

Characterization of a novel *Negevirus* and a novel *Bunyavirus* isolated from *Culex (Culex) declarator* mosquitoes in Trinidad

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Pools of mosquitoes were tested for insect-specific viruses using cytopathic effect (CPE) assays on *Aedes albopictus* (C6/36) cells. Illumina sequencing of RNA from pool TR7094, which produced extensive CPE 2 days post-infection, yielded the complete genome sequences of a previously unknown *Bunyavirus*, designated Cumuto virus (CUMV), and a second virus designated Wallerfield virus (WALV). WALV shared highest amino acid identity (60.1 %) with Dezidougou virus from Côte d'Ivoire, a positive-sense, single-strand RNA, insect-specific virus belonging to the newly proposed genus *Negevirus* associated with mosquitoes and phlebotomine sandflies. The S, M and L segments of CUMV were most closely related to those of Gouleako virus, also from Côte d'Ivoire (amino acid identities of 36 %, 38 % and 54 % respectively). Neither virus produced CPE on vertebrate cells, or illness in newborn mice. Isolation and characterization of these viruses increase our knowledge of the geographical distribution, diversity and host range of mosquito-specific bunyaviruses and negeviruses.

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Mosquitoes are vectors for many viruses of public and veterinary health importance. Mosquito-borne viruses consist primarily of members of the families *Flaviviridae*, *Togaviridae* and *Bunyaviridae* that are generally maintained in transmission cycles involving replication in both mosquito vectors and vertebrate hosts. In addition to these arthropod-borne viruses (arboviruses) of vertebrates, a number of novel mosquito-specific viruses have been identified recently. Most are members of the genus *Flavivirus* and are associated with *Aedes* and *Culex* spp. mosquitoes (reviewed by Cook *et al.*, 2012). However, more recently, the repertoire of mosquito-specific viruses has expanded (reviewed by Junglen & Drosten, 2013) to include flaviviruses from other mosquito genera such as Nakiwogo virus from *Mansonia* sp. (Cook *et al.*, 2009), Nounane virus from *Uranotaenia* sp. (Junglen *et al.*, 2009) and Aripo virus from *Psorophora albipes* (A. J. Auguste, M. Woodson, C. V. Carrington, B. G. Bolling, M. B. Sherman, H. Guzman, V. L. Popov, A. P. T. da Rosa, S. G.

Widen, T. G. Wood, R. B. Tesh & S. C. Weaver; unpublished data), as well as other virus families including *Bunyaviridae*, *Mesoniviridae*, *Reoviridae*, *Togaviridae* and the newly proposed taxon, *Negevirus* (Attoui *et al.*, 2005; Kuwata *et al.*, 2013; Lauber *et al.*, 2012; Marklewitz *et al.*, 2011; Nasar *et al.*, 2012; Vasilakis *et al.*, 2013; Zirkel *et al.*, 2013). This indicates that insect-specific viruses are more abundant and widely distributed than previously realized. However, little is yet known about their ecological niche, their place in the evolutionary history of their respective viral taxa, or to what extent they affect transmission of their vertebrate-pathogenic relatives. In particular, there is interest in their potential for use as vaccines, or for biological control of pathogenic virus vectors.

We screened a random sample of 300 *Culex* mosquito pools collected in Trinidad (Auguste *et al.*, 2010) for viral cytopathic effects (CPE) in cultures of C6/36 (*Aedes albopictus*) mosquito cells. One pool, TR7094, produced extensive CPE 2 days after inoculation. Electron microscopic examination of ultrathin sections of infected cells showed that the most prominent cytopathology was a tremendous expansion of perinuclear space and granular endoplasmic reticulum (Fig. 1a). These expansions were

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The GenBank/EMBL/DDBJ accession number for the WALV sequence is KF042857 and the CUMV sequences are KF543244–KF543246.

filled with vesicles approximately 20–40 nm in diameter (Fig. 1b) and tubular elements (Fig. 1a), suggesting some vesicles might represent cross-sections of the tubules. Additionally, large vacuoles were found containing spherical structures 40–60 nm in diameter at their inner periphery. These structures morphologically resembled alphavirus spherules but were also characteristic of the cytopathology observed with negevirus (Vasilakis *et al.*, 2013). Smaller vacuoles, which appeared to be Golgi derived, contained spherical enveloped particles ~75 nm in diameter with a dense core (Fig. 1c). These particles were similar in size and morphology to bunyavirus virions.

The isolates were subsequently cultured in larger quantities of C6/36 cells, precipitated with polyethylene glycol, and the pellet was resuspended in 250 µl of TEN buffer (10 mM Tris/HCl, pH 7.5; 1 mM EDTA, pH 8.0; and 0.1M NaCl). Total RNA was extracted and next-generation sequencing using the Illumina platform (Illumina) was used to determine the complete genome sequence. BLASTX searches (E-value cut off of $1E^{-10}$) indicated the presence of two viruses: (1) a bunyavirus designated Cumuto virus (CUMV; GenBank accession numbers KF543244–KF543246); and (2) a second virus, designated Wallerfield virus (WALV; GenBank accession number KF042857), which belongs to a newly described group of insect-specific viruses known as negevirus.

Neither virus subsequently produced CPE in Vero E6 (African green monkey kidney) or BHK-S (baby hamster kidney) cells or caused overt disease in intracerebrally inoculated, 2 day old CD1 mice, suggesting that they both were insect-specific viruses. The absence of cytopathology does not, however, rule out the possibility that these viruses can replicate in mammalian cells.

CUMV

CUMV was named after the town of Cumuto, which lies on the south-west boundary of the Aripo Savannas Scientific Reserve, close to the area where mosquito pool

TR7094 was collected. As indicated above, CUMV belongs to the family *Bunyaviridae*, and is most closely related to viruses in the genus *Phlebovirus*, which, like other bunyaviruses, have tripartite-segmented, negative (–) sense RNA genomes consisting of: (1) a large segment (L), which encodes an RNA-dependent RNA polymerase (RdRp) necessary for replication and transcription of genomic RNA; (2) a medium segment (M) encoding the viral surface glycoproteins Gn and Gc, and a non-structural protein NSm that occurs in the sandfly fever group of phleboviruses but not in the Uukuniemi group; and (3) a small segment (S) that also exhibits an ambisense coding strategy, which encodes the nucleocapsid (N) protein and a non-structural protein, NSs, found in some members of the family (Plyusnin *et al.*, 2011). The L, M and S segments of CUMV were found to be 6367 nt (GC content, 34.9%), 3269 nt (GC content, 39.2%) and 1176 nt (GC content, 41.5%) in length respectively, and were most similar to Gouleako virus (Marklewitz *et al.*, 2011) from Côte d'Ivoire in West Africa (amino acid identities of 54%, 38% and 36% respectively). Together with Gouleako virus, CUMV may represent a novel genus of insect-specific bunyaviruses that are evolutionarily related to phleboviruses.

The ORF for the RdRp gene within the CUMV L segment was 6216 nt in length (GC content, 31%), beginning at position 41. The 2071 amino acid RdRp gene product was estimated to be 240 kDa. The M segment ORF (2955 nt beginning at position 97) encoded the glycoprotein precursor (984 aa; 109.8 kDa) that is subsequently cleaved into Gn and Gc, with the predicted cleavage site between amino acids 491 and 492 (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). Four potential N-linked glycosylation sites were identified in Gn and three in Gc, which is similar to Uukuniemi group viruses that contain 4 N-linked glycosylation sites in both proteins (Kakach *et al.*, 1989; Rönnholm & Pettersson, 1987). In phleboviruses, the M segment ORF can have multiple in-frame AUG start codons, ranging from 1 in the Uukuniemi group to 13 in

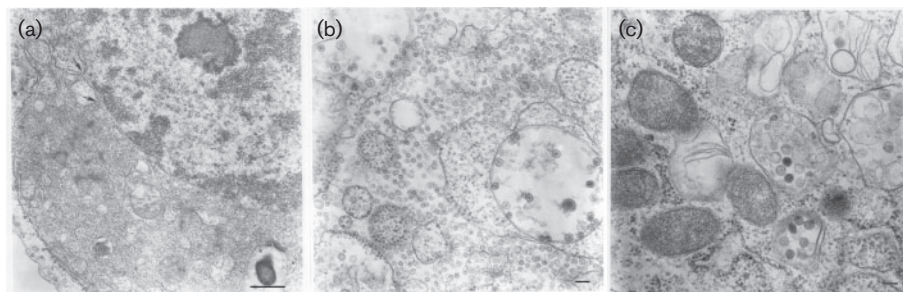


Fig. 1. Ultrastructure of C6/36 cells infected with Wallerfield and Cumuto viruses. (a) Expansions of perinuclear space and granular endoplasmic reticulum filled with tubular and vesicular structures. Arrows show points of perinuclear membrane expansion. Bar, 0.5 µm. (b) Large vacuoles with spherules of Wallerfield virus at their periphery and expansions of granular endoplasmic reticulum filled with vesicles 20–40 nm in diameter. Bar, 100 nm. (c) Two vacuoles with spherical enveloped particles ~75 nm in diameter possibly representing Cumuto virus. Bar, 100 nm.

the sandfly borne Punta Toro virus (Gerrard *et al.*, 2007; Kakach *et al.*, 1989; Matsuoka *et al.*, 1988; Moureau *et al.*, 2010). Similar to Gouleako virus, three were evident in CUMV, at nucleotide positions 97, 145 and 157. The NSm protein that occurs in the sandfly fever group is absent in Gouleako virus and the Uukuniemi group viruses (Marklewitz *et al.*, 2011), and was also not detected in CUMV.

The S segment of CUMV (which is ambisense in phleboviruses) was 1176 nt long (GC content, 41.5%). It contained 5 ORFs (>100 bp) including an N gene of 804 bp in length (267 aa; 29.3 kDa protein). The other four ORFs (two in reverse and two in forward orientation) were deemed too small to encode relevant proteins (all less than 60 aa) and none showed detectable homology to the putative 11.6kD protein identified in Gouleako virus (which has a molecular mass similar to the NSs protein of orthobunyaviruses). The absence of this 11.6 kDa protein from CUMV gives further credence to the suggestion that this protein may not exist in Gouleako virus (Marklewitz *et al.*, 2011). The NSs protein in phleboviruses is relevant for mammalian cell infection, because it contributes to the inhibition of type-I interferon synthesis (Bird *et al.*, 2008; Bouloy *et al.*, 2001; Ikegami *et al.*, 2006; Weber *et al.*, 2002). Previous work has shown that although the NSm and NSs proteins are dispensable for growth in cell culture, they play an important role in Rift valley fever virus pathogenesis (Gerrard *et al.*, 2007; Ikegami *et al.*, 2006). Absence of both NSm and NSs proteins in CUMV may therefore be consistent with the apparent inability

of CUMV to replicate in mammalian cells, although it should be noted that Rift valley fever virus can replicate in cell culture in the absence of a NSs protein.

Fig. 2(a) shows a maximum-likelihood (ML) phylogeny estimated from an alignment of CUMV amino acid sequences for the L segment together with corresponding sequences from other selected phleboviruses, as well as representatives of other *Bunyavirus* genera. Sequence alignment was performed using CLUSTAL W v2.0 (Larkin *et al.*, 2007) and the alignment trimmed to 3406 positions (including gaps) such that CUMV RdRp amino acid positions 25–2069 were represented. The ML phylogeny was estimated using the Blosum62 substitution model with six gamma-distributed rate categories and the proportion of invariant sites and gamma shape parameter estimated from the data (available from the authors on request). A BioNJ tree was used as the starting tree and the SPR search algorithm was employed. Bootstrap support was estimated from 100 replicates.

CUMV clustered with its closest relative, Gouleako virus, with 100% bootstrap support. Phylogenies generated based on the M segment and N gene also support this grouping with Gouleako virus with high bootstrap support (data not shown). These results together with the low sequence identity between CUMV and the other bunyaviruses suggest that CUMV and Gouleako viruses may represent a new genus in the family *Bunyaviridae* that is evolutionarily related to but distinct from phleboviruses. Consequently, we propose that the tentative name for this

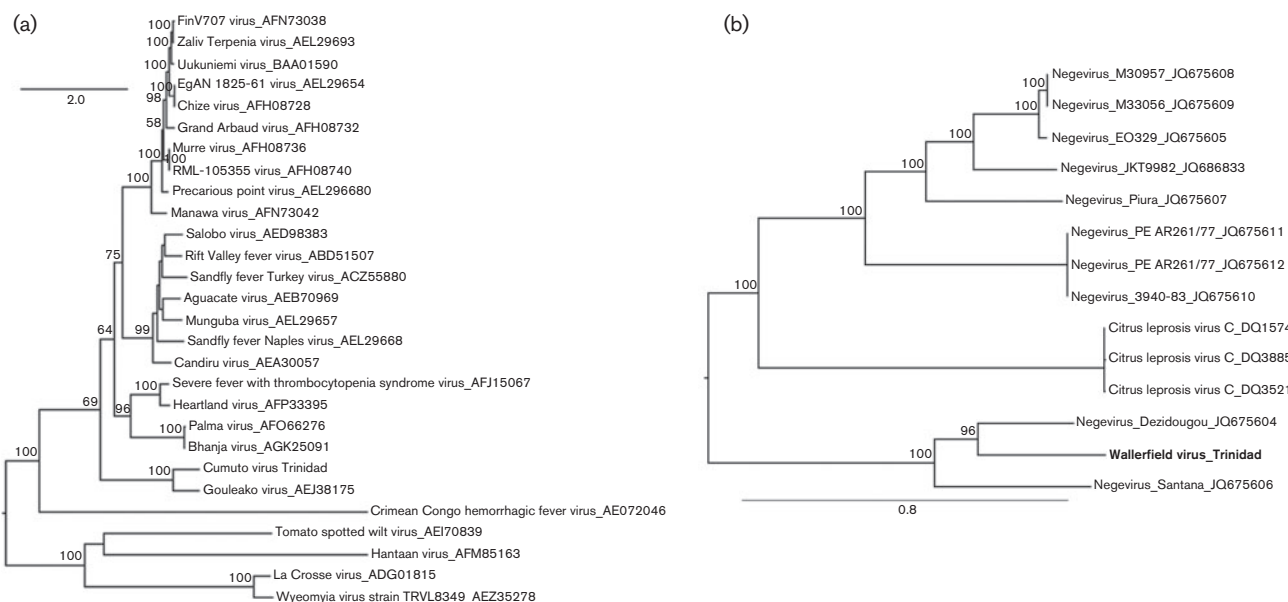


Fig. 2. (a) Midpoint rooted ML phylogeny estimated from amino acid sequences for phlebovirus L segment; (b) midpoint rooted ML phylogeny estimated from nucleotide sequences of negevirus RdRp genes. Bootstrap support values >50% indicated at relevant nodes. Taxon names include virus name, strain and corresponding accession numbers used for analysis. Scale bars represent percentage divergence.

genus should be *Gouleakovirus*, after the first virus to be detected in this group.

WALV

The WALV complete genome shared highest nucleotide (67%) and amino acid identity (60%) with the negevirus, Dezidougou virus from Côte d'Ivoire, followed by Santana virus (also a negevirus) from Brazil (65% and 49% respectively). The negeviruses are a recently described taxon of insect-specific viruses that have been isolated from mosquitoes and phlebotomine sandflies (Vasilakis *et al.*, 2013). Their closest but still distant genetic relationship is with viruses included in the unassigned genus *Cilevirus* (Vasilakis *et al.*, 2013), mite-transmitted viruses that cause disease in citrus plants (Locali-Fabris *et al.*, 2006; Pascon *et al.*, 2006). Negeviruses appear to have a wide host range among the Diptera, having been isolated from *Culex*, *Aedes*, and *Anopheles* mosquitoes, as well as *Lutzomyia* sandflies. They are also widely distributed geographically, having been isolated from insects in Israel, Indonesia, Ivory Coast, United States, Peru, Portugal and Brazil (Ferreira *et al.*, 2013; Vasilakis *et al.*, 2013).

The WALV genome is a 9023 nt long single-stranded, positive-sense RNA with a GC content of 35%, typical of negeviruses whose genomes are approximately 9–10 kb in length (Vasilakis *et al.*, 2013). Similar to other negeviruses, the WALV genome contains three ORFs flanked by non-coding regions, of which the 3' end is polyadenylated. Each ORF is one frame-shift downstream of the preceding one. ORF1 begins at nucleotide position 19 and is 6789 nt long. It encodes the putative non-structural proteins (2262 aa) with four functional domains identified by using Geneious v6.1 to search the Pfam database. These include a viral methyltransferase (amino acid positions 114–488), an S-adenosylmethionine-dependent methyltransferase (positions 795–978), a viral helicase (positions 1331–1602), and an RNA-dependent RNA polymerase (positions 1808–2240).

A signal peptide and potential cytoplasmic domain were identified in ORF2 (ORF2 positions are 6837–8105), which is 1269 nt (423 codons) long. In contrast to other negeviruses (Vasilakis *et al.*, 2013), no transmembrane regions were predicted in ORF2 of WALV, but three transmembrane domains were predicted in ORF3 transmembrane helix prediction based on hidden Markov models (TMHMM) (Krogh *et al.*, 2001). ORF3 (positions 8227–8826) is 600 nt (200 codons) long and thought to encode the negevirus envelope glycoproteins.

The 5' WALV UTR is 18 nt long and the 3' UTR is 197 nt. We inferred glycosylation sites using the NetNGlyc server 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) with a threshold value of 0.5 across the entire WALV genome and found 11 potential sites in ORF1, four in ORF2 and two in ORF3. These data suggest that the ORF3-encoded structural protein may undergo post-translational modification at two amino acid positions in the structural protein.

Fig. 2(b) shows a ML phylogeny estimated from alignments of the RdRp genes of all previously reported negeviruses and citrus leprosis virus C. WALV clusters with high bootstrap support with Santana and Dezidougou viruses (Fig. 2b); however, another analysis based on the helicase gene showed that its position in the two phylogenies was not distinct, such that the helicase phylogeny resulted in WALV grouping more closely with Santana virus with low bootstrap support (data not shown), and the RdRp phylogeny resulted in WALV grouping with Dezidougou virus, with high bootstrap support. These results suggest recombination, but further analyses with additional negevirus sequences are needed to obtain more robust evidence. Analysis of sequence identities suggested that WALV is more closely related to Dezidougou than Santana virus, supporting the phylogeny based on the RdRp sequences.

Conclusions

In summary, the complete genome sequences of two novel insect-specific viruses were derived from a single pool of *Culex declarator* mosquitoes collected in Trinidad. CUMV and WALV are novel insect-specific viruses belonging to the family *Bunyaviridae* and the genus *Negevirus*, respectively. Both are sufficiently genetically distinct to be considered new member species in their respective taxa. Their genetic characterization increases our knowledge of the known geographical distribution, diversity and host range of insect-specific bunyaviruses and negeviruses. Additionally, their close genetic relationship with West African viruses provides evidence that, like many other arboviruses, the progenitors of these viruses may have originated in the Old World. The complete genome sequences provided should be helpful in the development of specific detection methods for studying the distribution, patterns of global spread, and prevalence of these viruses in Diptera, as well as to better understand the evolutionary histories of these genera by using phylogenetic methods.

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