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Virome of > 12 thousand *Culex* mosquitoes from throughout California

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ABSTRACT

Metagenomic analysis of whole mosquitoes allows the genetic characterization of all associated viruses, including arboviruses and insect-specific viruses, plus those in their diet or infecting their parasites. We describe here the virome in mosquitoes, primarily *Culex pipiens* complex, *Cx. tarsalis* and *Cx. erythrothorax*, collected in 2016 from 31 counties in California, USA. The nearly complete genomes of 56 viruses, including 32 novel genomes, some from potentially novel RNA and DNA viral families or genera, were assembled and phylogenetically analyzed, significantly expanding the known *Culex*-associated virome. The majority of detected viral sequences originated from single-stranded RNA viral families with members known to infect insects, plants, or from unknown hosts. These reference viral genomes will facilitate the identification of related viruses in other insect species and to monitor changes in the virome of *Culex* mosquito populations to define factors influencing their transmission and possible impact on their insect hosts.

1. Introduction

Mosquitoes transmit numerous arboviruses, many of which result in significant morbidity and/or mortality in humans and animals (Ansari and Shope, 1994; Driggers et al., 2016; Gan and Leo, 2014; Reimann et al., 2008; Weaver and Vasilakis, 2009). The mosquito genus *Culex* is comprised of ~768 taxa, including some of the most ubiquitous and important vectors of human pathogens which, in the present context of environmental changes affecting their geographic range, pose particular concern (Harbach, 2011; Jansen et al., 2009; Lequime and Lambrechts, 2014; Li et al., 2010; Parra-Henao and Suarez, 2012; Wang et al., 2011). The human pathogens vectored by *Culex* mosquitoes currently include West Nile Virus (WNV), Japanese encephalitis virus (JEV), western equine encephalomyelitis virus (WEEV), and St. Louis encephalitis virus (SLEV). Filarial worms and avian malaria parasites are also transmitted by *Culex* mosquitoes.

Recent studies have shown the *Culex* virome to be quite diverse. An early viral metagenomics study revealed DNA viruses as well as mammalian papillomavirus and anellovirus and plant viruses (presumably from the ingested vertebrate blood and plant diet) present in 480 female mosquitoes from multiple mosquito species, including *Cx. erythrothorax* from 3 sites in southern California (Ng et al., 2011). Auguste et al. screened 300 *Culex* mosquito pools collected in Trinidad for induction of viral cytopathic effects (CPE) in cultures of C6/36 (*Aedes albopictus*) mosquito cells. CPE was initiated by one pool of *Cx. declarator* mosquitoes from which the complete genomes of two novel insect-specific viruses belonging to the family *Bunyaviridae* were sequenced (Auguste et al., 2014). Another study used RNA sequencing to characterize the viral communities associated with 3 mosquito species in northern California: *Cx. pipiens*, *Culiseta incidens*, and *Aedes sierrensis*. Viral sequences from the families *Bunyaviridae*, *Rhabdoviridae*, and *Narnavirus* were detected (Chandler et al., 2015). A new flavivirus was identified from *Mansonia genus* mosquitoes from Uganda after C6/36 *Aedes* cell line amplification (Cook et al., 2009). Using metagenomics, viral fragments from diverse families and 2 nearly complete RNA virus genomes were sequenced from unspecified mosquitoes from Southern France (Cook et al., 2013). Four novel RNA viruses and other previously sequenced viral genomes were amplified from Australian mosquito pools by cytopathic effect detection in vertebrate cells (Coffey et al., 2014). Novel RNA viruses amplified in C6/36 *Aedes* cells inoculated with different mosquito pools from Southeast Asia and the Americas were also sequenced describing the genome of three new reoviruses and previously described insect viral genomes (Sadeghi et al., 2017).

Insect-specific viruses (ISVs) and their potential role in disrupting

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pathogen transmission has been investigated during the last decade (Bolling et al., 2015; Calzolari et al., 2016; Nunes et al., 2017; Roundy et al., 2017; Vasilakis et al., 2013; Vasilakis and Tesh, 2015). 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) (Bolling et al., 2012; Goenaga et al., 2015; Hall-Mendelin et al., 2016; Hobson-Peters et al., 2013). The majority of ISVs have been described in mosquitoes, although they occur in other arthropod orders, including *Hemiptera and Parasitiformes* (Li et al., 2015a; Tokarz et al., 2014). ISVs belong to taxonomically diverse virus families including *Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae, Togaviridae, Birnaviridae, Nodaviridae,* and *Mesoniviridae* (Attoui et al., 2005; Auguste et al., 2014; Bolling et al., 2011; Calzolari et al., 2016; Ergunay et al., 2017; Fauver et al., 2016; Huang et al., 2013; Huhtamo et al., 2014, 2009; Kuwata et al., 2011, 2013; Nasar et al., 2012; Schuster et al., 2014).

It is also possible that medically important arboviruses evolved from ISVs that acquired the ability to infect vertebrates (Vasilakis and Tesh, 2015). Some viruses may also have adapted to animals or plants hosts replication without the need for an insect vector (Li et al., 2015a). Many insect-associated RNA viruses in the families *Bunyaviridae, Flaviviridae and Rhabdoviridae* belong to highly diverse lineages indicating they likely evolved and diversified with their insect hosts over extended time periods (Chandler et al., 2015; Cook et al., 2013; Marklewitz et al., 2015; Walker et al., 2015). That many of these insect viruses appear to be vertically transmitted without obvious negative fitness consequences may also be considered evidence of a long-term relationship with their insect hosts (Lequime et al., 2016; Marklewitz et al., 2015; Walker et al., 2015). Some viral genomes have also become endogenized in the genomes of their arthropod hosts (Ballinger et al., 2013; Crochu et al., 2004; Dennis et al., 2018; Fort et al., 2012). ISVs may also act as natural regulators of insect populations and may provide new avenues for developing vector control strategies. *Culex* mosquitoes including *Cx. quinquefasciatus* infected with the *Wolbachia* bacteria have been found to influence WNV transmission by lowering virus titers (Glaser and Meola, 2010).

We sought here to generate a more complete characterization of the *Culex* virome using deep sequencing of viral particle-enriched nucleic acids and by sampling a greater number of mosquitoes from a large geographic region. We identified and assembled nearly complete genomes of previously known as well as multiple previously uncharacterized RNA and DNA viruses, compared their genome organizations, and performed phylogenetic analyses. The geographic distribution of these viral genomes throughout California is also described. We estimate that the *Culex*-associated viruses in California belong to at least 21 viral families, as well as several newly described or still unclassified families of DNA and RNA viruses.

2. Materials and methods

2.1. Mosquito collection and screening for arboviruses

The mosquitoes analyzed here originated from mosquito control districts throughout California. Mosquitoes were initially collected in carbon dioxide-baited light or gravid traps, morphologically identified to species by mosquito control district staff, and female mosquitoes were pooled in groups of 1–50 individuals (except one pool of 167 *Culicoides sonorensis*). Pools were frozen at − 80 °C, then shipped on dry ice to the Davis Arbovirus Research and Training laboratory at the University of California, Davis (UC Davis). There, mosquitoes were thawed at room temperature, and two glass beads were added to each tube, along with diluent containing 10% fetal bovine serum and antibiotics (penicillin, streptomycin, and mycostatin). Each pool was then mechanically homogenized for three min using a dual mixer mill model 8000D (Spex SamplePrep, Metuchen, NJ) to release virus particles from mosquito carcasses. The resulting mosquito pool homogenate was centrifuged, and viral nucleic acids were then extracted from an aliquot of each mosquito pool's supernatant using a MagMAX Express-96 Deep Well Magnetic Particle Processor and then tested by RT-qPCR to detect viral RNAs for the three *Culex*-borne human-pathogenic viruses endemic to California, WNV, WEEV, and SLEV using a triplex assay (Brault et al., 2015). Pools that tested negative by RT-qPCR for WNV, WEEV and SLEV were selected for this study. These mosquitoes originated from 124 unique geographic locations and represented three different time periods corresponding to early, middle, and late summer of 2016 (Supplemental Table 1A). 410 such pools were assembled into 51 larger pools of pools (Supplemental Table 1B). The total number of mosquitoes from different species analyzed are listed (Supplemental Table 1B), along with the mosquito species in each pool (Supplemental Table 1C).

2.2. Deep sequencing

Mosquitoes from each pool were further homogenized after assembly into larger pools in 1 ml of mosquito diluent (PBS) with disruption beads (0.16 g of 2.3 mm Bead Size, Zirconia/Silica Beads, 3.7 g/cc Density) and centrifuged for 10 min using a tabletop microfuge. Supernatants were filtered and treated with nucleases prior to nucleic acid extraction (Sadeghi et al., 2017; Zhang et al., 2016). The viral supernatants (400 µl each) were first centrifuged at 12,000 × *g* for 5 min at 4 °C and supernatant then filtered through a 450 nM pore size filter (Millipore, Billerica, Massachusetts, USA) to further remove mosquito debris and bacteria. The filtrates were then treated with a nuclease mixture of 7 µl of 14U turbo DNase (Ambion, Life Technologies, Grand Island, NY, USA), 3 µl of 3U Baseline-ZERO (Epicentre, Chicago, IL, USA) and 2 µl of 20U RNase One (Promega, Madison, WI, USA) in 10 × DNase buffer (Ambion, Life Technologies, Grand Island, NY, USA) at 37 °C for 1.5 h to reduce background nucleic acids from host cells and bacteria. Viral nucleic acids (protected from nuclease digestion by viral capsids), were then extracted from ~ 300 µl resulting solutions by bead-based extraction kits (MagMAX Viral RNA Isolation Kit, Ambion, Inc., Austin, TX, USA) without the application of DNAse to the extract (Sadeghi et al., 2017; Zhang et al., 2016).

Viral cDNA synthesis was performed by incubation of 10 µl extracted viral nucleic acids with 100 pmol of a primer containing a fixed 18 bp sequence plus a random nonamer (GCCGACTAATGCGT AGTCNNNNNNNNN) at the 3′ end at 85 °C for 2 min. Next, 200U SuperScript III reverse transcriptase (Invitrogen, Waltham, Massachusetts, USA), 0.5 mM each of deoxynucloside triphosphate (dNTP), 10 mM dithiothreitol, and 1 × first-strand extension buffer were added to the mixture and incubated at 25 °C for 10 min, followed by 50 °C incubation for 1 h. The 2nd strand DNA synthesis was performed by incubation with 50 pmol of random primer at 95 °C for 2 min, 4 °C for 2 min, and then with 5U Klenow Fragment (New England Biolabs, Ipswich, MA, USA) at 37 °C for 1 h. The resulting products were PCR amplified by using 5 µl of the RT-Klenow dsDNA products and 2.5 µM primer consisting of the fixed 18 bp portion of the random primer (GCCGACTAATGCGTAGTC) with 1U AmpliTaq Gold DNA polymerase (Life Technologies, Grand Island, NY, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 × PCR Gold buffer in a reaction volume of 50 µl. Temperature cycling was performed as follows: 1 cycle of 95 °C for 5 min, 30 cycles of denaturing at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1.5 min (33, 34). An additional extension for 10 min at 72 °C was added to the end of the run. Using the random RT-PCR product DNA as target library preparation was performed using the Illumina XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) as previously described (Li et al., 2015b; Sadeghi et al., 2017; Zhang et al., 2016) with double index barcode labeling and a 15 cycle PCR according to the manufacturer's protocols. Library concentration was then measured using the KAPA library quantitation kit (KAPA-BIOSYSTEMS, San Diego, CA, USA) The resulting libraries of single-stranded DNA fragments were sequenced using the HiSeq. 4000

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