

# Commensal Viruses of Mosquitoes: Host Restriction, Transmission, and Interaction with Arboviral Pathogens



Roy A. Hall<sup>1</sup>, Helle Bielefeldt-Ohmann<sup>1</sup>, Breeanna J. McLean<sup>1</sup>, Caitlin A. O'Brien<sup>1</sup>, Agathe M.G. Colmant<sup>1</sup>, Thisun B.H. Piyasena<sup>1</sup>, Jessica J. Harrison<sup>1</sup>, Natalee D. Newton<sup>1</sup>, Ross T. Barnard<sup>1</sup>, Natalie A. Prow<sup>1,2</sup>, Joshua M. Deerain<sup>1</sup>, Marcus G.K.Y. Mah<sup>1,2</sup> and Jody Hobson-Peters<sup>1</sup>

<sup>1</sup>Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD, Australia. <sup>2</sup>QIMR Berghofer Medical Research Institute, Herston, QLD, Australia.

## Supplementary Issue: Evolution of the Insect Virome

**ABSTRACT:** Recent advances in virus detection strategies and deep sequencing technologies have enabled the identification of a multitude of new viruses that persistently infect mosquitoes but do not infect vertebrates. These are usually referred to as insect-specific viruses (ISVs). These novel viruses have generated considerable interest in their modes of transmission, persistence in mosquito populations, the mechanisms that restrict their host range to mosquitoes, and their interactions with pathogens transmissible by the same mosquito. In this article, we discuss studies in our laboratory and others that demonstrate that many ISVs are efficiently transmitted directly from the female mosquito to their progeny via infected eggs, and, moreover, that persistent infection of mosquito cell cultures or whole mosquitoes with ISVs can restrict subsequent infection, replication, and transmission of some mosquito-borne viral pathogens. This suggests that some ISVs may act as natural regulators of arboviral transmission. We also discuss viral and host factors that may be responsible for their host restriction.

**KEYWORDS:** mosquito-borne viruses, insect-specific viruses, flaviviruses, bunyaviruses, mesoniviruses, negeviruses

**SUPPLEMENT:** Evolution of the Insect Virome

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**CORRESPONDENCE:** roy.hall@uq.edu.au

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## Introduction

Mosquito-borne viruses are objects of intense research due to their complex biology, ecology, and evolution and their potential to produce large and unpredictable outbreaks of disease.<sup>1</sup> Indeed, explosive outbreaks of disease in many regions of the world have been caused by mosquito-borne viruses such as dengue virus (DENV), Zika virus (ZIKV), chikungunya virus (CHIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), Ross River virus (RRV), and Rift Valley fever virus. In the absence of safe and effective vaccines and antivirals against many of these viruses, new approaches are being explored to control these diseases and their transmissions.

Currently, there is a strong research focus on a novel group of “insect-specific” viruses (ISVs) that persistently infect mosquitoes, but do not infect vertebrates.<sup>2,3</sup> Importantly, persistent infection of mosquitoes with some ISVs appears to interfere with the replication and transmission of medically significant viruses, such as West Nile virus (WNV).<sup>4–7</sup> These findings suggest that ISVs may act as natural regulators of transmission of some arboviruses and may provide a new

avenue for developing vector control strategies. Furthermore, the potential to genetically manipulate ISVs to develop new platforms for the production of safe diagnostic antigens and vaccines for mosquito-borne pathogens is now recognized as an innovative and a viable strategy.<sup>8</sup>

In this article, we review the key research conducted on mosquito-borne ISVs with a focus on the recent isolation and characterization of several new ISVs that group within a range of virus families including flaviviruses, alphaviruses, bunyaviruses, mesoniviruses, negeviruses, and reoviruses. We also discuss mechanistic and evolutionary features that are likely to be associated with the adaption of ISVs to a mosquito-only transmission cycle and the potential applications of these viruses as novel model systems for research, recombinant technology platforms, and agents of biological control.

## Flaviviruses

The genus *Flavivirus* (family *Flaviviridae*) includes many important mosquito-borne human pathogens such as DENV,

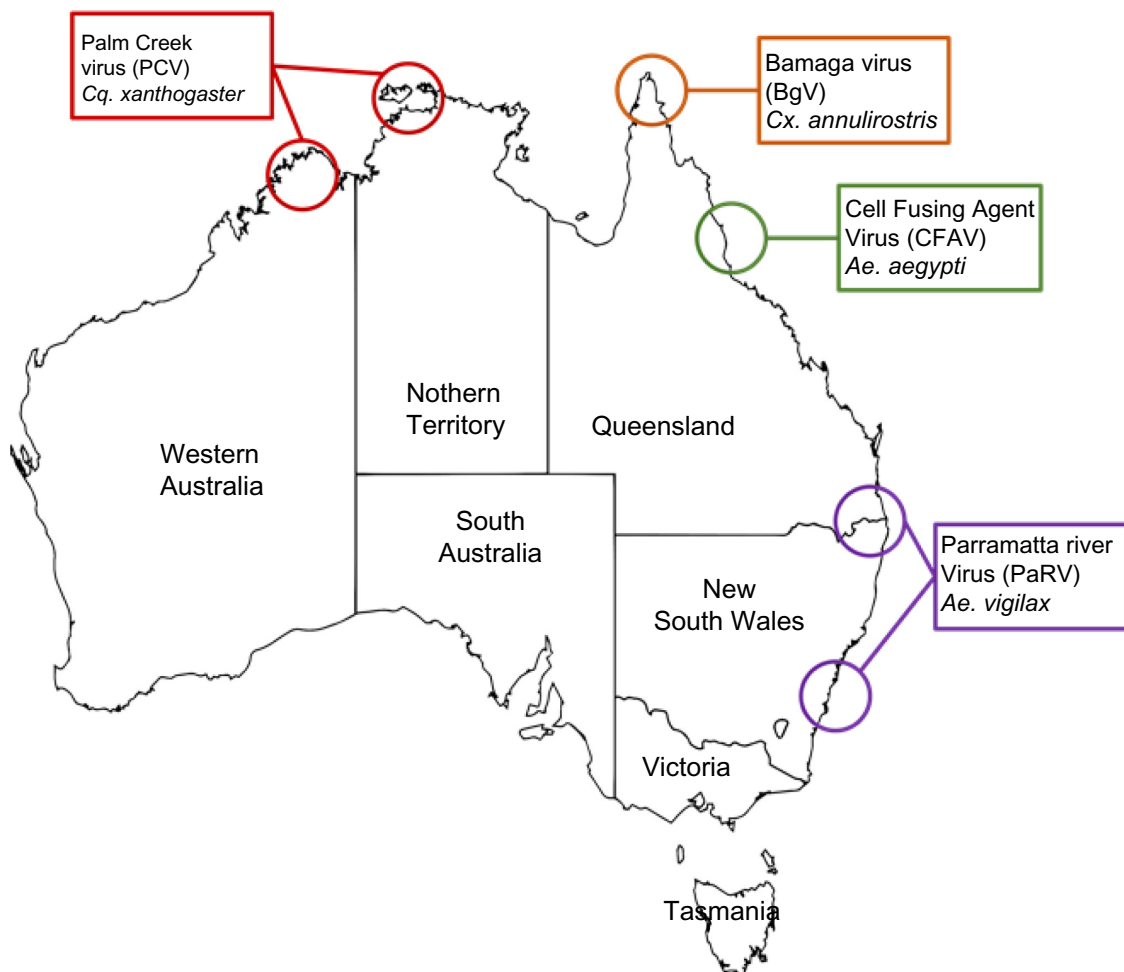
WNV, YFV, and ZIKV.<sup>9</sup> These viruses cycle between mosquitoes and human or animal hosts with replication in both the arthropod and the vertebrate required to maintain transmission and persistence of the virus. In contrast, insect-specific flaviviruses (ISFs) replicate only in mosquitoes and form an interesting subgroup within the *Flavivirus* genus.<sup>2</sup>

The first recognized ISF, cell-fusing agent virus (CFAV), was identified as an endogenous virus in a cell line derived from *Aedes aegypti* mosquito larvae.<sup>10</sup> However, it was not until almost 30 years later that ISFs, including CFAV and a related virus “Kamiti River virus”, were first isolated from mosquitoes in the wild and characterized.<sup>11–13</sup> Since then, many ISFs have been isolated or genetically detected in several mosquito species from different regions of the world.<sup>2</sup> Studies by Saiyasombat et al.<sup>14</sup> and Bolling et al.<sup>6</sup> indicate that ISFs are maintained in mosquito populations by vertical transmission – a process by which the progeny of infected female mosquitoes is infected via the egg.<sup>15</sup>

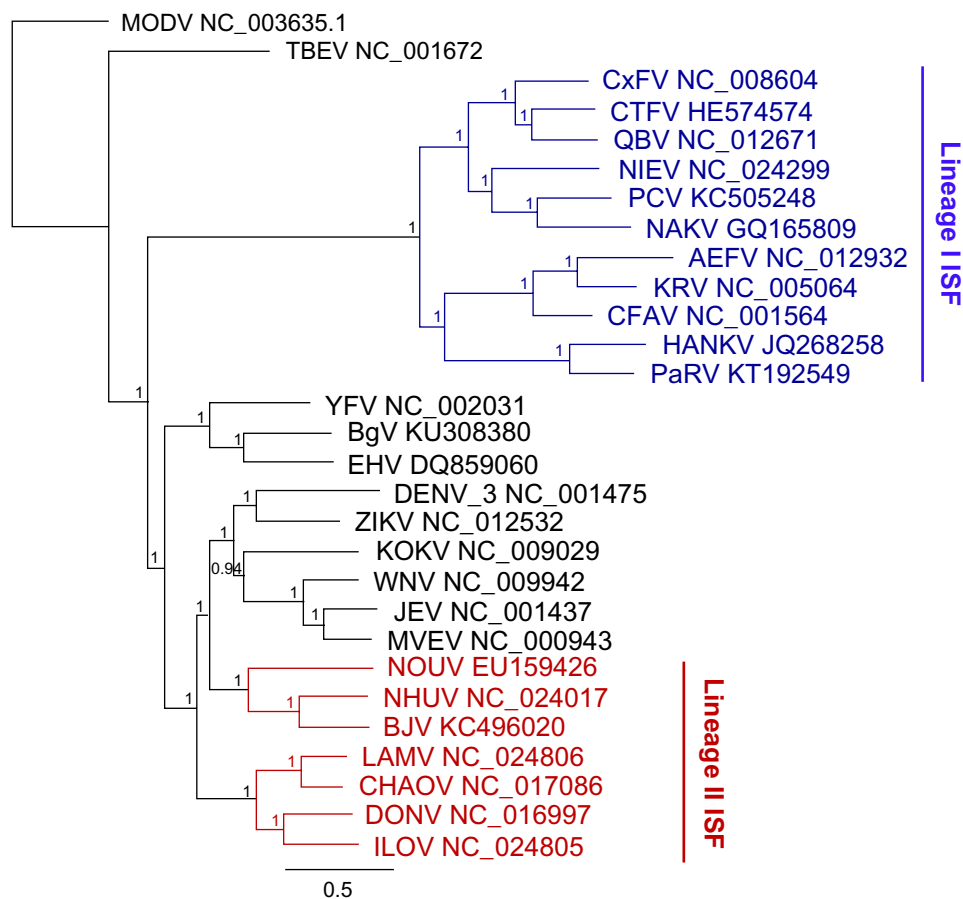
In 2010, our laboratory initiated a project to assess the biodiversity of ISFs in Australian mosquitoes. This was initially performed by the detection of flavivirus RNA directly in archival samples of homogenized mosquito pools or by

inoculation of the samples onto C6/36 cells prior to the detection by reverse transcription polymerase chain reaction and/or the presence of cytopathic effect (CPE) in the cells.<sup>5</sup> Subsequently, we enhanced the speed and sensitivity of the ISF isolation protocol by the detection of viral replicative dsRNA in inoculated mosquito cell culture by the use of anti-dsRNA mAbs in enzyme-linked immunosorbent assay – referred to as “MAVRIC” (*monoclonal antibodies against viral RNA intermediates in cells*).<sup>16</sup> This approach allowed the detection of novel and genetically diverse ISFs (as well as other novel RNA viruses, which are given in the following sections) in a sequence-independent manner and provided a relatively simple, cost-effective, and high-throughput protocol for the detection of infected cultures prior to sequencing and characterization of the isolates.

Several ISFs were subsequently isolated from Australian mosquitoes including Palm Creek virus (PCV) from *Coquillettidia xanthogaster* in northern Australia<sup>5,17</sup>; Parramatta River virus (PaRV) from *Aedes vigilax* from Sydney, Newcastle, and Brisbane<sup>17</sup>; and local isolates of CFAV from *Ae. aegypti* from Cairns (Harrison et al, unpublished data). Figure 1 illustrates the geographical distribution of these viruses



**Figure 1.** Map of Australia showing general locations of insect-specific flavivirus isolations.



**Figure 2.** Bayesian phylogenies of flaviviruses over the whole open reading frame nucleotide sequence. The tree was constructed in Geneious using MrBayes v3.2.2 under the Bayesian Marko chain Monte Carlo (MCMC) model with a general time reversible substitution model, gamma distribution (five discrete gamma categories), and invariant rates among sites. Horizontal branch lengths represent posterior probabilities. The tree has been rooted using the outgroup Modoc virus (MODV), a flavivirus with no known vector. The colored nodes represent insect-specific flaviviruses (ISFs), with Lineage I in blue and Lineage II in red.

in Australia, while Figure 2 shows the genetic relationship between these new viruses, other ISFs, and flaviviruses that infect vertebrates.

**ISF-like viruses.** Most of the ISFs reported to date belong to a group that is phylogenetically separate from the vertebrate-infecting flaviviruses and are described as “classical” ISFs.<sup>2</sup> For clarity, these viruses are referred to, in this article, as Lineage I ISFs (Fig. 2). However, a smaller subset of ISFs, termed dual-host affiliated ISFs, display an insect-specific phenotype but group phylogenetically with the mosquito-borne pathogenic flaviviruses.<sup>18–20</sup> These viruses are referred to, in this article, as Linage II ISFs (Fig. 2). While Lineage II ISFs have been assessed *in vitro* for growth in a range of vertebrate cell lines, with no replication detected, the phylogenetic position of these viruses suggests that they may have only recently evolved from a vertebrate-infecting phenotype to an insect-specific transmission cycle.<sup>2</sup> However, more studies are required to support this, including testing for growth in a more extensive panel of cell lines under variable growth conditions and additional *in vivo* experiments.

Another subset of ISF-like viruses shows some replication in vertebrate cells, but only in a limited range of cell

types or under specific growth conditions. Rabensburg virus, considered to be a strain of WNV, showed little to no replication in vertebrate cell lines or live birds.<sup>21</sup> However, further studies revealed that this virus replicates and causes CPE in vertebrate cells if they are incubated at temperatures below 35 °C.<sup>22</sup> A recent report from our laboratory also showed that a new Australian flavivirus named Bamaga virus (BgV), which groups phylogenetically with vertebrate-infecting flaviviruses in the YFV group, displays restricted replication in vertebrates, both *in vitro* and *in vivo*.<sup>23</sup> No replication of BgV could be detected in a range of vertebrate cells after 2 days of incubation, even when the cells were infected at a high multiplicity of infection. However, after 5 days of incubation, limited replication was detected in a subset of vertebrate cells tested.<sup>23</sup> When a range of viral doses were inoculated into weanling mice, none of the animals injected by the intraperitoneal route seroconverted, suggesting little or no replication *in vivo*. Indeed, mice showed no disease, and only a few animals seroconverted when the highest viral dose (10<sup>4</sup> infectious units) was inoculated directly into the brain.

The unusual phenotype described earlier for the two viruses may be indicative of their adaption to cryptic vertebrate hosts



with low optimal body temperature. Alternatively, the data may indicate that they are in transition between a vertebrate-infecting and an insect-specific transmission cycle.<sup>22,23</sup>

**Interference by ISFs with the replication and transmission of flaviviral pathogens.** Studies by Bolling et al.<sup>6</sup>, on a population of *Culex pipiens* naturally infected with the ISF *Culex* flavivirus (CxFV), revealed that these mosquitoes exhibited a delay in the transmission of WNV, compared to CxFV-free mosquitoes, when infected with WNV by the oral route. Suppression of WNV transmission has also recently been reported for some *Culex* species previously inoculated with Nhumirim virus<sup>7</sup> or PCV.<sup>4</sup> Furthermore, the latter study indicated that transmission interference probably occurred in the cells of the midgut; the exclusive site of localization of PCV replication as determined by immunohistochemistry labeling of mosquito sections<sup>4</sup> and the first tissue to be infected upon oral feeding with WNV.

The effect on DENV and ZIKV transmission by *Aedes* species carrying ISFs, such as CFAV or PaRV, has yet to be assessed; however, *in vitro* studies in our laboratory revealed that the replication of DENV-3 and WNV in *Aedes albopictus* cells (C6/36) was strongly inhibited, in a flavivirus-specific manner, by prior infection with PaRV (McLean et al, unpublished data).

The mechanism(s) involved in both *in vitro* and *in vivo* viral interferences by ISFs at the cellular level is unknown. Current theories suggest an upregulation of an antiviral host response by the initial ISF infection that subsequently inhibits the superinfecting virus or competition for cellular resources between the resident ISF and the second flavivirus.<sup>2,4</sup>

The evolutionary origin of ISFs is an enigma. Did they evolve from vertebrate-infecting flaviviruses by adapting to replication in mosquitoes or do they represent the ancestral lineage of the dual-host flaviviruses? While it has been proposed that Lineage I ISFs represent the precursors to dual-host flaviviruses, previous attempts to address this by bioinformatics analyses were inconclusive, due to the limited number of fully sequenced ISF genomes available at the time.<sup>24</sup> However, the discovery and full genome sequencing of several additional ISFs may now allow more meaningful bioinformatics to be undertaken to elucidate the evolutionary origins of the flavivirus genus.

The molecular basis for the restricted host range of ISFs is another poorly understood phenomenon. Alignment of the deduced amino acid sequences of Lineage I ISFs with vertebrate-infecting flaviviruses reveals significant changes in several genes including large conserved deletions in domain III of the envelope protein (EDIII).<sup>17</sup> These deletions are of particular interest since EDIII contains the putative flavivirus receptor-binding site.<sup>25</sup> Furthermore, four additional cysteines in the EDIII of ISFs suggest the formation of additional S–S bonds in this domain, which likely change the tertiary structure and may alter the ability of ISFs to recognize specific cell receptors or interfere with membrane fusion.<sup>17,26,27</sup>

The identification of conserved deletions in the NS5 gene and insertions in the 3' untranslated regions of some ISFs when compared to VIFs may also indicate that additional viral factors are associated with ISF host restriction.<sup>17,28</sup> These may be associated with the inability of ISFs to counteract the innate immune response in the vertebrate cell. Indeed, recent findings by Tree et al.<sup>29</sup> provide the first evidence that some ISFs can infect and replicate in vertebrate cells deficient in innate immune pathways.

The use of novel methods to manipulate infectious genomes of ISFs will allow the identification of viral and host factors that restrict the replication of ISFs to mosquitoes and help us to understand the complex dynamics of the transmission of mosquito-borne viruses<sup>30,31</sup> (Piyasena et al, unpublished data). These investigations will also provide valuable insights into the evolution of mosquito-borne viruses and underpin the future development of new strategies to genetically manipulate ISFs as new technological platforms to prevent mosquito-borne viral diseases and control their transmission.

## Alphaviruses

In contrast to the ever-expanding group of ISFs discussed earlier, only a single member of the *Alphavirus* genus has been shown to exhibit an insect-specific phenotype. While there are many mosquito-borne alphaviruses that infect vertebrate hosts and cause disease, including CHIKV, Sindbis virus (SINV), Western equine encephalitis virus (WEEV), and RRV, only Eilat virus (EILV) appears to be restricted to replication in mosquitoes. EILV was isolated from *An. constani* mosquitoes trapped in Israel.<sup>32</sup> The virus clusters phylogenetically with the WEEV complex of alphaviruses that also includes SINV and SINV-like viruses. While it shows a similar genome structure and virion morphology to other alphaviruses, it fails to infect and replicate in a range of mammalian and avian cell lines that are usually susceptible to alphavirus infection. While the precise mechanism of host restriction has yet to be defined, Nasar et al.<sup>33</sup> have used SINV–EILV chimeric viruses to demonstrate that EILV replication is inhibited at both precell and postcell entry stages of the viral life cycle.

The construction of chimeric viruses between EILV and CHIKV, with the viral structural protein derived from the latter and the replicase components from the former, has also yielded a viable virus that is antigenically similar to the CHIKV parental virus, but unable to replicate in vertebrate cells like EILV.<sup>8</sup> This represents a novel platform for the production of diagnostic antigens or vaccines for pathogenic alphaviruses without the need for inactivation.

Similar to findings with ISFs,<sup>4,6,7</sup> prior infection of mosquito cells with EILV also delayed replication and reduced viral titers of other alphaviruses, including SINV, WEEV, and CHIKV.<sup>34</sup> Furthermore, infection of *Ae. aegypti* with EILV prior to infection by CHIKV delayed dissemination of the latter by 3 days. These studies highlight the potential application of ISVs for the control of arbovirus transmission and disease.

## Bunyaviruses

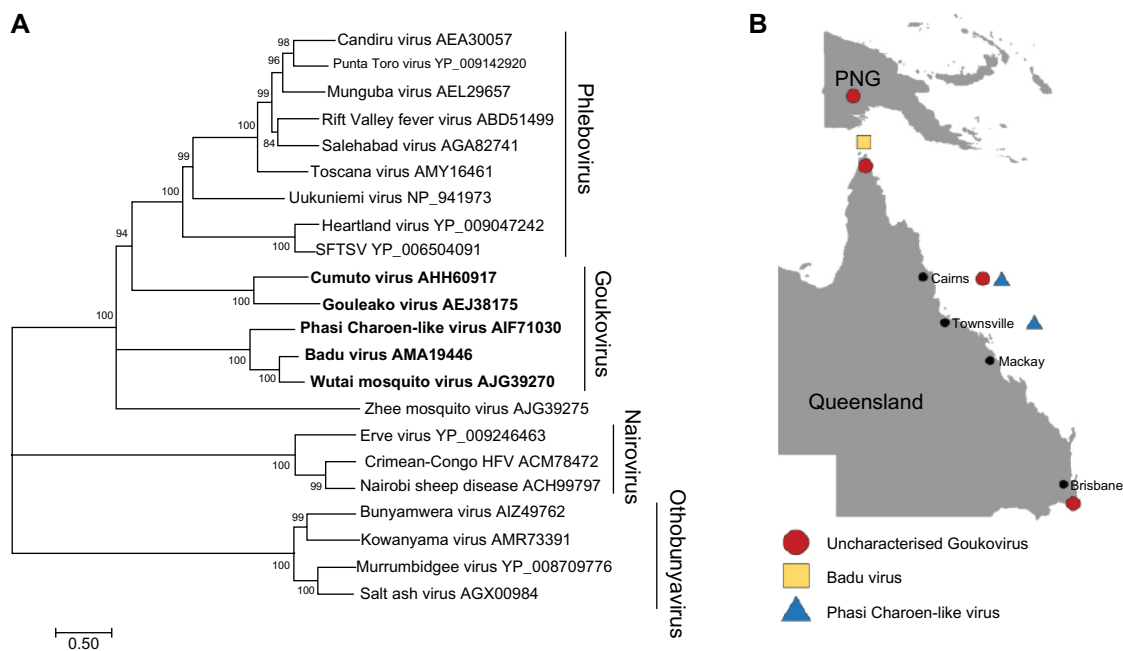
Recently, four divergent groups of bunyaviruses, which do not appear to replicate in vertebrate cells, have been identified in mosquitoes.<sup>35–37</sup> The first of these viruses to be isolated and characterized from mosquitoes collected in Cote d'Ivoire was Gouleako virus (GOLV).<sup>35</sup> The low genetic homology and lack of antigenic relatedness with other members of the *Bunyaviridae* family, in addition to its insect-restricted replication phenotype, led to the proposal of GOLV and other similar viruses to form a new genus named *Goukovirus*.<sup>35,38</sup> More recently, additional viruses likely to become members of the proposed *Goukovirus* genus have also been identified (Fig. 3), all with a basal phylogenetic relationship to the *Phlebovirus* genus.<sup>39</sup> These include Badu virus (BADUV), isolated in our laboratory from mosquitoes collected from Badu island in the Torres Strait between Australia and Papua New Guinea (PNG)<sup>40</sup>; Cumuto virus (CUMV), isolated from mosquitoes collected in Trinidad<sup>38</sup>; and sequences of viruses yet to be isolated – Zhee Mosquito virus (China), Phasi Charoen-like virus (PCLV; Brazil, Thailand), and Wutai mosquito virus (China).<sup>41–43</sup> More recently, we have obtained isolates of PCLV from *Ae. aegypti* mosquitoes from northern Australia (Harrison et al, unpublished data).

Although the three goukoviruses most studied to date (GOLV, CUMV, and BADUV) show restricted host range *in vitro*, *in vivo* studies have only been undertaken for CUMV. While inoculation of 2-day-old mice produced no apparent disease, no detailed analyses for evidence of virus replication in these animals were performed.<sup>38</sup> However, the associa-

tion of GOLV with fatal disease in pigs in Korea flags these new viruses as potential emerging pathogens.<sup>44</sup> Although a subsequent field study in Cote d'Ivoire found no evidence of GOLV infection in pigs,<sup>45</sup> further *in vivo* investigations are required to determine the actual host range and ecology of the different goukoviruses. In this context, we isolated BADUV from *Culex* mosquitoes collected during incursions of JEV into northern Australia.<sup>46</sup> In that outbreak, JEV was transmitted between *Culex* mosquitoes and pigs as part of the usual ecology for this virus. Whether BADUV simultaneously infected pigs is presently unknown. In addition to BADUV, we have recently isolated several novel bunyaviruses from northern Australia and PNG, which genetically cluster with the *Goukovirus* genus (Hobson-Peters et al, unpublished data; Fig. 3).

## Mesoniviruses

The order *Nidovirales* comprises four genetically diverse families of enveloped positive-sense single-stranded RNA viruses, including *Coronaviridae*, *Arteriviridae*, *Roniviridae*, and *Mesoniviridae*. The *Mesoniviridae* is a new ISV family that was established to accommodate Cavally virus, discovered in *Culex* mosquitoes captured in Côte d'Ivoire, West Africa, in 2004.<sup>47</sup> Independently, another group isolated Nam Dinh virus (NDiV) from *Culex* mosquitoes collected in Vietnam.<sup>48</sup> These viruses were later classified as different strains of *Alphamesonivirus-1*, the prototype species in the *Mesoniviridae* family.<sup>49</sup> Many other mesoniviruses have subsequently been discovered (Table 1).<sup>50–53</sup>



**Figure 3.** (A) Phylogenetic analysis of representative bunyaviruses showing the taxonomic position of goukoviruses. Maximum likelihood, midpoint rooted phylogenetic tree of Bunyavirus amino acid sequences over the complete RdRP open reading frame. The tree was constructed based on an MAFFT alignment via the CIPRES gateway and using Mega v7.0.14 tree builder with the Jones–Taylor–Thornton genetic distance model. Numbers on branches represent bootstrap values. Scale bar represents the number of substitutions per site. (B) The map shows distribution of new goukoviruses isolated in Australia and Papua New Guinea (PNG).



**Australian mesoniviruses.** In 2014, our laboratory reported the first mesonivirus discovered in Australia. Casuarina virus (CASV) was isolated from *Coquillettidia xanthogaster* mosquitoes collected in Darwin, Northern Territory.<sup>52</sup> Subsequently, we also isolated CASV from *Culex annulirostris* from Cairns in northern Queensland (Hobson-Peters et al, unpublished data). CASV was determined to be an ISV after the virus failed to replicate in a range of primate, rodent, human, and avian cell lines.<sup>52</sup> Alignment of the deduced amino acid sequences of the concatenated replicase domains between CASV and the *Alphamesonivirus-1* strains showed high identity of the RdRp (94.1%) and HEL (92.7–93.6%) domains. However, a lower identity (76.9–85.5%) was observed in the other four domains supporting assignment of CASV as a new species – *Alphamesonivirus-4*.<sup>52</sup> We further highlighted this proposed new assignment by mapping the evolutionary distance between the mesoniviruses (Fig. 4).

Following the discovery of CASV, the first Australian isolates of *Alphamesonivirus-1* (NDiV) were obtained from several mosquito species collected from various regions of the continent between 2007 and 2014 (Hobson-Peters et al, unpublished data).

## Reoviruses

The family *Reoviridae* comprises nonenveloped, segmented dsRNA viruses that include pathogens of a wide variety of

vertebrates and invertebrates including crustaceans, fish, insects, reptiles, and mammals. The genus *Orbivirus* is the largest genus within the family, containing 22 distinct virus species.<sup>54</sup> Orbiviruses are characterized by a 10-segment, double-stranded RNA genome. Parry's Lagoon virus (PLV) is a novel orbivirus that was isolated by our laboratory from *Cx. annulirostris* mosquitoes that were captured from northwestern Australia.<sup>55</sup> Phylogenetic analysis of each of the viral proteins demonstrated a moderate-to-high (72.6–95.3%) amino acid similarity to another orbivirus, Corriparta virus (CORV), supporting its classification as a member of the CORV serocomplex.<sup>55</sup> This conclusion was also confirmed by antigenic analysis that showed that PLV was recognized and cross-neutralized by CORV antisera.<sup>55</sup>

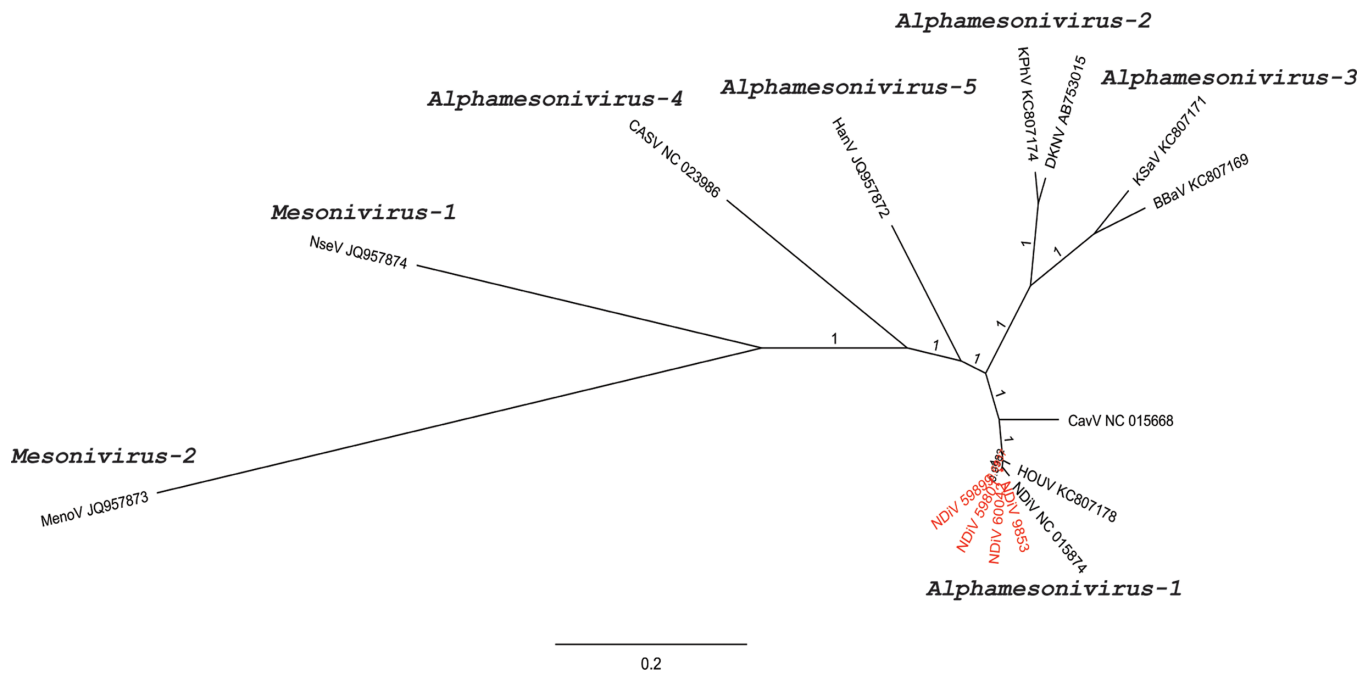
However, PLV shows a remarkably different phenotype in cell culture, failing to replicate in several mammalian and avian cell lines that supported efficient growth of CORV.<sup>55</sup> In contrast, PLV grew well in mosquito cells, suggesting that it has developed a restricted host range, indicative of a mosquito-only life cycle. Several other reoviruses also show a restricted host range (Table 2). *Aedes psuedoscutellaris* reovirus replicates in various mosquito cell lines, but failed to replicate in mammalian cell lines and *in vivo* in mice.<sup>56</sup> Similarly, Fako virus and Cimodo virus do not replicate *in vitro* in vertebrate cells.<sup>57,58</sup>

Another newly classified group of viruses in the *Reoviridae* family is the genus *Seadornavirus*.<sup>59</sup> The genus name refers

**Table 1.** Mesoniviruses published to date.

ISOLATE	SPECIES	ISOLATION REGION	COLLECTION DATE	MOSQUITO SPECIES OF ISOLATION	REFERENCES
Cavally	<i>Alphamesonivirus-1</i>	Côte d'Ivoire	2004	<i>Culex</i> spp. <i>Aedes</i> spp. <i>Anopheles</i> spp. <i>Uranotaenia</i> spp.	47
Nam Dinh Houston	<i>Alphamesonivirus-1</i> <i>Alphamesonivirus-1</i>	Vietnam U.S.A	2002 2004, 2010	<i>Culex vishnui</i> <i>Culex tritaeniorhynchus</i> <i>Culex quinquefasciatus</i> <i>Ae. albopictus</i>	48,53
Karang Sari (KSaV)	<i>Alphamesonivirus-2</i>	Indonesia	1981	<i>Cx. vishnui</i>	53
Bontag Baru (BBaV)	<i>Alphamesonivirus-2</i>	Indonesia	1981	<i>Cx. Vishnui</i> <i>Cx. tritaeniorhynchus</i>	53
Dak Nong (DKNV)	<i>Alphamesonivirus-3</i>	Vietnam	2007	<i>Cx. tritaeniorhynchus</i>	51
Kamphaeng Phet (KPhV)	<i>Alphamesonivirus-3</i>	Thailand	1984–85	Mosquito pool <sup>+</sup>	53
Casuarina (CASV)	<i>Alphamesonivirus-4</i>	Australia	2010, 2006	<i>Cq. xanthogaster</i> <i>Culex annulirostris</i>	52
Hana (HanV)	<i>Alphamesonivirus-5</i>	Côte d'Ivoire	2004	<i>Culex</i> spp. <i>Culex</i> spp.	50
Nsé (NseV)	<i>Mesonivirus-1</i>	Côte d'Ivoire	2004	<i>Aedes</i> spp. <i>Anopheles</i> spp. <i>Anopheles</i> spp.	50
Méno (MenoV)	<i>Mesonivirus-2</i>	Côte d'Ivoire	2004	<i>Culex</i> spp. <i>Uranotaenia chorley</i>	50

**Note:** <sup>+</sup>Mosquito species was not provided.



**Figure 4.** Phylogenetic tree showing the genetic relationship between the proposed species of the *Mesoniviridae* across the whole genome in nucleotides. The tree was constructed in Geneious using MrBayes v3.2.2 under the Bayesian Marko chain Monte Carlo (MCMC) model with a general time reversible substitution model, gamma distribution (five discrete gamma categories), and invariant rates among sites (Huelsenbeck and Ronquist, 2001). Horizontal branch lengths represent posterior probabilities. MenoV is used as an outgroup. Red text represents new Australian *Alphamesonivirus-1* isolates. Scale bar represents substitutions per site.

to the 12 segments of dsRNA comprising the viral genome and the geographical location of the first isolates.<sup>59</sup> Currently, there are three members of this genus: Banna virus (BAV), Kadapiro virus, and Liao ning virus (LNV). Only BAV has been directly associated with disease in humans.<sup>60</sup> LNV was first isolated in China in 1999. It was first detected in Australian mosquitoes by deep sequencing of mosquito samples containing unidentified viruses.<sup>61</sup> The first Australian isolates were made in our laboratory from *Ae. vigilax* mosquitoes collected in Sydney in 2007 and designated LNV<sub>Au</sub>. Since then, it has been isolated from four different mosquito genera (*Culex*, *Anopheles*, *Mansonia*, and *Aedes*) collected from all over Australia (Prow et al, unpublished data). In contrast to what was reported for the Chinese LNV isolates,<sup>62</sup> LNV<sub>Au</sub> strains do not replicate in vertebrate cell lines or in mice. Thus, at this stage, it would appear that LNV<sub>Au</sub> should be considered an ISV (Prow et al, unpublished data; Table 2). The mode of transmission between and within mosquito genera, however, still remains to be established.

### Negevirus

The taxon negevirus was originally described by Vasilakis et al.<sup>63</sup>, who reported the isolation and characterization of six viruses that formed an orphan group with no strong similarity to previously described families. Since this first report, at least six new species of negevirus have been discovered along with a number of reisolations of previously described negevirus often from different countries and host species (Table 3).

Bioinformatic analyses have indicated that the taxon negevirus is distantly related to the mite-transmitted, plant-infecting cileviruses and is likely to form two clades (*Nelorpivirus* and *Sandewavirus*). This group of viruses comprises a 9- to 10-kb positive-sense, single-stranded RNA genome with three open reading frames, two of which are believed to encode highly divergent structural proteins.<sup>63–65</sup>

Research into these viruses to date has been mostly *in vitro* and suggests that these viruses are restricted to insects with no growth reported in any vertebrate cell lines tested.<sup>63,64</sup> The discovery of a new species of negevirus, tentatively named Castlereia virus, from Australian mosquitoes is described in a separate article in this supplement (O'Brien et al, submitted for this supplement).

### Conclusions

With the application of deep sequencing approaches to virus discovery, many novel viral genomes have been detected in arthropod samples.<sup>42,61,66</sup> While the outcomes of these studies have redefined the taxonomy and phylogenetics of mosquito-borne viruses at the genus and family level and provided a wealth of data for the analysis of their evolution, additional efforts to isolate these new viruses are essential to determine their phenotypic properties. These properties include host range, mode of transmission, potential for pathogenesis, as well as interaction with the mosquito host and viral pathogens vectored by the host.

**Table 2.** Putative insect-specific reoviruses published to date.

ISOLATE	GENUS	ISOLATION REGION	COLLECTION DATE	MOSQUITO SPECIES OF ISOLATION	REFERENCES
Liao Ning <sup>#</sup>	<i>Seadornavirus</i>	China, Australia	1999,2007	<i>Cx. spp.</i> , <i>Ae. spp. Anopheles</i> , <i>Mansonia</i>	61, 62
Parry's Lagoon	<i>Orbivirus</i>	Australia	2010	–	55
<i>Aedes psuedoscutellaris reovirus</i>	<i>Dinovernavirus</i>	N/A	1974	Laboratory cell line isolation	56
Fako	<i>Dinovernavirus</i>	Cameroon	2010	<i>Ae. spp.</i> , <i>Eretmapodites dracaenae</i> , <i>inornatus</i>	57
Cimodo	N/A*	Côte d'Ivoire	2004	<i>Ae. spp.</i> , <i>Cx. spp.</i> , <i>An. spp. unclassified spp.</i>	58

**Notes:** <sup>#</sup>Australian Isolates shown to insect - specific. \*Currently unclassified. The authors concluded that Cimodo virus putatively defines a novel genus within the subfamily of *Spinareovirinae*.

**Table 3.** Negevirus published to date.

VIRUS	ISOLATION REGION	COLLECTION DATE	ISOLATION HOST	REFERENCE
<b>Nelorpivirus</b>				
Brejeira	Brazil	Not specified	<i>Culex spp.</i> <i>Psorophora ferox</i> *	67
Castlereia	Australia	1988–2015	<i>Aedes spp.</i> <i>Anopheles spp.</i> <i>Culiseta spp.</i> <i>Coquillettidia spp.</i> <i>Anopheles albimanus</i>	(O'Brien et al, <i>submitted for this supplement</i> )
Loreto	Peru	1977, 1983	<i>Culex spp.</i> <i>Lutzomyia spp.</i>	63
Negev virus	Israel U.S.A Portugal	1983, 2008	<i>Anopheles coustani</i> <i>Culex quinquefasciatus</i> <i>Culex coronator</i> <i>Culex univittatus</i>	63,64
Ngewotan ochlerotatus	Indonesia	1981	<i>Culex vishnui</i>	63
Caspian negevirus	Portugal	2009	<i>Ochlerotatus caspius</i>	68
Okushiri	Japan Republic of Korea	2010, 2012	<i>Aedes spp. (larvae)</i> <i>Culex pipiens</i>	69,70
Piura	Peru Mexico	1996, 2008	<i>Culex spp.</i> <i>Mansonia spp.</i> <i>Wyeomyia spp.</i> <i>Trichoprosopon spp.</i> <i>Coquillettidia spp.</i> <i>Psorophora spp.</i>	63,71
<b>Sandewavirus</b>				
Dezidougou	Côte d'Ivoire	1987	<i>Aedes aegypti</i>	63
Santana	Brazil	1992	<i>Culex spp.</i> <i>Culex spp.</i>	–
Wallerfield	Trinidad Brazil	2007–2009	<i>Psorophora ferox</i> *	38,67
Goutanap	Côte d'Ivoire	2004	<i>Culicidae spp.</i> <i>Culex quinquefasciatus</i>	71
Tanay	Philippines	2005	<i>Culex spp.</i> <i>Armigeres spp.</i>	65

Our studies and those of several other groups demonstrate that many ISVs are carried by mosquitoes and occur at high prevalence in some populations, apparently transmitted vertically. Furthermore, some of these viruses can regu-

late the transmission of pathogenic arboviruses in coinfecting mosquitoes. Future studies should be directed at determining the mechanisms by which ISVs interfere with arbovirus transmission and the viral and host factors associated with



their restriction to mosquito hosts and efficient vertical transmission. These studies will also require the development of essential ISV-specific research tools, including antibodies, molecular detection reagents, and reverse genetics systems.

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## Author Contributions

Conceived and designed the experiments: RAH, HBO, NAP, JH-P. Analyzed the data: RAH, HBO, BJM, CAO, AMGC, JJH, NDN, NAP, JMD, MGM, JH-P. Wrote the first draft of the manuscript: RAH. Contributed to the writing of the manuscript: RAH, HBO, CAO, AMGC, TBHP, JJH, NDN, RTB, NAP, JH-P. Agreed with the manuscript results and conclusions: RAH, HBO, BJM, CAO, AMGC, TBHP, JJH, NDN, RTB, NAP, JMD, MGM, JH-P. Jointly developed the structure and arguments for the article: RAH, HBO, CAO, AMGC, JJH, NDN, NAP, JH-P. Made critical revisions and approved the final version: RAH, HBO, BJM, CAO, AMGC, TBHP, JJH, NDN, RTB, NAP, JMD, MGM, JH-P. All the authors reviewed and approved the final manuscript.

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