

Upon analysis of the pairwise alignments for each of the predicted BADUV proteins, the RdRp identities returned the highest values, as expected. As the relationship between the viruses across the other proteins analysed was not substantially different, there is no evidence of reassortment of the BADUV tripartite genome with that of another virus. (Table 4).

*Analysis of BADUV proteins*

To analyse the major structural proteins of BADUV, a potassium tartrate-purified virus preparation was assessed by SDS-PAGE. Three proteins with apparent molecular masses (in unglycosylated form) of approximately 56, 52 and 25 kDa were resolved (Fig. 4a). Each of the bands were excised and analysed by mass spectrometry. The 25 kDa band (band 3) was confirmed to correspond to the putative nucleocapsid protein, with the identification of 28 unique peptides, covering 87% of the predicted nucleocapsid protein sequence (Fig. 4, S2). As expected, this protein was not glycosylated, as determined by PNGase F digestion (Fig. 4a), consistent with other bunyavirus nucleocapsid proteins.

We hypothesise that the BADUV M segment-encoded polyprotein is post-translationally cleaved into the Gn and Gc glycoproteins. Two signal peptidase cleavage sites were identified as well as putative transmembrane domains, potential glycosylation motifs and the Gc cytoplasmic tail and are summarised in Fig. 4c. The predicted molecular mass of BADUV Gc (unglycosylated and including the cytoplasmic tail) was 56 kDa, consistent with the migration of band 1 (Fig. 4a). Mass spectrometry analysis of band 1 revealed that while some unique peptides corresponding to the N-terminal region of the polyprotein were detected, a higher proportion of the peptides hits were restricted to the C terminal region of the glycoprotein polyprotein (Fig. 4c).

Upon analysing the M segment ORF for signal peptide cleavage sites and transmembrane domains, we predicted that the Gn protein would have a molecular mass of 80.6 kDa in unglycosylated form based on *in silico* protein mass estimation. This estimation was at odds with the resolution of a 51–52 kDa protein, presumably Gn, following SDS-PAGE analysis of the PNGase F-treated protein. The putative BADUV Gn sequence was analysed for potential alternative translation initiation sites, but no additional signal peptides that would ensure translocation of the protein to the endoplasmic reticulum were found following methionines. Of note is a furin cleavage motif present at aa 258 (RKKR) of the M segment ORF.

Analysis of virion protein band 2 (Fig. 4) by mass spectrometry, revealed that while there was once again overlap of peptides from both glycoproteins (most likely due to carry-over of protein consistent with the limitations of a 1D gel), there was selective enrichment of unique peptides from the region predicted for Gn. Of importance was that peptides preceding the predicted furin cleavage site were not resolved for either bands 1 or 2 (the first peptide detected for these bands begins with amino acid 262), but were observed in small quantities in the nucleocapsid band (band 3, Fig. 4c). In an attempt to detect the unprocessed N-terminal region of the BADUV glycoprotein, a BADUV-infected C6/36 cell lysate was probed with BADUV-immune serum in Western blot. The mouse serum clearly detected the two glycoproteins, as well as higher molecular weight proteins that were susceptible to cleavage by furin (Fig. 5). We hypothesise that the doublet with an apparent molecular mass of approximately 140 to 150 kDa could represent the entire glycoprotein propeptide. Alternatively, in unreduced form, this protein may also be a dimer of the unprocessed (by furin) N-terminal end of the glycoprotein polyprotein precursor. In any case, this protein was undoubtedly cleavable by furin. Together, these data clearly support the conclusion that post-translational processing of Gn by furin cleavage is occurring.

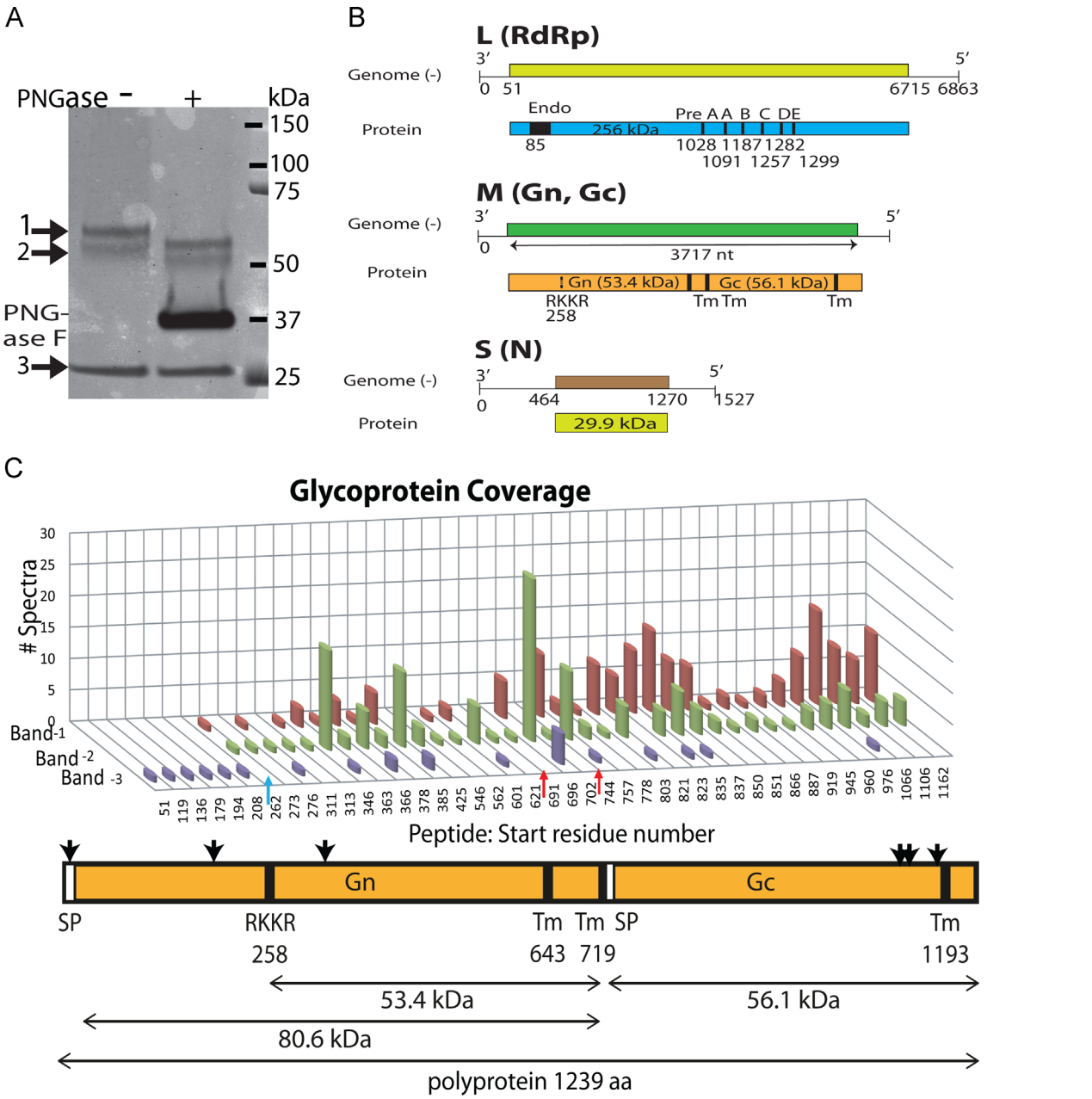


**Fig. 4.** BADUV genome organisation and analysis of structural proteins. (a) SDS-PAGE analysis of gradient-purified BADUV virions. The virion proteins were assessed for the presence of N-linked glycans by treatment with PNGase F (+) or left untreated (−), (b) schematic diagram of the BADUV genome, (c) tryptic digests of BADUV virion proteins 1, 2 and 3 (as highlighted in panel A, but excised from a duplicate gel) were assessed by mass spectrometry. The relative number of spectra for each peptide identified within a band indicates the region of the protein predominant in each digest. In the schematic representation of the M segment ORF. Putative signal peptide (white box, SP), transmembrane helices (black box, first residue of helice indicated), and glycosylation sites (arrow) are indicated as determined by signalP, TMHMM and NetNGlyc respectively. The predicted size of each protein as determined by http://web.expasy.org/cgi-bin/protparam/protparam is indicated.

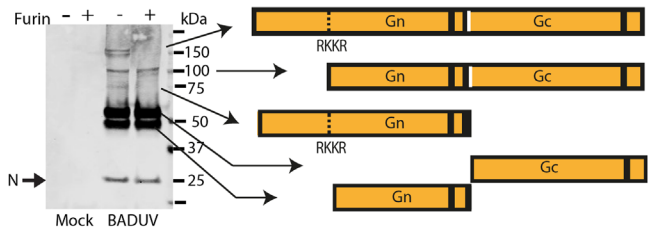


**Fig. 5.** Furin digestion of BADUV M segment polyprotein. Mock and BADUV-infected C6/36 cell were lysed and left untreated (−) or treated (+) with furin. The lysates were assessesd by Western blot and probed with anti-BADUV immune mouse serum. The hypothesised identity of each of the bound proteins is provided in the schematic to the right of the Western blot.

Post-translational processing by furin is not predicted for *goukovirus* group viruses (GOLV and CUMV), nor for phleboviruses, but is used by Crimean–Congo haemorrhagic fever virus (CCHFV) to process the Gn protein (Sanchez et al., 2006).

### *Serological analysis*

Preliminary antigenic distinction of BADUV from the *Phlebovirus* genus was assessed using BADUV mouse antiserum and CMCV (phlebovirus of the UUKV serocomplex and the closest relative of BADUV available to us)-inoculated cells. Analysis of the antiserum against BADUV proteins as cell lysate by Western blot revealed the presence of antibodies reactive to both BADUV

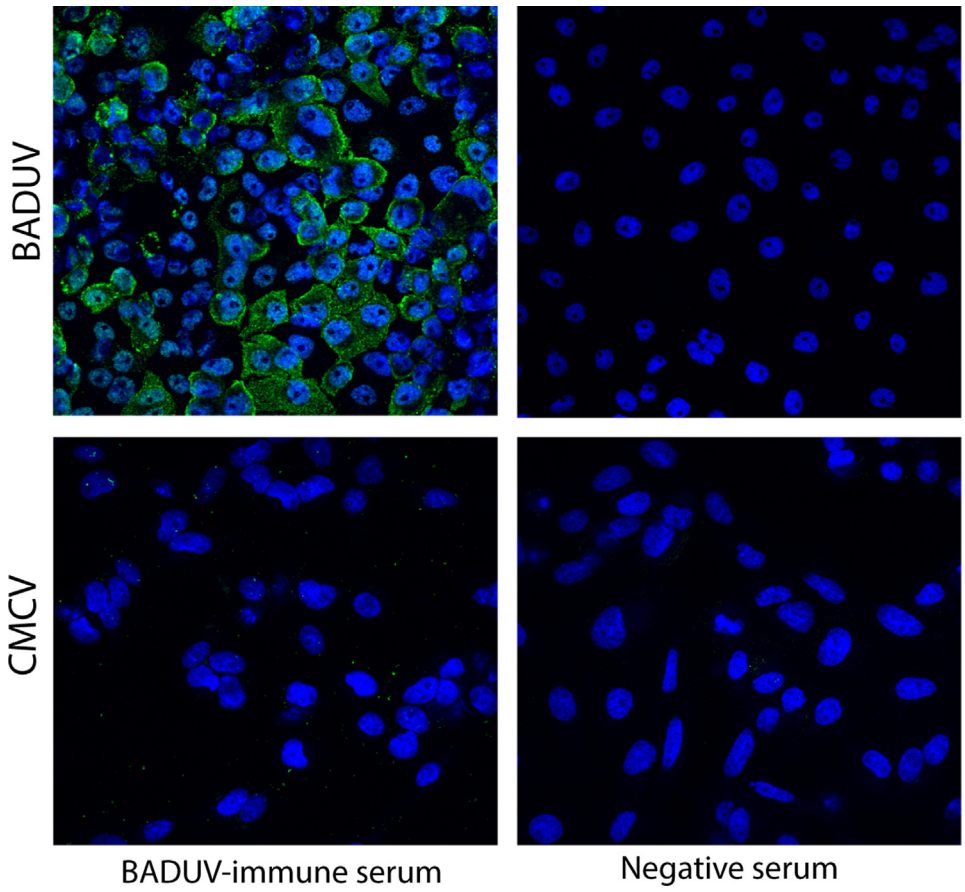


**Fig. 6.** Immunofluorescence assay with BADUV and phlebovirus-infected cells. Mosquito cell (C6/36) monolayers were infected with BADUV and Vero cell monolayers were infected with CMCV at an MOI of 0.1. Four days post-infection, the cells were fixed and probed with either BADUV-immune mouse serum or negative mouse serum. The nucleus of each cell was stained with Hoechst. Images were taken using a x40 objective lens.

glycoproteins and the nucleocapsid protein (Fig. 5). Due to the inability of CMCV to replicate in mosquito cells, Vero cells were inoculated with CMCV and infection confirmed by the observation of clear CPE and subsequent determination of infectious particles in the culture supernatant. No staining of CMCV-infected cells was observed by IFA upon probing with the BADUV antiserum, while clear staining was observed for BADUV-infected C6/36 cells (Fig. 6), indicating that BADUV is serologically distinct from a related bunyavirus.

## Discussion

We have identified a novel bunyavirus in mosquitoes collected from northern Australia. The virus was named Badu virus (BADUV) after the location of the first isolate on Badu Island in the Torres Strait. The virus replicated in a range of *Culex* and *Aedes* mosquito cell lines, but failed to grow in mammalian and avian cell lines, indicating that its tropism is likely to be limited to insects. This virus represents the first mosquito-specific bunyavirus to be found in Australia and is distinct from all other bunyavirus isolates characterized to date.

Phylogenetic analysis clearly showed that BADUV is related to, but distinct from, viruses of the *Phlebovirus* genus. This is consistent with a lack of cross-reactivity of polyclonal sera raised to BADUV against a representative member of the tick-borne Uukuniemi group, CMCV, the most genetically similar lineage of the phleboviruses. However, serological analysis must be performed with other phleboviruses to confirm this observation. Indeed, BADUV appears to be genetically closer to the recently identified mosquito-borne, insect-specific members of the proposed *Goukovirus* genus. However, while BADUV clusters most closely with these other insect-specific bunyaviruses, the low amino acid identities over all proteins analysed indicate that BADUV is divergent from GOLV and CUMV. More recently, a bunyavirus sequence (PCLV) has been obtained from *Aedes aegypti* in Thailand and from colony mosquitoes of the same species from Brazil, that was phylogenetically related to *goukoviruses* and phleboviruses (Aguiar et al., 2015; Chandler et al., 2014). As no virus isolate was obtained in these particular studies, its relevance to bunyavirus taxonomy has yet to be confirmed. However, when each of the BADUV protein sequences were aligned with those of PCLV, it was clear that these two viruses are more closely related to each other than to other *goukoviruses*.

Previous studies have used the terminal sequences of the bunyavirus genomic segments to assist in classifying the viruses into the various genera (Elliott and Schmaljohn, 2013). In this context it is worth noting that while the L segment terminal sequence of BADUV resembles that of the *Phlebovirus* genus, a mismatch is present in the terminal sequence of the S segment. A similar observation was also noted for GOLV where the L segment terminal sequence conformed to that of the phleboviruses, whereas two mismatches were identified in the termini of the S and M segments (Marklewitz et al., 2011). Sequences of the 5' and 3' termini of other recently identified (or sequenced) phleboviruses, such as SFTSV and Lone Star virus, also deviate from the traditional consensus sequences (as summarised in Elliott and Brennan, (2014)), highlighting that not all viruses placed in the *Phlebovirus* genus adhere to the traditional classification.

While functional and phylogenetic analysis of BADUV indicates it is most closely related to viruses of the recently proposed *Goukovirus* genus, the larger genome size of BADUV is more similar to that of the phleboviruses. BADUV appears to be different from the phleboviruses and the *goukovirus* members, GOLV and CUMV, in the post-translational processing of one of the two glycoproteins that results in a shorter protein than predicted. The presence of a furin cleavage motif within the elucidated sequence, supportive mass spectrometry data and detection of viral proteins sensitive to digestion with furin strongly supports the utilisation of this site. Indeed this form of glycoprotein processing by a furin-like protease has been proposed for CCHFV, a member of the *Nairovirus* genus (Sanchez et al., 2006). Interestingly, an identical RKKR motif is present at aa 257 in the M segment ORF of PCLV. Similar sequences are also present in the M segment sequences of GOLV (DKKR at aa 259) and CUMV (GKKR at aa 177), although these sequences do not fulfil the minimum furin recognition consensus sequence of R-X-X-R.

While the glycoproteins of the other *goukoviruses* are yet to be analysed, phleboviruses are known to employ a variety of strategies in processing the M segment gene product. Translation of the RVFV M segment gene product is complex. From five in-frame translation initiation codons, three polyprotein precursors are produced that are cleaved by cellular signalases to generate 4 proteins – NSm, Gn, Gc and a 78 kDa glycoprotein which is a fusion of NSm and Gn (Kakach et al., 1988; Suzich and Collett, 1988; Suzich et al., 1990). In this context, the N-terminal region of the BADUV M segment ORF was analysed for alternative signal sequences that would ensure the translocation of the polyprotein into the endoplasmic reticulum. Apart from the signal peptide following the first methionine, no others were predicted in the sequence upstream of the elucidated peptides of BADUV virion protein bands 1 and 2 by mass spectrometry.

Like RVFV, other phleboviruses from the phlebotomus fever group-Punto Toro virus and Toscana virus, are also predicted to produce a polyprotein precursor from which NSm is cleaved, upstream of the two glycoproteins (Gro et al., 1997; Ihara et al., 1985). Whether the amino-terminal region of the BADUV M segment ORF similarly produces an NSm protein warrants further investigation. While RVFV NSm has been shown to be a virulence factor in vertebrates (Kreher et al., 2014), recent studies have shown that deletion of NSm and the 78 kDa NSm-Gn fusion protein lowers the virus infection and dissemination rates in the mosquito (Crabtree et al., 2012; Kading et al., 2014) and that the 78 kDa Nsm-Gn fusion protein that is the major determinant that affects the virus dissemination in the mosquito (Kreher et al., 2014). Given that insect-specific viruses are likely to be transmitted vertically and not via a blood-meal, the relevance of this information in the context of insect-specific viruses is unclear and warrants further investigation.

The inability of BADUV to replicate in vertebrate cells was consistent with the lack of the NSs gene, which is present within the S segment of phleboviruses, but is absent in the genome of the other insect-specific bunyaviruses, apart from the recently characterised Jonchet and Ferak viruses (Elliott and Schmaljohn, 2013; Marklewitz et al., 2011, 2015, 2013). While NSs was shown to be unnecessary for replication of a RVFV infectious clone in two mammalian cell lines (Ikegami et al., 2006), there is evidence that it is associated with virulence due to its function as an interferon antagonist (Bouloy et al., 2001; Vialat et al., 2000). Due to the additional role of NSs as a host cell transcription inhibitor (Le May et al., 2004), the lack of this gene may also be instrumental in establishing persistent bunyavirus infections in the mosquito (Leger et al., 2013).

In general, the structures of the S segments of the family *Bunyaviridae* have a diverse transcriptional strategy, coding capacity (one or two ORFs) and size. Preliminary analysis of the *Goukovirus* group S segment reveals a single open reading frame (ORF) encoding the nucleocapsid protein (N), differing from the most closely related genus, the phleboviruses, by encoding only one ORF in its S segment and lacking an ambisense transcriptional strategy to produce NSs. Interestingly, the S segment of BADUV is longer (1.513 kb) than both the Cumuto (1.176 kb) and Gouleako viruses (1.087 kb) and closer in size to the S segment of the phleboviruses. This might indicate that the most recent common ancestor of both the phleboviruses and the proposed *goukoviruses* had an S segment of approximately 1.5 kb; subsequently, the segment decreased in size following divergence of CUMV and GOLV viruses. It should also be noted that upon comparison of the S segments of BADUV with GOLV and CUMV, all the S segments encode similarly sized N proteins of 269, 267 and 261 and amino acids, respectively. Hence, the viruses primarily differ in the size of their S segment by the length of their untranslated regions. Interestingly, this is particularly the case with the 463 nt 5' untranslated region of the basally branching BADUV, which is considerably larger than 86 and 81 nt for CUMV and GOLV viruses, respectively. It was noted by Chandler et al. (2014) that PCLV was also predicted to have a large non-coding region prior to the nucleocapsid ORF. In light of studies performed by Moutailler et al. (2011) showing that serially passaged RVFV in mosquito cells acquired large nucleotide deletions in the NSs gene and that such mutations did not occur upon alternating the passaging between vertebrate and mosquito cells, it is tempting to speculate that the most recent ancestor of BADUV did possess an ORF for a functional NSs that was subsequently lost upon repeated transfer of the virus from one mosquito to the next without the involvement of a vertebrate host. However, the detection of expressed putative NSs protein for the insect-specific Jonchet virus from an ORF upstream of the N ORF highlights the requirement for future analysis in this area since rigorous examination of expressed proteins of GOLV, CUMV and PCLV are yet to be performed and the lack of an encoded NSs by these viruses needs to be validated (Marklewitz et al., 2015).

At 131.9 nm, the BADUV virions observed by electron microscopy following negative staining are larger than the typical 80–120 nm diameter of bunyaviruses (Elliott and Schmaljohn, 2013) and display a more uniform morphology in comparison to GOLV (Marklewitz et al., 2011). Treatment of BADUV virions with glutaraldehyde did, however, cause the virions to adopt a shape more similar to that presented for GOLV. We have also observed differences in the apparent size of virions previously that were attributable to the fixation and staining protocols (Warrilow et al., 2014).

BADUV was found in high prevalence in two species of mosquitoes on Badu Island, with 100% and 39% of *Cx. sitiens* and *Cx. annulirostris* pools positive, respectively. Although we have demonstrated that there is a high prevalence rate in members of the *Cx. sitiens* subgroup, it is possible that it may be found in other *Culex* spp., as well as in mosquitoes of other genera. Determining if this virus is also present in mosquito populations of PNG and mainland Australia is also of interest.

The inability of BADUV to replicate in vertebrate cells suggests that this virus may be maintained in nature via a vertical or venereal transmission cycle. Indeed, other viruses with insect-restricted tropism, such as the insect-specific flaviviruses, have been isolated from mosquito eggs and larvae, indicative of vertical transmission (Bolling et al., 2012). Whether BADUV and the other recently isolated mosquito-associated bunyaviruses are transmitted vertically warrants further investigation, particularly in light of the fact that Herbert, Tai and Kibale viruses could not be detected in male mosquitoes, despite their inability to replicate in vertebrate cells (Marklewitz et al., 2013).

In conclusion, genomic sequencing of BADUV and its phylogenetic clustering with GOLV, CUMV and PCLV, along with its insect cell-restricted replication, suggests that it should be classified as a member of the newly proposed *Goukovirus* genus. Phylogenetic analysis, along with investigations of the gene products, indicates that BADUV may have ancestral links with members of the *Phlebovirus* genus. The discovery of additional insect-specific bunyaviruses and thorough investigations into the ecology of these viruses will provide a greater understanding into the biological significance of these viruses and an insight into the evolution of pathogenic bunyaviruses.

## Materials and methods

### *Mosquito collection and processing*

Details on the mosquito collections undertaken on Badu Island in March 2003 have been provided previously (van-den-Hurk et al., 2008). Due to their status as the primary vectors throughout the geographical range of JEV (van den Hurk et al., 2009), only members of the genus *Culex* were retained and processed for virus detection from Badu Island in 2003.

Mosquitoes were collected using Centres for Disease Control light traps baited with $CO_2$ alone or in combination with 1-octen-3-ol. Mosquitoes were killed by freezing and transported in liquid nitrogen shippers or on dry ice to Cairns, Australia for storage at −70 °C. On a refrigerated table, mosquitoes were identified to species group and placed into pools of ≤200 mosquitoes in 5 mL screw cap vials. Depending on the pools size, 2.5–5 mL of growth media (containing 2% foetal bovine serum, and antibiotics and antimycotics) and 5–8 sterile glass beads were added to each vial. Pools were homogenised in a SPEX 8000 mixer/mill (Spex Industries, Edison, NJ). Prior to inoculation onto cells, the homogenates were filtered through a 0.8/0.2 μm filter into a sterile 1.5 mL flip-top tube.

### *Cell culture*

*Aedes albopictus* C6/36 cells were cultured in RPMI 1640 with 5–10% foetal bovine serum (FBS) and incubated at 28 °C. The vertebrate cells, African Green Monkey kidney (Vero), baby hamster kidney (BHK-21), human adeno carcinoma (SW-13) and chicken embryo fibroblast (DF-1) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2–10% FBS, while the Chao Ball (*Culex tarsalis*) and HSU (*Culex quinquefasciatus* both obtained from Dr Robert Tesh, UTMB) (Hsu, 1971) cells were grown in Leibovitz's L-15 medium supplemented with 10% tryptose phosphate broth and 15% FBS. All vertebrate cells were incubated at 37 °C with 5% $CO_2$, while the insect cells were maintained at 28 °C. All media were supplemented with 50 U penicillin mL-1, 50 μg streptomycin mL-1 and 2 mM L-glutamine.

### *BADUV detection and isolation from mosquito homogenates*

Initial virus isolation was performed from mosquito homogenate that was inoculated onto monolayers of the C6/36 cells and incubated at 28 °C for 5–7 days. Culture supernatant was collected (passage 0) and aliquots of this culture supernatant were then stored at −70 °C for further analysis. For the detection of BADUV, RNA was extracted from 150 μL of the culture supernatant using the Machery Nagel Viral RNA extraction kit as per the manufacturer's instructions. RT-PCR was performed using the primer pair Bunya8M (5'-CAAACTTTGATTGCGTAGAGC-3') and Bunya9M (5'-GTGTCACTGATATTCCGTTATCC-3'), 5 μL RNA and the Superscript III One-step RT-PCR system with Platinum Taq DNA polymerase (Life Technologies). The cycling conditions were 45 °C/30 min, 94 °C/2 min, followed by 40 cycles of 94 °C/30 s, 45 °C/30 s, 68 °C/45 s and final 68 °C extension for 5 min. The amplicons were purified by agarose gel electrophoresis and extracted using the Nucleospin Gel and PCR clean-up kit (Macherey Nagel). The purified DNA fragments were sequenced at the Australian Genome Research Facility (Brisbane, Queensland).

### *Virus culture*

Successive passaging of BADUV was performed by inoculation onto monolayers of C6/36 cells and incubation at 28 °C for 5–7 days before harvesting the supernatant. Stocks of vertebrate-infecting bunyaviruses were prepared by inoculating monolayers of BHK-21 or Vero cells, incubating at 37 °C and harvesting the supernatant after 3–5 days. The viruses used were Catch-Me-Cave virus (CMCV) isolate I2 (Major et al., 2009); Finch Creek virus (FCV) isolate EB-6 (Major et al., 2009); and Akabane virus (AKAV) strain A661. The viral titres were determined by 50% tissue culture infective dose ($TCID_{50}$) assays using the method of Reed and Muench (Reed and Muench, 1938). Virus stocks were titrated by serial 10-fold dilution and inoculated onto monolayers of Vero cells (for CMCV, FCV and AKAV) or C6/36 cells (for BADUV) as described elsewhere (May et al., 2006). After 5 days incubation, wells exhibiting CPE were observed by microscopic analysis for CMCV, FCV and AKAV. To determine the titre of BADUV, infected wells were identified either by the detection of BADUV RNA in the supernatant using the RT-PCR defined above, or by fixing the cells and performing ELISA using BADUV-immune mouse serum as described above (Clark et al., 2007).

### *Genome sequencing*

Initial sequences of the prototype BADUV isolate (TS6347) were obtained using sequence-independent amplification as described in detail previously (Warrilow et al., 2014). The virus genome assembly was completed by designing primers to bracket the sequence gaps, enabling the amplification of products by RT-PCR, using Superscript III reverse transcriptase (Life Technologies) and Phusion high fidelity DNA polymerase (Finnzymes), followed by standard Sanger sequencing. The 5' and 3' ends of the S and L segments were amplified using the GeneRacer kit (Life Technologies) according to the manufacturer's instructions, except that the 3′ terminal sequences were determined by ligating an arbitrary primer to the viral RNA using T4 RNA ligase. The 5' and 3' ends of the M segment ORF were obtained using sequence-specific primers paired with a primer designed to the expected conserved terminal sequence (shown in bold below), with additional arbitrary nucleotides as described elsewhere (Palacios et al., 2013) Primer Bunya11 5'- CAA CGC AGA GTA C**AC ACA AAG** -3'. The purified DNA fragments were sequenced at the Australian Genome Research Facility (Brisbane, Queensland). Where necessary, the 5' and 3' RACE products were confirmed following cloning into a TOPO vector (Life Technologies) and the sequencing performed directly on the purified plasmid.

### *Phylogenetic analysis*

Amino acid sequences were aligned with the MAFFT plug-in of GeneiousPro 5.6 (Kearse et al., 2012) using a Blosum 62 scoring matrix and a gap open penalty of 1.53 and offset value of 0.123. The output file was imported into MEGA5.03 (Tamura et al., 2011) and used to construct a tree by a maximum likelihood [Jones–Taylor–Thornton with Freqs (+F) model] and gamma distributed rates with 5 discrete categories. The internal branches were statistically verified using 1000 bootstraps.

*Genome analysis*

*In silico* analysis of putative BADUV amino acid sequences was performed using the following programmes: Identification of the cleavage sites of the signal peptides was performed using SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/); identification of furin cleavage motif was determined using ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/); estimation of protein mass was performed using http://web.expasy.org/cgi-bin/protparam/protparam; predication of transmembrane domains was done by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/); determination of potential N-linked glycosylation motifs was performed using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). Pairwise amino acid identity determination was performed using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) using default settings (Blosum62, Gap Open 10, Gap extend 0.5).

*Electron microscopy*

BADUV isolate TS6347 was inoculated onto C6/36 cells and the culture supernatant harvested 6 days post-infection by centrifugation at 2000xg, for 15 min at 4 °C. Virus purification and electron microscopy was performed as described previously (Warrilow et al., 2014). The purified virus was buffer exchanged into NTE (12 mM Tris at pH 8, 120 mM NaCl, 1 mM EDTA pH8) prior to negative staining with 1% uranyl acetate and then viewed using a Tecnai T12 120 keV TEM operating at 120 kV.

*Viral replication kinetics*

Growth kinetics analysis was performed by infecting C6/36 cells at a M.O.I of 0.1 for 2 h at 28 °C. Cells were washed three times with PBS and the appropriate culture medium was then added. Growth kinetics were performed in triplicate, allowing independent wells for each time point harvested. Culture supernatants were harvested at time point 0 and then daily for 7 days for BADUV and 0, 1, 3, 5 and 7 days post-infection for West Nile virus (WNV; Kunjin strain MRM61C). Samples were stored at −70 °C and viral concentrations were determined by $TCID_{50}$ assays on C6/36 cells as described above (except that 4 wells were used for each dilution) and detecting positive wells by ELISA using BADUV-immune mouse serum or WNV-reactive monoclonal antibody for BADUV and WNV assays respectively.

*Generation of antiserum to BADUV*

All animal procedures had received prior approval from The University of Queensland Animal Ethics Committee and where necessary were performed under ketamine:xylazil anaesthesia. Six-week old BALB/c mice (Animal Resources Centre, Murdoch, Western Australia, Australia) were immunised sequentially via the subcutaneous route with purified BADUV, along with Titre-Max Gold (Sigma Aldrich) that was diluted according to the manufacturer's instructions. Mice were kept on clean bedding and given food and water *ad libitum*. Immunised mice were bled via the tail vein at least two weeks following immunisation and the sera tested for evidence of seroconversion to BADUV using a fixed cell ELISA as previously described (Clark et al., 2007). The titre of the antiserum used for IFA analysis was determined to be $>1/4000$ by fixed cell ELISA.

*Insect and vertebrate cell infection studies*

Monolayers of each cell line were inoculated with either BADUV, CMCV, AKAV or FCV at a MOI of 0.1. After incubation for 1 h, the inoculum was removed and the monolayer washed three times with sterile PBS. Cell-specific growth medium was added to each well and the cultures incubated for five days. Two additional blind passages were performed for BADUV and CMCV by inoculating freshly seeded cell monolayers with a 1/10 dilution of the previous culture at five day intervals. The monolayers were observed for morphological changes daily and the culture supernatant harvested at each passage was stored immediately at −70 °C. RNA extraction from the supernatants was achieved with the Nucleospin RNA virus kit (Machery-Nagel). Extracted RNA from the passage 3 supernatants were tested for BADUV and control CMCV viral RNA by RT-PCR using the Badu SF_10 (5'-AAGCCGATAACTTTAGGGACACTGC-3'/Badu SR_9 (5'- ACCTTGTCCTGCAACTGGTAATTGG-3') or CMCV_S_Fwd (5'- TGTGTGAGGATAGTGTCACTGG-3')/CMCV_S_Rev (5'- AAAGGAGAAGCCACCAAGG-3') primer sets, respectively. The BADUV RT-PCR was performed with the Rotor-Gene SYBR green RT-PCR kit (Qiagen). The cycling conditions were 55 °C/10 min, 95 °C/5 min, followed by 40 cycles of 95 °C/5 s, 60 °C/10 s. The CMCV RT-PCR was performed with the Superscript III One-step RT-PCR system with Platinum Taq DNA polymerase (Life Technologies) and cycling conditions of 45 °C/30 min, 94 °C/2 min; followed by 40 cycles of 94 °C/30 s, 54 °C/30 s, 68 °C/45 s and final 68 °C extension for 5 min. The PCR products were analysed following gel electrophoresis.

For IFA analysis, cell monolayers that were grown on glass coverslips were inoculated with BADUV or WNV at an MOI of 10 or 0.01, or mock-infected as described above. After 3 days (M.O.I 0.01) or 2 days (M.O.I 10), the cells were fixed in acetone and probed with BADUV-immune or WNV-immune mouse serum (diluted 1/50) using methods described previously (Hobson-Peters et al., 2013).

*IFA Cross-reaction studies*

C6/36 and Vero cell monolayers were inoculated with BADUV and CMCV respectively at an M.O.I of 0.1. After 4 days, the cells were fixed in acetone and probed with a 1/50 dilution of BADUV-immune mouse serum or negative control mouse serum as detailed above. Infection of Vero cells with CMCV was confirmed by the observation of clear CPE and determination of infectious particles in the culture supernatant ($10^{5.9}$ $TCID_{50}$ units per mL).

*BADUV structural protein analysis*

Analysis for protein glycosylation was performed using gradient-purified BADUV virions that were resuspended in the glycoprotein denaturing and reaction buffers (NEB) as per the manufacturer's instructions, with or without the addition of 500 units of PNGase F. Each preparation was incubated at 37 °C for 1 h, prior to the addition of LDS sample buffer (Life Technologies) and separation on a 4–12% Bis-Tris SDS-PAGE gel (Life Technologies). The gel was stained using Sypro Ruby stain (Life Technologies) according to the manufacturer's protocol and the protein bands were visualised on the Typhoon phosphoimager (GE Healthcare).

For mass spectrometry analysis, the gradient-purified BADUV virions were incubated at 90 °C for 10 min in LDS sample buffer (Life Technologies) and 0.1 M Dithiothreitol (DTT) before separation on a 4–12% Bis-Tris SDS-PAGE gel (Life Technologies) and staining with a Coomassie blue stain. Individual bands were excised, destained then reduced and alkylated in 40 mM ammonium bicarbonate (20 mM DTT at 37 °C for 2 h, 50 mM iodoacetamide at 22 °C for 40 min). Dried gel slices were digested overnight with trypsin at 37 °C as described in detail previously (Hastie et al., 2012). Acidified digests were subjected to NanoHPLC-MS/MS analysis using a nanoAcquity nanoHPLC system (Waters, MA, USA) interfaced with a linear ion-trap (LTQ)- Orbitrap Elite hybrid mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) as

described previously with changes as follows (Dave et al., 2014). The digests were loaded onto the trap and washed with 98% A (0.1% (v/v) aqueous formic acid) 2% B (100% (v/v) ACN, containing 0.1% (v/v) formic acid) for 3 min, the trap was switched in-line with the main column and peptides separated using a sequence of linear gradients: to 5% B over 1 min; to 40% B over 29 min and to 95% B over 4 min. The heated capillary temperature was set to 275 °C. The LTQ- Orbitrap Elite was controlled using Xcalibur 2.2 SP1.48 software (Thermo FisherScientific). Charge state filtering with unassigned precursors and singly charged ions not selected for fragmentation, and a dynamic exclusion duration of 30 s were used. Maximum ion injection times were 250 ms for survey full scans and 100 ms for MS/MS scans.

Data Analysis: Tandem mass spectra were processed using Proteome Discoverer (version 1.4, Thermo Fisher Scientific) and searched against a database (17698 entries) generated from the BADUV sequences described in this paper and the following Uniprot search on the 26 February 2014 (organism:aedes AND organism:aegypti AND keyword:1185 OR (organism:aedes AND organism:albopictus)). The internal build of Sequest and Mascot (version 2.2.06, Matrix Science) were used to assign the spectra. Fixed modification: carbamidomethyl-cysteine, Variable modifications: deamidation (asparagine, glutamine); oxidation (methionine). Enzyme: trypsin, 2 missed cleavages, MS tolerance 10 ppm, MSMS tolerance 0.6 Da. Scaffold (4.4.1.1, Proteome Software, Portland, Oregon, USA; (Searle, 2010)) was used to validate Mascot protein identifications. Scaffold probabilistically validates these peptide identifications using PeptideProphet (Keller et al., 2002). Criteria of 2 peptides with a 95% probability and protein probability of 99.9% were used.

*Furin cleavage analysis of BADUV glycoproteins*

Monolayers of C6/36 cells were infected with BADUV at an MOI of 3 or mock infected. After 24 h, the wells were washed twice with sterile PBS, before culturing for a further 24 h in RPMI medium containing 2% FBS and 50 mM $NH_4Cl$. The cell monolayers were washed 3 times with ice-cold PBS and then harvested into a solution containing 10 mM Tris–HCl pH7.4, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% triton X-100, 0.1% SDS, inhibitor cocktail (Sigma Aldrich) and 1 x phosphatase inhibitor 2 cocktail (Sigma Aldrich). The lysates were then diluted into a furin protease reaction buffer (0.1 M Hepes pH 7.5, 0.5% triton X-100, 1 mM $CaCl_2$) and incubated with or without furin protease (NEB) at 25 °C overnight. Western blot was performed by adding LDS sample buffer (Life Technologies) and separation on a 4–12% Bis-Tris SDS-PAGE gel (Life Technologies), followed by transfer to nitrocellulose membrane (Whatman). BADUV proteins were detected by incubation with BADUV-immune mouse serum followed by IRDye® 800 CW Donkey anti-Mouse IgG (H+L) (Li-Cor) and visualised on the Odyssey imaging system (Li-Cor).

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.003.

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