Aedes anphevirus (AeAV): an insect-specific virus distributed worldwide in *Aedes aegypti* mosquitoes that has complex interplays with *Wolbachia* and dengue virus infection in cells

Rhys Parry and Sassan Asgari*

Australian Infectious Disease Research Centre, School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

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*Corresponding author: Sassan Asgari; Tel: +617 3365 2043; Fax: +617 3365 1655;
s.asgari@uq.edu.au

Abstract

Insect specific viruses (ISVs) of the yellow fever mosquito *Aedes aegypti* have been demonstrated to modulate transmission of arboviruses such as dengue virus (DENV) and West Nile virus by the mosquito. The diversity and composition of the virome of *Ae. aegypti*, however, remains poorly understood. In this study, we characterised Aedes anphevirus (AeAV), a negative-sense RNA virus from the order *Mononegavirales*. AeAV identified from *Aedes* cell lines were infectious to both *Ae. aegypti* and *Aedes albopictus* cells, but not to three mammalian cell lines. To understand the incidence and genetic diversity of AeAV, we assembled 17 coding-complete and two partial genomes of AeAV from available RNA-Seq data. AeAV appears to transmit vertically and be present in laboratory colonies, wild-caught mosquitoes and cell lines worldwide. Phylogenetic analysis of AeAV strains indicates that as the *Ae. aegypti* mosquito has expanded into the Americas and Asia-Pacific, AeAV has evolved into monophyletic African, American and Asia-Pacific lineages. The endosymbiotic bacterium *Wolbachia pipientis* restricts positive-sense RNA viruses in *Ae. aegypti*. Re-analysis of a small RNA library of *Ae. aegypti* cells co-infected with AeAV and *Wolbachia* produces an abundant RNAi response consistent with persistent virus replication. We found *Wolbachia* enhances replication of AeAV when compared to a tetracycline cleared cell line, and AeAV modestly reduces DENV replication *in vitro*. The results from our study improve understanding of the diversity and evolution of the virome of *Ae. aegypti* and adds to previous evidence that shows *Wolbachia* does not restrict a range of negative strand RNA viruses.

Importance

The mosquito *Aedes aegypti* transmits a number of arthropod-borne viruses (arboviruses) such as dengue virus and Zika virus. Mosquitoes also harbour insect-specific viruses that may affect replication of pathogenic arboviruses in their body. Currently, however, there are only a handful of insect-specific viruses described from *Ae. aegypti* in the literature. Here, we characterise a novel negative strand virus, Aedes anphevirus (AeAV). Meta-analysis of *Ae. aegypti* samples showed that it is present in *Ae. aegypti* mosquitoes worldwide and

is vertically transmitted. *Wolbachia* transinfected mosquitoes are currently being used in biocontrol as they effectively block transmission of several positive sense RNA viruses in mosquitoes. Our results demonstrate that *Wolbachia* enhances the replication of AeAV and modestly reduces dengue virus replication in a cell line model. This study expands our understanding of the virome in *Ae. aegypti* as well as providing insight into the complexity of the *Wolbachia* virus restriction phenotype.

Introduction

The yellow fever mosquito *Aedes aegypti* is a vector of medically important viruses with worldwide distribution within the tropical and subtropical zones (1). *Ae. aegypti* is the principal vector of both dengue virus (DENV) and Zika virus (ZIKV) (Family: *Flaviviridae*) with estimates suggesting up to 390 million incidences of DENV infections a year (2), and approximately 400,000 cases of ZIKV during the 2015–2016 Latin American ZIKV outbreak (3).

The ability of mosquitoes to transmit viruses is determined by a complex suite of genetic and extrinsic host factors (4-6). One developing area is the contribution of insect-specific viruses (ISVs), demonstrated not to replicate in mammalian cells, in the vector competence of individual mosquitoes (7, 8). ISVs can suppress the anti-viral RNAi response as shown in *Culex-Y* virus (CYV) of the *Birnaviridae* family (9), or enhance the transcription of host factors; cell fusing agent virus (CFAV) (Family: *Flaviviridae*) infection of *Ae. aegypti* Aa20 cells upregulates the V-ATPase-associated factor RNASEK allowing more favourable replication of DENV (10). ISVs have also been shown to suppress or exclude replication of arboviruses; prior infection of *Aedes albopictus* C6/36 cells and *Ae. aegypti* mosquitoes with Palm Creek virus (PCV) (Family: *Flaviviridae*) has been shown to suppress replication of the zoonotic West Nile virus (WNV) and Murray Valley encephalitis virus (Family: *Flaviviridae*) (11, 12). Also, it has recently been demonstrated in *Aedes* cell lines that dual infection with Phasi Charoen-like virus (Family: *Bunyaviridae*) and CFAV restricts the cells permissivity to both DENV and ZIKV infection (13).

Metagenomic and bio-surveillance strategies have proved invaluable in describing the virome diversity of wild-caught *Culicinae* mosquitoes (14, 15). To date, six ISVs have been identified and

characterised from wild-caught and laboratory *Ae. aegypti*; CFAV (16, 17), Phasi Charoen-like virus (Family: *Bunyaviridae*) (18), Dezidougou virus from the Negevirus taxon (19), Aedes densoviruses (Family: *Parvoviridae*) (20) and the unclassified Humaita-Tubiacanga virus (HTV) (21). Recently, transcriptomic analysis of wild-caught *Ae. aegypti* mosquitoes from Bangkok, Thailand and Cairns, Australia suggested possible infection of the mosquitoes with up to 27 insect-specific viruses, the majority of which currently uncharacterized (22). This represents a narrow understanding of the diversity of the circulating virome harboured by *Ae. aegypti* mosquitoes.

In this study, we identified and characterised a novel negative-sense RNA *Anphevirus*, putatively named Aedes anphevirus (AeAV), from the order *Mononegavirales* in *Ae. aegypti* mosquitoes. According to the most recent International Committee on Taxonomy of Viruses (ICTV) report (23), Xīnchéng mosquito virus (XcMV), assembled as part of a metagenomic analysis of *Anopheles sinensis* mosquitoes in Xīnchéng China, is the only member of the *Anphevirus* genus and closely related to members of *Bornaviridae* and *Nyamiviridae* (24). Originally thought to only encode for four ORFs, presence of a number of closely related viruses to XcMV from West African *Anopheles gambiae* mosquitoes (15) and West Australian *Culex* mosquitoes (25) suggests that members of this taxon encode for six ORFs with a genome size of approximately 12kb.

The endosymbiotic bacterium *Wolbachia pipientis* was first shown to restrict RNA viruses in *Drosophila melanogaster* (26, 27). Transinfection of *Wolbachia* into *Ae. aegypti* restricted DENV and Chikungunya virus (Family: *Togaviridae*) replication in the host (28). In *Ae. aegypti* Aag2 cells, stably transinfected with a proliferative strain of *Wolbachia* (wMelPop-CLA), the endosymbiont restricts CFAV (29), but has no effect on the negative sense Phasi Charoen-like virus (Family: *Bunyaviridae*) (30). In addition to characterising AeAV, we also studied the effect of *Wolbachia* on AeAV replication and co-infection of AeAV and DENV in *Ae. aegypti* cells.

Results

Identification and assembly of the full Aedes anphevirus (AeAV) genome from *Wolbachia*-infected *Aedes* cells

During replication of RNA viruses in *Ae. aegypti* mosquitoes, the RNA interference (RNAi) pathway cleaves viral dsRNA intermediates into 21nt short interfering RNAs (vsRNAs) (31, 32). Using this fraction of reads from RNA-Seq data, it is possible to *de novo* assemble virus genomes (21, 33).

The previously sequenced small RNA fraction of embryonic *Ae. aegypti* Aag2 cells and Aag2 cells stably infected with *Wolbachia* (wMelPop-CLA strain) (34) was trimmed of adapters, filtered for 21nt reads and *de novo* assembled using CLC Genomics Workbench with a minimum contig length of 100nt. The resulting contigs were then queried using BLASTX against a local virus protein database downloaded from the National Centre for Biotechnology Information (NCBI). In the Aag2.wMelPop-CLA assembly, four contigs between 396-1162nt were found to have amino acid similarity (E value 9.46E-51) to proteins from two closely related *Mononegavirales* viruses: Culex mononega-like virus 1 (CMLV-1) and Xīnchéng mosquito virus (XcMV); the type species for the *Anphevirus* genus. No contigs from the *Wolbachia* negative *Ae. aegypti* Aag2 dataset showed any similarity to CMLV-1 or XnMV.

Subsequent RT-PCR analysis between RNA samples from Aag2 and Aag2.wMelPop-CLA cell lines indicated that this tentative virus was exclusive to the Aag2.wMelPop-CLA cell line (Fig. 1A). We hypothesised that the presence of any putative virus may have been the result of contamination during *Wolbachia* transinfection. The *Wolbachia* wMelPop-CLA strain was isolated from the *Ae. albopictus* cell line RML-12 and transinfected into Aag2 (35) and the *Ae. albopictus* C6/36 (C6/36.wMelPop-CLA) cell lines (36). RT-PCR analysis of RNA extracted from RML-12 and C6/36 cells, as well as the *Ae. aegypti* cell line Aa20, showed that the putative virus was present only in RML-12 cells (Fig. 1A).

To recover the remainder of the virus genome, transcriptome RNA-Seq data from RML-12.wMelPop-CLA and C6/36.wMelPop-CLA cells were downloaded (37, 38) and *de novo* assembled with automatic bubble and word sizes using CLC Genomics Workbench. BLASTN analysis of assembled contigs indicated that a complete 12,940 contig from the C6/36.wMelPop-CLA cells and two contigs (9624 and 3487nt) from RML-12.wMelPop-CLA were almost identical (99-100% pairwise nucleotide identity) to the virus like contigs assembled from Aag2.wMelPop-CLA. We were then able to use this

reference to recover the full genomes from Aag2.wMelPop-CLA and RML-12.wMelPop-CLA consensus mapping to this reference. To re-validate that AeAV was only present in Aag2.wMelPop-CLA cells, reads from the *Wolbachia*-negative Aag2 cells were mapped to the representative genome, and only four reads were identified in the data. The result from here and the RT-PCR analysis above (Fig. 1A) also confirm that the virus found in the *Wolbachia* transinfected cells originate from RML-12 cells in which wMelPop-CLA was originally transinfected and subsequently transferred to other cell lines.

Characterisation of *Aedes anphevirus* (AeAV)

AeAV genomes assembled in this study were between 12,455 to 13,011 nucleotides in length with a %GC content of 46.8% and encode for 7 non-overlapping ORFs (Fig. 1B). Phylogenetic analysis of the RNA-dependent RNA polymerase protein places AeAV within a well-supported clade of the unassigned *Anphevirus* genus, which are from the order *Mononegavirales* and closely related to members of *Bornaviridae* and *Nyamiviridae* (Fig. 1C).

All members of *Mononegavirales* have a negative-stranded RNA genome encapsidated within the capsid and the RNA polymerase complex (39). The RNA genome is used as the template by the RNA polymerase complex to sequentially transcribe discrete mRNAs from subgenomic genes. mRNA from each gene is capped and polyadenylated. To analyse the transcriptional activity of AeAV, we used the poly-A enriched RNA-Seq libraries prepared from the Cali, Colombia laboratory strain (40). Read mapping and coverage analysis of the AeAV genome showed that AeAV follows the trend of reduced transcriptional activity seen in other *Mononegavirales* species (41) with approximately 50% reduction between ORF1 and ORF2 but an increased transcription between ORF2 and ORF3 (Fig. 1B). The reduction in transcriptional activity of AeAV genes is conserved for each sequential ORF with the least transcriptional activity for ORF7/L protein, that is conserved in all AeAV strains in Poly(A) enriched RNA-Seq libraries (Fig. S1).

ORF1 of AeAV encodes a predicted 49kDa nucleoprotein with no transmembrane domains and closest pairwise amino acid identity (26%) to the nucleoprotein gene from *Culex mononega*-like virus 1 (CMLV-1) from *Culex* mosquitoes in Western Australia (25). Protein homology analysis using

133 HHPred showed that ORF1 was a likely homolog of the p40 nucleoprotein of the Borna disease virus
 134 (Probability 98.66%, E- value: 7.1e-10). ORF2 encodes an 11kDa protein with two transmembrane
 135 domains in the N-terminus of the protein with no similarity to any proteins within the non-redundant
 136 protein database or homologs as predicted by HHPred. ORF3 and ORF4 encode putative
 137 glycoproteins, 64kDa and 72kDa, respectively. ORF3 has no pairwise amino acid similarity to any
 138 virus protein or homologs as per HHPred analysis. ORF4 was predicted to have a signal peptide in
 139 the N-terminus followed by a heavily O- and N-linked glycosylated outside region as well as two
 140 transmembrane domains in the C-terminus of the protein. ORF4 is most closely related to the
 141 glycoprotein from the Gambie virus identified from West African *An. gambiae* mosquitoes with 45%
 142 pairwise amino acid identity (15). Protein homology analysis predicted ORF4 to be a homolog of the
 143 Human Herpesvirus 1 Envelope Glycoprotein B (Probability 99.88%, E-value 2.2e-22).

144 The presence of a Zinc-like finger (ZnF) domain in a small ORF proximal to the L protein previously
 145 reported in closely related viruses (15) (Fig. 2A and B), was identified in AeAV based on sequence
 146 alignment (Fig. 1B). Re-analysis of putative ORFs from CMLV-1 and CMLV-2 (25) showed the
 147 presence of this GATA-like ZnF domain in both of these viruses and the genus type species XcMV
 148 identified from *An. sinesis* (24) (Fig. 2C).

149 ORF6 encodes for a small 4kDa protein that has a single transmembrane domain in the C-terminus.
 150 This protein was almost missed in the prediction of ORFs due to having only 37 amino acids,
 151 however, it has a strong transcriptional coverage in Poly-A datasets and exists in all assembled
 152 strains (Fig. 1B and Fig. S1). It was predicted to share no structural homology or amino acid identity
 153 with any previously reported peptide. In addition to this, we were able to identify small
 154 transmembrane domain containing proteins proximal to or overlapping with the ZnF protein in CMLV-
 155 1, CMLV-2 and XcMV (Fig. 2A), suggesting that this protein may be a conserved feature of
 156 anpheviruses.

157 ORF7 encodes for the 226kDa L protein, has 41% pairwise amino acid identity with the RNA
 158 dependent RNA polymerase from CMLV-1. Protein domain analysis of the L protein showed the
 159 highly conserved *Mononegavirales* RNA dependent RNA polymerase, mRNA capping domain and

160 a mRNA (guanine-7-) methyltransferase (G-7-MTase) domain conserved in all L proteins in
161 *Mononegavirales* (42).

162 **AeAV *cis*-regulatory elements**

163 For identification of *cis*-regulatory elements in the AeAV genome, we used MEME (Multiple Em for
164 Motif Elicitation) to search for overrepresented 5-50nt motifs (43). Using a 0-order Markov model,
165 one 32nt motif 3'-UUVCUHWUAAAAACCCGCYAGUUASAAAUCA-5' was considered statistically
166 significant (E-value: 4.2e-010). Importantly the motif was proximal to each predicted virus gene ORF,
167 suggesting it may be a potential promoter (Fig. 3A and C). No motif was found between ORF 5 and
168 6 in AeAV suggesting that these two genes may be under the control of a single *cis*-regulatory
169 element. Interestingly, the complement of this motif appeared twice on the anti-genome suggesting
170 that it may be used in an anti-genome virus intermediate. We noticed that these motifs localised to
171 partial palindromic repeats and predicted that they may form stable secondary RNA structures. Using
172 RNAfold, we were able to visualise and predict the MFE structure 20nt upstream and downstream
173 of the motif (44). All predicted *cis*-regulatory motifs formed partial or complete stable secondary stem
174 loops and hairpins with high base-pair probabilities (Fig. 3B). The exception was predicted element
175 3, which is proximal to the second ORF; as this second gene is transcribed less than ORF3
176 irrespective of its similarity to the motif, the lack of a stable stem loop structure may be a novel
177 transcriptional regulatory mechanism. The presence of two conserved homopolymeric triplets in the
178 overrepresented motif is very similar to "slippery" -1 ribosome frame shifting (RFS) sites XXX YYY
179 Z (X=A, G, U; Y=A, U; Z=A, C, U) (45). It has been previously demonstrated that similar 'slippery'
180 sequence motifs followed by a predicted stem-loop structure is a feature of rhabdovirus gene overlap
181 regions (46). In AeAV, this feature appears in the intergenic space and is unlikely to represent
182 ribosomal frame shifting event and subsequent extension of a protein. We also searched for
183 additional slippery motifs in the AeAV genome. The genomic context for each predicted "slippery
184 motif" did not extend or produce additional ORFs.

185 **AeAV infection is widespread in *Ae. aegypti* laboratory colonies, wild-caught mosquitoes and** 186 **cell lines**

187 Taking advantage of the currently published RNA-Seq data, we performed a meta-analysis of global
 188 incidence and genetic diversity of this virus. We were able to show that AeAV is ubiquitous in
 189 laboratory colonies, cell lines and wild-caught *Ae. aegypti* mosquitoes. During preparation of this
 190 manuscript a partial AeAV genome of 5313nt (Accession: MG012486.1) was deposited into NCBI
 191 nucleotide database from a study characterising the evolution of piRNA pathways across arthropods
 192 (47). Using our AeAV genome reference, we were able to complete the CDS portion of the genome
 193 and also 16 additional strains of AeAV with two additional incomplete genomes (Fig. 4) (Table S1).

194 AeAV was present in colonies of *Ae. aegypti* established from eggs collected in Bakoumba, Gabon
 195 (48) and also from Rabai, Kenya (designated K2, K14) as well as four mated hybrid strains (49). In
 196 colonies wild caught from locations in the Americas (47), full genomes of AeAV were assembled
 197 from Miami, USA , Cali, Colombia (40), and Chetumal, Mexico (50, 51) laboratory strains. Partial
 198 genomes of AeAV were assembled from Cayenne and St-Georges, French Guiana (52). AeAV was
 199 identified in colonies established from eggs collected in Chaiyaphum and Rayong, Thailand (49, 53)
 200 as well as Jinjang, Malaysia (54). AeAV was also identified from the widely used Bora-Bora reference
 201 strain from French Polynesia (55). AeAV was also present in eight pools of wild-caught *Ae. aegypti*
 202 mosquitoes used for ZIKA bio-surveillance in Miami, Florida (56) as well as Nakhon Nayok, and
 203 Bangkok, Thailand (18, 22).

204 In *Aedes* cell lines, AeAV was assembled from RNA-Seq data from the larval *Ae. aegypti* line CCL-
 205 125 originally produced in Pune, India (57) and sequenced by the Arthropod Cell Line RNA-Seq
 206 initiative, Broad Institute (broadinstitute.org). With the exception of RNA-Seq data from the three
 207 *Aedes* cell lines stably infected with *Wolbachia* (RML-12.wMelPop-CLA, C6/36.wMelPop-CLA and
 208 Aag2.wMelPopCLA), AeAV was not identified in any other available C6/36 or Aag2 RNA-Seq
 209 libraries.

210 **Genetic variation and evolution of AeAV strains**

211 To assess relatedness and evolution between AeAV strains, a Maximum likelihood phylogeny
 212 (PhyML) was undertaken of the CDS region of all strains with complete genomes (Fig. 5A). The

unrooted radial phylogenetic tree indicated three strongly supported monophyletic lineages associated with the geographic origin of the sample. We have designated these lineages of AeAV as African, American and Asia-Pacific (Fig. 5B).

In the American lineage of AeAV, all strains that are associated with *Wolbachia*-infected *Aedes* cell lines (RML-12.wMelPop-CLA, C6/36.wMelPop-CLA and Aag2.wMelPopCLA) are almost identical (99.55-99.86% identity), supporting the hypothesis that contamination of C6/36 and Aag2 cell lines infected with *Wolbachia* is likely from the original RML-12 cell line. AeAV from the eight wild-caught pools of *Ae. aegypti* mosquitoes from Florida, USA (56) and the laboratory colony established from wild collected samples Florida (47) were almost identical (99.86% pairwise identity) with only 17nt differences over the CDS region. The three African lineage strains of AeAV were slightly closer in pairwise nucleotide identity to the American strains (92.65-93.15%) than the Asia/Pacific strains (91.63%-91.74%). All samples that originated from Thailand form a monophyletic group and are closely related to other Thai strains (99.23-99.62%).

We hypothesised that AeAV may have been harboured as part of the virome of *Ae. aegypti* mosquitoes as *Ae. aegypti* expanded from its sub-Saharan African location into the Americas and Asia-Pacific (58). Phylogenetic studies of the *Ae. aegypti* genome support the origin of *Ae. aegypti* from Africa into the New World (Americas) and a subsequent secondary invasion of *Ae. aegypti* from the New World to the Asia-Pacific region (59, 60). Comparing the evolution of the *Ae. aegypti* nuclear genome with the evolution of AeAV indicates that the Asia-Pacific strains of AeAV have not evolved from the currently circulating American strain lineage. This may indicate that the virus was established independently in both the New-world Americas and also in the Asia-Pacific (Fig. 5B).

Anphevirus-like insertions into the *Ae. aegypti* genome

The *Ae. aegypti* genome has a large repertoire of virus genes and partial viral genomes, termed Endogenous Viral Elements (EVEs) (61, 62). To explore the possibility of anphevirus-like insertions within the *Ae. aegypti* genome, we queried the most recent Liverpool genome (Aaegl5) with the

239 Aag2.wMelPop-CLA AeAV reference strain using the VectorBase BLASTN suite
 240 (<https://www.vectorbase.org/blast>). There were numerous hits of nucleotide similarity (67-70%) of
 241 500-1704nt regions dispersed throughout the *Ae. aegypti* genome. EVEs are acquired through
 242 recombination with long terminal repeat (LTR) retrotransposons (62). We present one ~20kb portion
 243 of Chromosome 2 of the *Ae. aegypti* genome (Fig. 6) with four anphevirus-like insertions and close
 244 proximity to LTR retrotransposable fragments in unidirectional orientation. This suggests insertion of
 245 viral elements through LTR retrotransposases and a long evolutionary history of challenge with
 246 anphevirus-like species in *Ae. aegypti*.

247 **Aedes Anphevirus (AeAV) replicates in Aedes cell lines but does not replicate in three** 248 **mammalian cell lines**

249 Supernatant of Aag2.wMelPop-CLA cells was infectious to both *Ae. aegypti* cells (Aa20), and *Ae.*
 250 *albopictus* C6/36 cells over a five-day time course through RT-qPCR analysis (Fig. 7A). Generally,
 251 there was significantly more relative AeAV genome copies detected in C6/36 cells at 1 and 5 dpi
 252 compared to Aa20 cells. There were also significantly more anti-genome copies of AeAV in C6/36
 253 cells over the five-day time course. The higher replication of AeAV in C6/36 cells as compared to
 254 Aa20 cells is not unexpected since C6/36 cells are RNAi deficient and generally RNA viruses
 255 replicate more efficiently in the cells (63-65).

256 We assumed that AeAV is an insect-specific virus based on its phylogenetic position, however, to
 257 test if AeAV can replicate in mammalian cells, we inoculated human hepatocellular carcinoma cells
 258 (Huh-7), African green monkey cells (Vero), and baby Hamster Kidney (BSR) cells with medium from
 259 AeAV-infected cells and performed RT-PCR for AeAV RNA genome abundance over a 7-day time
 260 course. While AeAV RNA (most likely from the inoculum) could be detected by RT-PCR at day 1 and
 261 3 after inoculation, it did not increase over time and was visibly reduced in the mammalian cells by
 262 5/7 dpi (Fig. 7B). AeAV was also not detected in the *An. gambiae* cell line MOS-55 transinfected with
 263 wMelPop-CLA from RML-12-wMelPop-CLA (36) sequenced by the Arthropod Cell Line RNA-Seq
 264 initiative, Broad Institute (broadinstitute.org). Taken together, the results suggest that AeAV infection
 265 may be restricted within the subfamily *Culicinae* or even the *Aedes* genus and is insect specific.

***Wolbachia pipientis* infection in *Ae. aegypti* cells enhances AeAV replication**

As *Wolbachia* is being deployed in the field to reduce dengue transmission, we were interested to find out if it has any effect on replication of AeAV. We extracted RNA from Aag2.ϖMelPop-CLA cells and a previously tetracycline cured Aag2.ϖMelPop-CLA cell line (66), and tested the effect of *Wolbachia* infection on AeAV genome and anti-genome copies. AeAV genomic RNA copies were significantly greater in *Wolbachia*-infected (Aag2.ϖMelPop-CLA) cells than those in tetracycline cleared (Aag2.ϖMelPop-CLA.Tet) *Ae. aegypti* cells, however, there was no statistically significant difference between the relative anti-genome copies between the two cell lines (Fig. 8A).

To explore the host small RNA response to AeAV, clean reads from previously prepared sRNA libraries from Aag2.ϖMelPop-CLA and Aag2 (34) were mapped to the AeAV genome. In the cytoplasmic fraction of the Aag2.ϖMelPop-CLA sample 870,012 of 4,686,954 reads (18.56%) mapped to AeAV. In the nuclear fraction 420,215 of 11,406,324 reads (3.68%) mapped to the genome. In the combined Aag2 sRNA library, of 8,600,821 clean reads only four reads mapped to the AeAV genome. The mapping profile of 18-31nt reads mapped from the Aag2.ϖMelPop-CLA library to AeAV indicates a higher proportion 27–31nt viral derived PIWI RNAs (vpiRNAs) than the 21nt vsiRNAs (Fig. 8B).

Analysis of the profile of mapped AeAV vsiRNAs fairly ubiquitously targeted the AeAV genome and anti-genome (Fig. 8C). Hotspots in the vpiRNA mapping profile appeared to target the 3'UTR and ORF1 and the 5'UTR of the AeAV anti-genome (Fig. 8D). Biogenesis of vpiRNAs are independent of Dicer-2 with a bias for adenosine at position 10 in the sense position and a uracil in the first nucleotide of antisense polarity (67). This “ping-pong” characteristic signature was apparent in the vpiRNA reads from the cell line (Fig. 8E and F).

Persistent infection of AeAV in Aa20 cells modestly reduces replication of DENV-2 genomic RNA

Recently, it has been demonstrated that in *Aedes* cell lines experimentally infected with two ISVs replication of DENV and ZIKV was reduced (13). To test if there was any interaction between AeAV

and the subsequent challenge of cells with DENV, we generated an Aa20 cell line inoculated with medium from RML-12 and maintained it for three passages. Aa20 cells persistently infected with AeAV were challenged with 0.1 and 1 multiplicities of infection (MOI) of DENV-2 ET-100 strain. RT-qPCR analysis of DENV-2 genomic RNA showed that accumulation was on average less in AeAV infected Aa20 cells as compared to the control (Fig. 9A and B). This reduction in DENV-2 genome copies was statistically significant at MOI of 0.1 at both three and five days post infection. No AeAV-related RT-qPCR product was detected in mock-infected Aa20 cells (data not shown). We also examined the effect of DENV infection on AeAV RNA levels in the AeAV persistently infected cells. RT-qPCR analysis showed no significant effect on AeAV levels between 1 and 3 days post-DENV infection, however, AeAV genomic RNA levels significantly declined at 5 days-post DENV infection (Fig. 9C). There was no significant difference in the results between 0.1 and 1 MOI of DENV.

Evidence for vertical transmission of AeAV

We were fortunate to explore the potential vertical transmission of AeAV by using RNA-Seq data of uninfected and infected mated individuals from a study characterising the genetic basis of olfactory preference in *Ae. aegypti* (49). Briefly, McBride and colleagues used eggs from a number of *Ae. aegypti* species in Rabai, Kenya to establish laboratory colonies for RNA-Seq analysis. We identified AeAV in the domestic K2 and K14 colonies, which was seemingly absent from the other Rabai (K18, K19, K27) colonies. The K27 colony was interbred with the strain K14, which we found to be AeAV positive. In all the four resultant hybrid colonies, which were subjected to RNA-Seq analysis, we were able to *de novo* assemble identical K14 AeAV strain genomes (Fig. 10).

The possibility of vertical transmission also is supported by the presence of AeAV in both RNA-Seq data from the sperm of adult male mosquitoes (54) and the female reproductive tract (53).

Discussion

The ability of AeAV to propagate in *Ae. aegypti* and *Ae. albopictus* cell lines but not in the three mammalian cell lines suggests that AeAV is most likely an ISV, although this needs to be further confirmed using cell lines from other species. To the best of our knowledge, this is the first

comprehensive characterisation of any *Anphevirus* species within *Mononegavirales* and the first *Mononegavirales* virus species within *Ae. aegypti*. Although as this manuscript was under revision, the complete genome sequence of AeAV and its phylogenetic relationship with other ISVs was published in a short communication (68). While we have demonstrated that AeAV is spread worldwide in *Ae. aegypti* mosquitoes, we have limited understanding as to how prevalent AeAV is in individual mosquitoes in wild populations, its tissue tropism, or potential impacts on the host. Although it is likely that AeAV is maintained in wild populations of *Ae. aegypti* mosquitoes through vertical transmission, it is possible that AeAV could be maintained through venereal transmission as we were able to identify the whole AeAV genome from a dataset prepared from the sperm of adult male *Ae. aegypti* mosquitoes (54).

To our knowledge, the oldest continually maintained colony of laboratory mosquitoes with AeAV present comes from Jinjang, Malaysia, which was established from wild-collected samples from as early as 1975 (54). This suggests vertical transmission rates of AeAV are high or a high incidence of AeAV within the colony. In comparison, in both French Guiana colonies, we were unable to recover the complete genomes from these strains. It is unlikely that this is due to insertion of AeAV into the nuclear *Ae. aegypti* genome as numerous reads mapped to the ORF7/L region of the reference strain; however, there was not enough coverage to reach consensus of the full genome. As these libraries were prepared from homogenates of mosquitoes, it seems likely that the incidence of infection within these colonies may be lower; however, further analysis would have to be conducted.

In our analysis, AeAV was not detected in any of the widely used Liverpool (LVP) and Rockefeller/UGAL, as well as derived “white eye mutant” strains of *Ae. aegypti*. Analysis of published ncRNA-Seq data from Australian Townsville and Cairns colonies of wild-caught Australian mosquitoes (69, 70) suggests that there is no RNAi response or presence of AeAV in these mosquitoes. Evidence from this study and others suggests that widely used laboratory strains of *Ae. aegypti* harbour a diverse and heterogeneous virome composition and may contribute to the variable vector competence between these colonies.

344 In our analysis, the geographic origin of the RNA-Seq samples matched the resulting phylogenetic
345 relationship of each strain. The presence of AeAV in the *Ae. albopictus* cell line RML-12, presumably
346 the origin of the AeAV contamination in other *Aedes* cell lines transinfected with adapted wMelPop
347 strain, was the only *Ae. albopictus* sample in our analysis. During our analysis, we queried all of the
348 266 currently available *Ae. albopictus* RNA-Seq datasets (TaxonID: 7160) uploaded to the SRA,
349 none of which indicated presence of AeAV. We hypothesise that AeAV from RML-12 is likely due to
350 contamination as the cell line is often mischaracterised as originating from *Ae. aegypti* (71, 72). In
351 many laboratories that study arbovirus interactions, more than one *Aedes* cell line is maintained. As
352 the RML-12 AeAV strain is genetically placed within the American lineage, and the namesake of the
353 cell line, Rocky Mountain Laboratories, (71) located in Montana, USA suggests possible
354 contamination from domestic *Ae. aegypti* mosquito samples. While no other *Ae. albopictus* RNA-
355 Seq data were positive for AeAV in this study, we cannot rule out the possibility that AeAV could
356 exist in American populations of *Ae. albopictus*. *Ae. aegypti* and *Ae. albopictus* co-exist in North
357 America and compete for larval habitats of discarded tires and other artificial containers (73).

358 RNAi response is commonly observed in mosquitoes against RNA viruses. This response includes
359 miRNA, siRNA and piRNA pathways (74, 75). Similarly, we found a large number of 21nt vsRNAs
360 produced against AeAV in infected cells that were evenly mapped to both sense and antisense
361 strands, indicating that dsRNA intermediates produced during replication must be the target of the
362 host cell RNAi response. In addition, a large number of vpiRNAs were found mapped to the 5'UTR,
363 ORF1 and the 3'UTR of the AeAV genome. These vpiRNAs had the typical ping-pong signature (U₁-
364 A₁₀) of secondary piRNAs. This signature has also been found in vpiRNAs produced during
365 alphavirus (76) and bunyavirus (63, 77) infections, but not in vpiRNA-like small RNAs in most
366 flaviviruses, such as DENV (78), ZIKV (79, 80) and an insect-specific flavivirus (81). We found a
367 higher proportion of small RNAs from Aag2-wMelPop-CLA cells that mapped to AeAV are vpiRNAs
368 (about 50%) as compared to less than 10% vsRNAs. Literature suggests that when the siRNA
369 pathway is compromised more vpiRNAs are produced. This has been shown in RNAi deficient C6/36
370 cells when infected with Sindbis virus, Rift Valley fever virus or La Crosse virus (63-65). The RNA-

Seq data from C6/36. *w*MelPop-CLA cells were for long transcripts rather than small RNAs, therefore we were not able to confirm if in those cells there are higher proportion of vpiRNAs than vsiRNAs. The over-representation of vpiRNAs in respect to vsiRNAs has also been demonstrated in negative-sense *Bunyavirales* members PCLV and RVFV (30, 65) in Aag2 cells for all segments of the genome. It remains to be seen however if the higher vpiRNA to vsiRNA ratio in Aag2. *w*MelPop-CLA could be due to suppression of the siRNA pathway by AeAV, or alternatively, *Wolbachia* may have an effect on the siRNA pathway. It seems however more likely that as negative sense RNA viruses produce less dsRNA replicative intermediates, these could be simply less targeted by the siRNA pathway and are unable to be resolved by sRNA profiling. These possibilities require further investigations, and it remains to determine the role of the vpiRNAs in AeAV replication or host anti-viral response.

The effects of *Wolbachia* on virus restriction are variable and depend on *Wolbachia* strain, virus family and transinfected host (82). *Wolbachia* was shown to enhance AeAV replication in *Ae. aegypti* cells in this study. Recent studies have demonstrated that *Wolbachia* has no effect on multi-segmented negative-sense RNA viruses; for example, Phasi Charoen-like virus (PCLV) (Family: *Bunyaviridae*) in *w*MelPop-CLA strain infected Aag2 cells (30), La Crosse virus (Family: *Bunyaviridae*) and vesicular stomatitis virus (Family: *Rhabdoviridae*), which is in the same order as AeAV, in *w*Stri strain in *Ae. albopictus* C710 cell line (83), and the Rift Valley fever virus (RVFV) (Family: *Phenuiviridae*), in *Culex tarsalis* mosquitoes transinfected with a somatic *Wolbachia* (strain *w*AlbB) had no effect on RVFV infection or dissemination (84), respectively. RVFV and PCLV belong to the *Bunyavirales* order and AeAV belongs to *Mononegavirales*, distinct orders of negative strand RNA viruses. All three viruses have conserved features that may provide insight into how they might be protected from restriction by *Wolbachia*. The genome of all negative-sense ssRNA viruses is both encapsidated within the nucleoprotein (85, 86) and is attached to RNA dependent RNA polymerase within the virion (87). The RNA dependent polymerase complex carries out both transcription of virus genes and replication of the genome.

Wolbachia has been shown to restrict a number of positive-sense RNA virus species from the *Togaviridae* and *Flaviviridae* families (82). After fusion and entry into the host cell, the genomes of

398 *Togaviridae* and *Flaviviridae* species are released into the cytoplasm and translated directly into
 399 polypeptide protein(s). These polypeptide proteins are processed by viral and cellular proteases to
 400 generate the mature structural and non-structural proteins which are then used to replicate the
 401 genome (88). While the exact mechanism for RNA virus restriction in *Wolbachia*-infected insects has
 402 remained elusive, it has been shown that restriction of RNA viruses by *Wolbachia* happens early in
 403 infection (89, 90). In the *Ae. albopictus* cell line C710 stably infected with wStri, the polypeptide of
 404 ZIKV is not produced as determined by immunoblot at one day post-infection (90). Additionally,
 405 *Wolbachia* exploits host innate immunity to establish a symbiotic relationship with *Ae. aegypti* (91).
 406 Perhaps the combination of protection of the RNA nucleocapsid genome or genome segments when
 407 released in the cytoplasm or activity of the RNA-dependent RNA polymerase may aid in evasion of
 408 host immune response enhanced by *Wolbachia* or *Wolbachia* effector molecules (92). However, a
 409 recent study suggested increases in infection of *Ae. aegypti* mosquitoes by insect-specific
 410 flaviviruses when they harbour *Wolbachia* wMel strain (93).

411 The ability of AeAV to modestly reduce DENV-2 genomic RNA in a persistently infected cell line was
 412 unexpected. While it has previously been shown that members of the same virus family can provide
 413 super exclusion of additional viruses (12, 94), little work has been undertaken to look at cross viral
 414 family exclusion effects. Our results showed that less DENV replication occurred in the presence of
 415 AeAV, with the difference particularly significant at lower MOI. If this suppressive effect also occurs
 416 in mosquitoes, enhancement of AeAV in *Ae. aegypti* mosquitoes infected with *Wolbachia* may be
 417 beneficial in terms of DENV suppression.

418 As *Ae. aegypti* is perhaps the most important vector of arboviruses worldwide, further work should
 419 be undertaken in understanding and characterising the virome of this mosquito and effects on
 420 mosquito life-history traits. Our findings provide new insights into the evolution and genetic diversity
 421 of AeAV across a wide geographic range as well as providing valuable insights into the virus features
 422 and families restricted by *Wolbachia* in mosquito hosts and its effects on arboviruses they transmit.

423 **Materials and Methods**

424 **Cell lines maintenance and experimental infection with AeAV**

425 *Ae. aegypti* cell line (Aag2) stably infected with *Wolbachia* (denoted Aag2.wMelPop-CLA) as
 426 previously described for the C6/36.wMelPop-CLA (35), with its previously generated tetracycline-
 427 treated line (66), and both *Ae. albopictus* C6/36 cell line (57) and RML-12 cell lines were maintained
 428 in 1:1 Mitsuhashi-Maramorosch and Schneider's insect medium (Invitrogen) supplemented with 10%
 429 Fetal Bovine Serum (FBS, Bovogen Biologicals). Aa20 cells established from *Ae. aegypti* larvae (95)
 430 were maintained in Leibovitz's L15 medium supplemented with 10% FBS (France – Biowest) and
 431 10% Tryptose phosphate broth at 27°C. African green monkey cells (Vero) were maintained in Opti-
 432 MEM I Reduced-Serum Medium supplemented with 2% FBS and 10 mL/L Penicillin-Streptomycin
 433 (Sigma-Aldrich). Human hepatocellular carcinoma cells (Huh-7) were maintained in Opti-MEM I
 434 Reduced-Serum Medium supplemented with 5% FBS and 10 mL/L Penicillin-Streptomycin (Sigma-
 435 Aldrich). BSR cells (a clone of Baby Hamster Kidney-21) were maintained in Dulbecco's Modified
 436 Eagle Medium (Gibco), 2% FBS and 10 mL/L Penicillin-Streptomycin (Sigma-Aldrich). All
 437 mammalian cells were kept at 37°C with 5%CO₂.

438 For experimental infection of cells, 10⁶ cells of *Aedes* or mammalian cells were seeded in a 12-well
 439 plate. Subsequently, supernatant from Aag2.wMelPop-CLA cells was collected, centrifuged at
 440 2150xg for 5 min to remove cells and debris, and used as an AeAV inoculation source. One Aa20
 441 cell line was experimentally inoculated with RML-12 cell supernatant and kept as a persistently
 442 infected AeAV cell line. Cells were collected at 1, 3 and 5 days post-inoculation for *Aedes* cell lines
 443 for RT-qPCR analysis and 1, 3, 5 and 7 days for mammalian cell lines for RT-PCR analysis.

444 ***Aedes anphevirus* (AeAV) and dengue virus (DENV-2) interaction assay**

445 The third passage of Aa20 cells persistently infected with AeAV (denoted Aa20_{AeAV}) and Aa20 mock
 446 were seeded at the density of 3×10⁵ cells in 12-well plates overnight. Cells were then infected with
 447 the East Timor (ET-100) DENV-2 strain at a multiplicity of infection (MOI) of 0.1 and 1, cells were
 448 rocked for an hour at room temperature and supernatant was discarded and replaced with fresh
 449 medium. Cells were collected at 0, 1, 3 and 5dpi after infection with mock collected at 5 dpi. Cells

were subjected to RNA extraction to quantify the DENV-2 genomic RNA levels by RT-qPCR as described below.

Assembly and identification of AeAV strains from RNA-Seq data

For detection of AeAV in previously published RNA-Seq data, we used the assembled RML-12 AeAV genome as a BLASTN query for all available *Ae. aegypti* (taxonID: 7159) RNA-Seq data within the Sequence Read Archive (SRA) on NCBI. SRA run files with positive hits of 90-100% identity and an E value $<2E-30$ were downloaded and converted to fastq using the NCBI SRA toolkit <https://www.ncbi.nlm.nih.gov/sra/docs/toolkitsoft/> for further analysis. FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used for quality checking of fastq files and adapter identification. Fastq files were then imported into CLC Genomics Workbench (10.1.1) and were adapter and quality trimmed (<0.02 ; equivalent Phred quality score of 17, ambiguous nucleotides: 2).

Two strategies were used to assemble strains of AeAV; fastq files from the same source of *Ae. aegypti* were pooled and *de novo* assembled using the CLC Genomics Workbench assembly program with automatic bubble and word sizes. This was sufficient to assemble the full coding sequences (CDS) of most strains of AeAV. Table S1 contains *de novo* assembly statistics from each dataset used.

If *de novo* assembly did not produce the complete AeAV genome, to complete further sections of the AeAV genome clean reads were mapped to the C6/36.wMeIPop-CLA strain of AeAV with stringent alignment criteria (Match score:1 Mismatch cost:2 length fraction 0.89 and similarity fraction 0.89) to exclude false-positive mapping that derives from Endogenous Viral Elements (EVEs). To confirm accuracy of assembly, the largest contigs of consensus mapping were extracted and then used as a reference for re-mapping and manually checked. Final sequences of the virus genomes were obtained through the majority consensus of the mapping assembly and were given Coding Complete (CC) or Standard Draft (SD) genome quality ratings (96).

RNA isolation, strand specific cDNA synthesis and RT-qPCR

476 Total RNA was extracted from mosquito cells using QIAzol Lysis Reagent (QIAGEN) and treated
477 with Turbo DNase (Thermo Fisher Scientific) as per manufacturer's instructions. RNA quality and
478 quantity were evaluated using a BioTek Epoch Microspot Plate Spectrophotometer. For the
479 production of AeAV genome and anti-genome cDNA, two cDNA reactions were generated using
480 600ng of RNA and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The genome
481 cDNA strand was synthesised using a forward primer to AeAV (AeAVGenome-RT 5'-
482 AGACTTCTAAGCCTGCCCACA -3'), and the AeAV anti-genome cDNA strand was synthesised
483 using a reverse orientation primer (AeAVAntiGenome-RT 5'- ACACTTGCCATGTGCTCAG -3').
484 *Aedes* Ribosomal protein subunit 17 (RPS17) primers (*Ae. aegypti*: AeRPS17-qR 5'-
485 GGACACTTCGGGCACGTAGT-3' and *Ae. albopictus* AalRPS17q-R 5'-
486 ACGTAGTTGTCTCTCTGCGCTC -3') were used for reference gene cDNA synthesis. Following RT,
487 qPCR with AeAV primers (AeAV-qF 5'-GACAATCGCATTGGCTGCAT-3' and AeAV-qR 5'-
488 CCCGAGACAATCGGCTTCTT - 3') as well as primer pairs for the RPS17 genes (*Ae. aegypti*:
489 AeRPS17-qF 5'- CACTCCGAGGTCCGTGGTAT -3' and *Ae. albopictus*: AalRPS17-qF 5'-
490 CGCTGGTTTCGTGACACATC -3') were undertaken. RPS17 was used for normalizing data as
491 described previously in *Ae. aegypti* cells (97).

492 For quantitation of DENV-2 genome copies in Aa20 cells, two SuperScript III reverse transcription
493 (Thermo Fisher Scientific) reactions with 1000ng of RNA were prepared. For DENV-2 genome
494 copies reaction, the reverse primer (DENV2-qR 5'- CAAGGCTAACGCATCAGTCA -3') and in a
495 separate cDNA synthesis reaction the *Ae. aegypti* RPS17 primers as described above.
496 Subsequently, qPCR for DENV-2 was carried out using the DENV-2 primer pair (DENV2-qF 5'-
497 GGTATGGTGGGCGCTACTA -3' and DENV2-qR), and RPS17 was used as a normalising control
498 as also described above.

499 Each time-point for experimental infection was run with three biological replicates and two technical
500 replicates. Platinum SYBR Green Mix (Invitrogen) was used for qPCR with 20ng of RT products in
501 a Rotor-Gene thermal cycler (QIAGEN) as described above. The relative abundance of AeAV RNA

502 and DENV-2 to the host reference gene was determined by qGENE software using the $\Delta\Delta C_t$ method
503 and analyzed using GraphPad Prism.

504 To test for the replication of AeAV in mammalian cells, 1000ng of total RNA extracted from the cells
505 was extracted and used for first stand synthesis with SuperScript III reverse transcriptase with the
506 AeAVGenome-RT primer. qPCR was subsequently carried out using AeAVGenome-RT primer and
507 the qPCR primer AeAVqR2 5'-ATGAAAGTATGGATACACACCGCG-3'. Products were then
508 visualised on a 1% agarose gel.

509 **Virus genome annotation**

510 Potential ORFs of AeAV were analysed using NCBI Open Reading Frame Finder
511 (<https://www.ncbi.nlm.nih.gov/orffinder/>) with a minimal ORF length of 150. ORFs were cross
512 referenced with mapping from Poly(A) enriched transcriptomes (Fig. S1) to reduce false positive
513 identification of ORFs. For determination of putative domains in AeAV, ORFs were translated and
514 searched against the Conserved Domain Search Service (CD Search)
515 (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). For protein homology detection, we used
516 the HHPred webserver on translated AeAV ORFs (98).

517 To best discriminate N-terminus transmembrane domains from signal peptides, we used the
518 consensus TOPCONS webserver (99). Glycosylation sites were predicted by the NetNGlyc 1.0/
519 NetOGlyc 4.0 Server (<http://www.cbs.dtu.dk/services/>).

520 **Phylogenetic analysis**

521 For placement of AeAV within the order *Mononegavirales*, ClustalW was used on CLC Genomics
522 Workbench to align amino acid sequences of 30 L proteins of the most closely related
523 *Mononegavirales* species as determined by BLASTp of the NCBI non-redundant database. A
524 Maximum likelihood phylogeny (PhyML) was constructed using the Whelan and Goldman (WAG)
525 amino acid substitution model with 1000 bootstraps. Accession numbers and associated hosts used
526 to produce the phylogenetic tree are available in Table S1.

To determine relatedness between different strains of AeAV, genomes that were coding complete and greater than 30x coverage were trimmed of 3'UTR and 5'UTR regions and aligned using the ClustalW algorithm on CLC Genomics Workbench. A Maximum likelihood phylogeny (PhyML) was constructed. A Hierarchical likelihood ratio test (hLRT) with a confidence level of 0.01 suggested that the General Time Reversible (GTR) +G (Rate variation 4 categories) and +T (topology variation) nucleotide substitution model was the most appropriate. 1000 bootstrap replicates were performed with 95% bootstrap branching support cut-off.

Statistical analysis

Statistical analysis Unpaired t-test was used to compare differences between two individual groups, while One-way ANOVA with Tukey's post-hoc test was carried out to compare differences between more than two groups. Data that did not pass the normality test were re-analysed by the non-parametric Wilcoxon test indicated in their relevant figure legends.

Accession numbers

All the complete and incomplete virus genome sequences generated in this study have been deposited in Genbank under the accession numbers (MH037149 and MH430648-MH430666).

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874 **Figure legends:**

875 **Figure 1. Presence of Aedes anphevirus (AeAV) in insect cell lines, and genome**
876 **organisation and phylogeny of the virus. A)** RT-PCR analysis of *Aedes* cell lines Aag2,
877 Aag2. wMelPop-CLA (Pop), wMelPop-CLA.Tet (Pop-T), Aa20, RML-12, and C6/36 for the
878 presence of AeAV. RPS17 was used as a loading control. **B)** Genome organisation of the
879 Cali, Colombia AeAV genome strain and subgenomic gene transcription profile.
880 Transmembrane domains (TMD) are depicted as boxes with dashed lines and signal peptide
881 is depicted as a blue box. NP, nucleoprotein; G, glycoprotein; ZnF, zinc-like finger; RdRP,
882 RNA dependent RNA polymerase. **C)** AeAV is a member of the *Anphevirus* genus (red),
883 related to members of the *Nyamiviridae* (pink) and *Bornaviridae* (purple) in an unassigned
884 family within the order *Mononegavirales*. A multiple sequence alignment of the RNA
885 dependent RNA polymerase and the mRNA capping domain was used to create a Maximum
886 likelihood phylogeny. The phylogeny is arbitrarily rooted. 1000 bootstraps were performed
887 and branches with bootstrap values greater than 85% are highlighted. Branch length

888 represent expected numbers of substitutions per amino acid site. Genbank protein
889 accession numbers are Bolahun virus variant 1 (AOR51366.1), Culex mononega-like virus
890 1 (CMLV1) (ASA47369.1), Culex mononega-like virus 2 (CMLV2) (ASA47322.1), Gambie
891 virus (AOR51379.1), Xincheng Mosquito Virus (XcMV) (YP_009302387.1), Borna disease
892 virus (YP_009269418.1), Canary bornavirus 1 (YP_009268910.1), Loveridges garter snake
893 virus 1 (YP_009055063.1), Parrot bornavirus 1 (AEG78314.1), Variegated squirrel
894 bornavirus 1 (SBT82903.1), Midway nyavirus (YP_002905331.1), Nyamanini nyavirus
895 (YP_002905337.1), Sierra Nevada virus (YP_009044201.1), Soybean cyst nematode
896 socyavirus (YP_009052467.1), Farmington virus (YP_009091823.1), Beihai rhabdo-like virus
897 3 (APG78650.1), Beihai rhabdo-like virus 5 (YP_009333422.1), Beihai rhabdo-like virus 6
898 (YP_009333413.1), Drosophila unispina virus 1 (AMK09260.1), Hubei diptera virus 11
899 (YP_009337182.1), Hubei orthoptera virus 5 (YP_009336728.1), Hubei rhabdo-like virus 7
900 (YP_009337121.1), Orinoco virus (ANQ45640.1), Sanxia Water Strider Virus 4
901 (YP_009288955.1), Shuangao Fly Virus 2 (AJG39135.1), Wenling crustacean virus 12
902 (YP_009336618.1), Wenzhou Crab Virus 1 (YP_009304558.1), Wenzhou tapeworm virus 1
903 (YP_009342311.1), Wuchan romanomermis nematode virus 2 (YP_009342285.1).

904 **Figure 2: Conservation of GATA-like Zinc finger (ZnF) domain and small transmembrane**
905 **domain containing protein between tentative members of the *Anphevirus* taxon. A)** Genome
906 orientation of previously discovered viruses within the *Anphevirus* taxon, and **B)** two viruses within
907 a closely related clade. Predicted ORF encoding the ZnF domain is indicated by a black square.
908 Predicted ORFs containing transmembrane domains are indicated by dashed lines. Genbank
909 accession numbers are shown below virus name. NP, nucleoprotein; G, glycoprotein; ZnF, zinc-
910 like finger; RdRP, RNA dependent RNA polymerase. **C)** Alignment of predicted GATA-like ZnF
911 protein sequence (C-X(2)-C-X(17-20)-C-X(2)-C) between three representative strains of AeAV
912 (Miami, USA ; Pune, India; Rabai, Kenya) and predicted ZnF domain proteins from Figure 2A and
913 B.

914 **Figure 3. *Aedes anphevirus* (AeAV) *cis*-regulatory elements.** **A)** Location and orientation of
 915 predicted *cis*-regulatory element in AeAV indicated by numbered red arrows; downwards indicating
 916 genome and upwards arrow indicating anti-genome. **B)** Predicted minimum free energy (MFE) RNA
 917 structure of the region surrounding the motif for each element using the RNAfold web server. Colour
 918 indicates probability of base-pairing and motif is indicated by the black line. **C)** Sequence of the
 919 conserved motif as predicted by MEME as well as location and the statistical confidence of the motif.
 920 Sequences are written 3' to 5' and anti-genome motif sequences 1 and 7 are depicted as reverse
 921 complement for visual clarity.

922 **Figure 4. *Aedes anphevirus* (AeAV) has worldwide distribution in *Ae. aegypti* laboratory**
 923 **colonies, cell lines and wild-caught mosquitoes.** Locations of mosquito collection from RNA-
 924 Seq data that were positive for AeAV (Table S1). Points refer to collection sites from American
 925 (orange), Asia-Pacific (blue) and African (green) locations.

926 **Figure 5. *Aedes anphevirus* (AeAV) strains have evolved into African, Asia-Pacific and**
 927 **American lineages.** **A)** Maximum likelihood phylogeny (PhyML) between AeAV strains using a
 928 General Time Reversible (GTR) + G +T model with 1000 bootstraps. Branch lengths represent
 929 expected numbers of substitutions per nucleotide site. For visual clarity, the RML-12 clade and Miami
 930 clades were collapsed and single examples were shown. **B)** Evolutionary history of worldwide
 931 sampling of *Ae. aegypti* adapted from (59, 60) from 1504 SNPs species. Bootstrapped neighbour-
 932 joining network based on population pairwise chord-distances from with node support over 90% is
 933 shown on relevant branches. New World (American) populations in yellow, and Asia-Pacific
 934 populations are shown in light blue. We have truncated the tree and rooted to the *Ae. aegypti*
 935 *formosus* (Aef) shown as a red branch.

936 **Figure 6. Genomic context for anphevirus-like insertions into the *Ae. aegypti* genome.** A 21,
 937 242nt portion of chromosome 2 depicting anphevirus insertions (red) with predicted ORFs that
 938 encode for LTR retrotransposase elements (yellow).

939 **Figure 7. *Aedes anphevirus* (AeAV) is infectious to *Aedes* cell lines but does not replicate in**
 940 **Huh-7, Vero and BSR vertebrate cell lines. (A)** RT-qPCR of AeAV genome and anti-genome in a
 941 five-day time course in *Ae. aegypti* Aa20 cells, and *Ae. albopictus* C6/36 cells. Error bars represent
 942 the SEM of three biological replicates. **(B)** RT-PCR of AeAV genome in a seven-day time course in
 943 Human hepatocellular carcinoma cells (Huh-7), African green monkey cells (Vero), Baby Hamster
 944 Kidney (BSR). M, Mock infected cells.

945 **Figure 8. *Aedes anphevirus* (AeAV) genome replication is enhanced by *Wolbachia* infection**
 946 **in *Ae. aegypti* cells and produces abundant vsiRNAs and vpiRNAs. A)** RT-qPCR of the AeAV
 947 genomic (gRNA) and antigenomic RNA in tetracycline cured Aag2. *w*MelPop-CLA cells (Pop-tet) and
 948 Aag2. *w*MelPop-CLA cells (Pop) relative to RPS17. Error bars represent the SEM of six (genome)
 949 and three (antigenome) biological replicates. n.s, not significant; **, $p < 0.01$. **B)** Mapping profile of
 950 pooled small RNA fraction in Aag2. *w*MelPop-CLA cells. **C)** Alignment of the 21-nt sRNA reads
 951 (representing siRNAs), and **D)** the 26-31nt reads (representing piRNAs) mapped to the AeAV
 952 antigenome (blue) and genome (red) in Aag2. *w*MelPop-CLA cells. Relative nucleotide frequency
 953 and conservation of the 28nt small RNA reads that mapped to the **E)** genome, and the **F)** anti-
 954 genome of AeAV in Aag2. *w*MelPop-CLA cells.

955 **Figure 9. *Aedes anphevirus* (AeAV) reduces dengue virus replication in Aa20 cells.** Aa20 cells
 956 persistently infected with AeAV were infected with **(A)** 0.1 and **(B)** 1 MOI of dengue virus serotype 2
 957 (DENV-2). Total RNA was extracted at 0, 1, 3 and 5 days following DENV-2 inoculation and analysed
 958 by RT-qPCR. **(C)** RT-qPCR analysis of AeAV persistently infected Aa20 cells infected with 0.1 and
 959 1 MOI of DENV-2 using specific primers to the AeAV genome. Error bars represent the SEM of three
 960 biological replicates. n.s, not significant; *, $p < 0.05$; **, $p < 0.01$.

961 **Figure 10. *Aedes anphevirus* (AeAV) is potentially vertically transmitted. A)** Diagram showing
 962 the parental (K14, K27) and hybrid strains (GP1, GP2, HP1, HP2) from (49). **B)** Table showing
 963 assembly statistics and BLASTN similarity of AeAV assembled from K14 and K27 hybrid strains.

A

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Parental Strains		K14	K27	
Assembled AeAV genome		13,012	-	
Hybrid Strains	GP1	GP2	HP1	HP2
Assembled AeAV genome	13,059	13,034	13,010	7,191; 5,962
BLASTN identity to K14	100%	100%	100%	100%