

Detection of novel and recognized RNA viruses in mosquitoes from the Yucatan Peninsula of Mexico using metagenomics and characterization of their *in vitro* host ranges

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Abstract

A metagenomics approach was used to detect novel and recognized RNA viruses in mosquitoes from the Yucatan Peninsula of Mexico. A total of 1359 mosquitoes of 7 species and 5 genera (*Aedes*, *Anopheles*, *Culex*, *Mansonia* and *Psorophora*) were sorted into 37 pools, homogenized and inoculated onto monolayers of *Aedes albopictus* (C6/36) cells. A second blind passage was performed and then total RNA was extracted and analysed by RNA-seq. Two novel viruses, designated Uxmal virus and Mayapan virus, were identified. Uxmal virus was isolated from three pools of *Aedes* (*Ochlerotatus*) *taeniorhynchus* and phylogenetic data indicate that it should be classified within the recently proposed taxon *Negevirus*. Mayapan virus was recovered from two pools of *Psorophora ferox* and is most closely related to unclassified *Nodaviridae*-like viruses. Two recognized viruses were also detected: *Culex* flavivirus (family *Flaviviridae*) and Houston virus (family *Mesoniviridae*), with one and two isolates being recovered, respectively. The *in vitro* host ranges of all four viruses were determined by assessing their replicative abilities in cell lines of avian, human, monkey, hamster, murine, lepidopteran and mosquito (*Aedes*, *Anopheles* and *Culex*) origin, revealing that all viruses possess vertebrate replication-incompetent phenotypes. In conclusion, we report the isolation of both novel and recognized RNA viruses from mosquitoes collected in Mexico, and add to the growing plethora of viruses discovered recently through the use of metagenomics.

INTRODUCTION

The advent of next-generation sequencing (NGS) has revolutionized virus discovery [1–5]. This technology allows for the rapid and inexpensive detection of nucleic acids in diverse biological and environmental samples and offers distinct advantages over conventional virus detection techniques. Unfortunately, NGS is not always complemented by virus isolation experiments and therefore many newly discovered viruses are known solely from sequence data [6, 7]. Although many viruses cannot be easily propagated in cell culture, the importance of virus isolation cannot be understated because a newly discovered virus cannot be phenotypically characterized if an isolate is unavailable. Information on host range, transmissibility, pathogenesis, antigenicity and epidemiology are essential for the development and implementation of effective virus surveillance, diagnosis, control and prevention strategies.

A rapidly increasing number of novel RNA viruses have been identified in mosquitoes by NGS in recent years [8–13]. One of the first mosquito-associated RNA viruses to be discovered using this technology was Negev virus, the prototype virus of a newly proposed taxon, designated *Negevirus*, which also includes Dezydougou virus (DEZV) and Wallerfield virus (WALV) [8, 12]. Negeviruses have been isolated exclusively from mosquitoes and phlebotomine sandflies and are assumed to have insect-restricted host ranges [8, 12, 14–21]. Phylogenetic analyses have shown that negeviruses separate into two monophyletic clades (*Nelorpivirus* and *Sandewavirus*) and the amount of genetic diversity between viruses of different clades is sufficient to allow each clade to be elevated to the level of genus [20]. Negeviruses have a close phylogenetic relationship with plant viruses of the genera *Cilevirus*, *Higrevirus* and *Blunervirus*, some of which are arthropod-borne, and therefore it

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Abbreviations: 2'OMTase, 2'-O-methyltransferase; CPE, cytopathic effect; CT, *Culex tarsalis*; CxFV, *Culex* flavivirus; DEZV, Dezydougou virus; Hel, heli-case; HOUV, Houston virus; MTase, methyltransferase; MYPV, Mayapan virus; NGS, next-generation sequencing; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; RNAi, RNA interference; RT-PCR, reverse transcription/polymerase chain reaction; UTR, untranslated region; UXMV, Uxmal virus; WALV, Wallerfield virus.

has been postulated that negevirus possess the capacity to replicate in plants, although direct experimental evidence is lacking [12, 19, 20, 22].

Shi and colleagues recently discovered over a thousand novel putative RNA viruses in the largest NGS investigation of viruses ever performed on invertebrates [23]. This groundbreaking study provides valuable insights into virus biodiversity and evolution. More than 220 species of terrestrial and marine invertebrates from 9 metazoan phyla collected in China were examined. Among the numerous viruses discovered were multiple unclassified RNA viruses designated as Hubei noda-like virus 1 to 26. Phylogenetic studies revealed that Hubei noda-like viruses are closely related to viruses in the family *Nodaviridae*, a group of non-enveloped, bipartite RNA viruses that are classified according to their host associations. The family contains two established genera, *Alphanodavirus* (insect-associated) and *Betanodavirus* (fish-associated), and one proposed genus, *Gammanodavirus* (decapod-associated), besides a large number of other divergent but unclassified proposed members [23–25].

In the present study, mosquitoes of multiple species from the Yucatan Peninsula of Mexico were tested for the presence of novel and recognized RNA viruses. Mosquitoes were assayed by virus isolation in mosquito cell culture and then total RNA was extracted from all cultures, regardless of whether cytopathic effect (CPE) occurred, and examined for viral sequences using metagenomics. The replicative abilities of each novel and recognized virus were assessed using multiple vertebrate and invertebrate cell lines to determine their *in vitro* host ranges.

RESULTS

Mosquito collections and virus discovery

This study was performed using 1359 mosquitoes of 7 species and 5 genera collected in the Yucatan Peninsula of Mexico in 2007 and 2008 (Table 1). The mosquitoes were sorted into 37 pools and homogenized, and then an aliquot of each homogenate underwent 2 blind passages in *Aedes albopictus* (C6/36) cells. Eight homogenates caused cell death and seven others caused mild to severe clumping or

rounding of cells. Total RNA was extracted from all second passage cultures, regardless of whether CPE was observed, and analysed by RNA-seq for viral sequences. Two novel viruses, designated Uxmal virus (UXMV) and Mayapan virus (MYPV), were discovered (Table 2). The viruses were named after archaeological ruins in the Yucatan Peninsula of Mexico. Two recognized viruses were also isolated: *Culex flavivirus* (CxFV; genus *Flavivirus*, family *Flaviviridae*) and Houston virus (HOUV; a variant of the species *Alphamesonivirus-1* of the genus *Alphamesonivirus*, family *Mesoniviridae*). An acronym has not been previously assigned to Houston virus, but HOUV is used in this paper. Virus sequences were not recovered from several samples that produced CPE, indicating that the metagenomics analysis did not identify all viruses.

Uxmal virus

UXMV was isolated from three pools of female *Aedes* (*Ochlerotatus*) *taeniorhynchus* collected in Merida (Table 2). All second-passage C6/36 cell cultures displayed 40–60% CPE at 3 to 4 days post-inoculation, as indicated by the presence of elongated cells, clumping of cells into a chain-like formation and cell death. The *in vitro* host range experiments confirmed that C6/36 cells are permissive to UXMV replication and revealed that *Culex tarsalis* (CT) cells also support replication (Table 3). Unlike for the C6/36 cells, CPE was not observed in the CT cells. *Anopheles*, lepidopteran and vertebrate cells did not support virus replication. Homogenates prepared from 268 male *Ae. taeniorhynchus* in 15 pools were also tested by reverse transcription/polymerase chain reaction (RT-PCR) for UXMV, and all were negative.

The genome of one isolate (designated UXMV-M985) was fully sequenced (GenBank accession no. MH719095). The sequence consists of 9227 nt, excluding the polyadenylate tail, and is most closely related to the genomes of WALV (69.5% nt identity with 67.4% coverage) and DEZV (66.9% nt identity with 70.0% coverage). The genomic organization of UXMV is characteristic of viruses in the taxon *Negevirus*, with the genome predicted to encode three major open reading frames (ORFs) that are separated by short (22 to 119 nt) intergenic regions and flanked by 5' and 3' untranslated regions (UTRs) of 235 nt and 286 nt, respectively (Fig. 1a).

ORF1 encodes a putative replicase protein of 2239 residues that is most closely related to the corresponding translation products of WALV (60.2% identity and 77.6% similarity, with 100% coverage) and DEZV (60.8% identity and 77.2% similarity, with 98.6% coverage). The putative protein contains predicted viral methyltransferase (pfam01660), 2'-O-methyltransferase (pdb3R24), helicase (pfam01443) and RNA-dependent RNA polymerase (RdRp; pfam00978) domains at residues 82 to 413, 775 to 965, 1312 to 1579 and 1783 to 2220, respectively. These domains are highly conserved among the replicase proteins of negeviruses [12, 19].

Table 1. Mosquitoes tested by virus isolation in cell culture and viral metagenomics

Species	No. of mosquitoes	No. of pools
<i>Aedes aegypti</i>	62	4
<i>Aedes taeniorhynchus</i>	764	16
<i>Aedes trivittatus</i>	30	2
<i>Anopheles vestitipennis</i>	16	1
<i>Culex quinquefasciatus</i>	305	10
<i>Mansonia titillans</i>	89	2
<i>Psorophora ferox</i>	93	2
Total	1359	37

Table 2. Novel and recognized RNA viruses isolated from mosquitoes collected in the Yucatan Peninsula of Mexico

Virus	No. of isolations	Isolate name(s)	Taxonomic classification	Study site	Mosquito species
Culex flavivirus	1	CxFV-T1123	Flavivirus (Flaviviridae)	Tixkokob	<i>Cx. quinquefasciatus</i>
Houston virus	2	HOUV-M742, HOUV-M2668	Alphamesonivirus (Mesoniviridae)	Merida	<i>Cx. quinquefasciatus</i> , <i>Ae. taeniorhynchus</i>
Mayapan virus	2	MYPV-H44, MYPV-H56	^a Nodaviridae?	Tzacacab	<i>Ps. ferox</i>
Uxmal virus	3	UXMV-M985, UXMV-M1000, UXMV-M2038	Negevirus	Merida	<i>Ae. taeniorhynchus</i>

a, The closest known relatives of Mayapan virus are unclassified viruses that phylogenetically group with viruses in the family *Nodaviridae*.

ORF 2 is predicted to encode a membrane-associated protein of 413 residues. The putative protein is most closely related to the corresponding translation products of WALV (44.9% identity and 64.7% similarity, with 100% coverage) and DEZV (35.3% identity and 54.4% similarity, with 98.3% coverage). The putative protein is predicted to contain an amino-terminal signal peptide (residues 1 to 16), in addition to three carboxy-terminal transmembrane domains (residues 337 to 356, 368 to 389 and 395 to 410) and two carboxy-terminal cytoplasmic domains (residues 357 to 367 and 411 to 413), which is consistent with the ORF 2-encoded translation products of other negevirus [16, 17, 26]. Three potential N-linked glycosylation sites are also present (residues 178, 244 and 269).

ORF 3 is predicted to encode a membrane-associated protein of 200 residues. The putative protein is most closely related to the corresponding translation products of WALV (63.0% identity and 82.5% similarity, with 100% coverage) and DEZV (62.1% identity and 75.6% similarity, with 99.5% coverage). The putative protein is predicted to

contain four transmembrane domains (residues 49 to 71, 101 to 121, 133 to 158 and 165 to 183) and two cytoplasmic domains (residues 72 to 100 and 159 to 164). Two potential N-linked glycosylation sites are also present (residues 42 and 72). An additional ORF, which we have called ORF3a, spans nucleotide positions 8236 to 8448. The ORF overlaps the 5' end of ORF3 and is conserved in sandewaviruses (except the divergent Hubei virga-like virus 7 and Lodeiro virus) and encodes a cysteine-rich putative protein of 70 residues (Fig. 2a). ORF3a mostly fills a previously presumed non-coding gap between ORFs 2 and 3 (Fig. 1a).

A phylogenetic tree was constructed with Bayesian methods using the deduced amino acid sequence of the RdRp of UXMV and the corresponding regions of related sequences (Fig. 3). UXMV is most closely related phylogenetically to WALV and DEZV, and the posterior support for this topological arrangement is 1.0. Most of the viruses used in the analysis belong to one of two previously established clades: *Nelorpivirus* (group I) and *Sandewavirus* (group II). UXMV is a group II negevirus.

Table 3. *In vitro* host ranges of novel and recognized viruses isolated from mosquitoes in the Yucatan Peninsula of Mexico

Cell line	Virus			
	Culex flavivirus	Houston virus	Mayapan virus	Uxmal virus
<i>Mosquito cells</i>				
C6/36	^a +	^a +	+	^a +
CT	–	+	–	+
Sua 4.0	–	–	–	–
<i>Lepidopteran cells</i>				
High Five	–	–	–	–
Sf9	–	–	–	–
<i>Vertebrate cells</i>				
BSR-T7/5	–	–	–	–
DEF	–	–	–	–
HeLa	–	–	–	–
LLC-MK2	–	–	–	–
Murine microglia	–	–	–	–
Vero	–	–	–	–

a, CPE was observed.

Mayapan virus

MYPV was isolated from two pools of female *Psorophora ferox* collected in Tzacacab (Table 2). CPE was not observed in the first- or second-passage C6/36 cell cultures. The *in vitro* host range experiments revealed that MYPV has a narrow host range because only C6/36 cells were permissive to virus infection (Table 3). Male *Ps. ferox* were not tested for MYPV because none were available in our archived collections.

The genomic organization of MYPV has similarities with that of viruses in the family *Nodaviridae*. Viruses classified within this family have positive-sense, single-stranded, bipartite RNA genomes and the two segments are denoted as RNA1 and RNA2 [27]. RNA1 (3.1 kb) encodes the RdRp and RNA2 (1.4 kb) encodes the capsid protein. A subgenomic RNA (sgRNA3; 0.4 kb) is produced from RNA1 and encodes one or two small proteins, B1 and B2. The function of B1, which is identical to the C-terminus of the RdRp, is unknown, and B2 is a suppressor of RNA interference (RNAi) [28]. An additional subgenomic RNA (0.3 kb) is uniquely produced from RNA1 of mosinivirus, a nodavirus isolated from *Culicidae* spp. mosquitoes in Cote d'Ivoire [29].

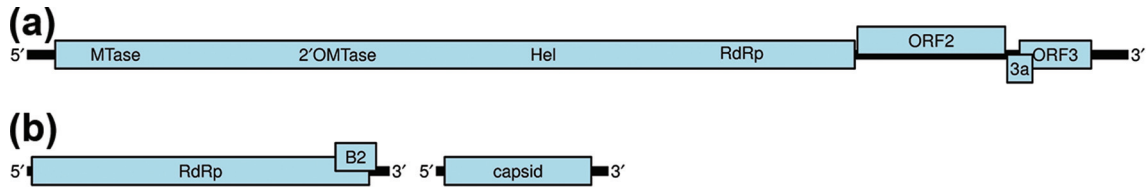


Fig. 1. Predicted genomic organization of (a) Uxmal virus and (b) Mayapan virus. The predicted viral methyltransferase, 2'-O-methyltransferase, helicase and RNA-dependent RNA polymerase domains located within ORF 1 of Uxmal virus are denoted as MTase, 2'OMTase, Hel and RdRp, respectively.

Two non-overlapping MYPV sequences were recovered, indicating that the virus has a bipartite genome (GenBank accession nos MH719096 and MH719097; Fig. 1b). A less likely explanation is that we recovered genome segments from two distinct but related viruses. RNA1 consists of 3045 nt and is most closely related to the corresponding region of Hubei noda-like virus 5 (82.3 % identity with 98.6 % coverage), an unclassified *Nodaviridae*-like virus detected in spiders and insects in China [23]. The detection of Hubei

noda-like virus 5 in spiders suggests that the virus naturally infects spiders or was acquired from recently eaten insects. RNA1 of MYPV is predicted to encode a large ORF flanked by 5' and 3' UTRs of 39 and 171 nt, respectively. The putative protein consists of 944 residues and is most closely related to the corresponding translation product of Hubei noda-like virus 5 (90.8 % identity and 95.2 % similarity, with 97.6 % coverage), followed by mosinovirus (43.2 % identity and 60.7 % similarity, with 96.3 % coverage). Domains

(a)	
Tanay virus (KF425261)	MN ST EAI C IF-----V CL TVVALIV I INKLFYF L EHLIVENGSK THD -SR
Goutanap virus (KF588035)	M CF VVSI I VVLFV GF -----L LS LIT DC N-----E FD CT S AYERY YG
Biggievirus (MF281708)	--MFY CL L ILL -----V CF IVY LL Y-PSK PS YV-----PA KE K-Y G
Culex negev-like virus (MF176277)	MFY WS V LL -----I CF VVY LL -Y PS KPDY V -----SA KE K-Y G
Uxmal virus	M N LQTTLD F L AY -----L ALL WYV S FV F N L I H S L L E ---R K NG S AA Y W-Y G
Wallerfield virus (KF042857)	M N IV D CV T I IL W S H F -----L IR LY LS LR K F Y AFV Y I Y -----N Y G PA AY W -Y G
Dezidougou virus (JQ675604)	M IL T I CV V V L -----F LY LS VD CV Y H T Y LL -----R K NG Y AS Y W-Y G
Santana virus (JQ675606)	M S IV ILL PL LL TY L I Y LV Y H PR FI Y DL T I I Y L IS Y LI IK FID S IV N H L TR ND GS AS Y W - H G
Bustos virus (LC103139)	M F PL LA AV V LL Q SE S ---Y IL Q IF G R I CL LFY IV D CV F N RI QL F LL ---R Y H GP Q AY W-Y G
Biratnagar virus (KX518758)	M Y PL IL AV V LL Q SE F ---P LY Q V F GR V CL CL CI VD CI I ER I Y L FL ---R H H GP Q AY R-Y G
Tanay virus (KF425261)	RS Q GD L F Q NR RS S RES N-----R DS RY SE EN RY Q D Q W Q D ---
Goutanap virus (KF588035)	CT SL T -Y G SE D CC CP PP W LL Q D---S AST CP ARG ST TT D S ST QT -
Biggievirus (MF281708)	CT AST-Y G AE G IC P ST AW LL Q L AD AE ASH Y NG ND R S ATT SS AE TS
Culex negev-like virus (MF176277)	CP AAT-Y G SE G CL P ST AW LL Q L AD AE ASH D IGH DC P ATT ST ETT
Uxmal virus	CE SA H -Y GL PE CT CP-----Q E AY Y Q ES L G SD P -----
Wallerfield virus (KF042857)	CE AS C - CC SP D CL CP -----P H SH CE GE AD PT S G IG ES---
Dezidougou virus (JQ675604)	CE AK N -Y G DE G CV CP SN-----K D PP C -----
Santana virus (JQ675606)	CT AE F -Y E AED CV CP-----K A EE IS V LR H G IE-----
Bustos virus (LC103139)	CS AC FD CP SC GY CC P-----R E K S GE T S ER I RE -----
Biratnagar virus (KX518758)	CS PH ND V S SC CC CC CP -----F E K S CE S GE E FR E -----
(b)	
Hubei noda-like virus 5 (KX883080)	M SL T KN Q S EL V AA V Q R Q NY K T AE AL R R LV TD H L ARG KV LE D RL AK S GL R K PL AP D H Y KK N
Hubei noda-like virus 5 (KX883161)	M SL T KN Q S EL V AA V Q R Q NY K T AE AL R R LV TD H L ARG KV LE D RL AK S GL R K PL AP D H Y KK N
Mayapan virus	M SL T KN Q S EL V AA V Q R Q NY K T AE AL R R LV TD H L ARG KV LE D RL T K AG L R T PL G PG Q V Y KN
	*****:****:*:*:*
Hubei noda-like virus 5 (KX883080)	ER P V RM V N EM TE LL TV HP DL P V G AD Q PA V P Q Y D K CD P N WD PP K SP S GP P VP HE
Hubei noda-like virus 5 (KX883161)	ER P V RM V N EM TE LL TV HP DL P V G AD Q PA V P Q Y D K CD P N WD PP K SP S GP P VP HE
Mayapan virus	DR S I WR V DK M T DM L TV HP DL PD G AD PP AV P Q Y DR CD P N WD PP K SP S GP S PT PH -
	:*:*:*:*:*:***** ** * * * * * . * * * * * . * * *

Fig. 2. Alignment of (a) the translation products potentially encoded by ORF3a of Uxmal virus and selected related viruses and (b) deduced amino acid sequences of the putative B2 proteins of Mayapan virus and Hubei noda-like virus 5.

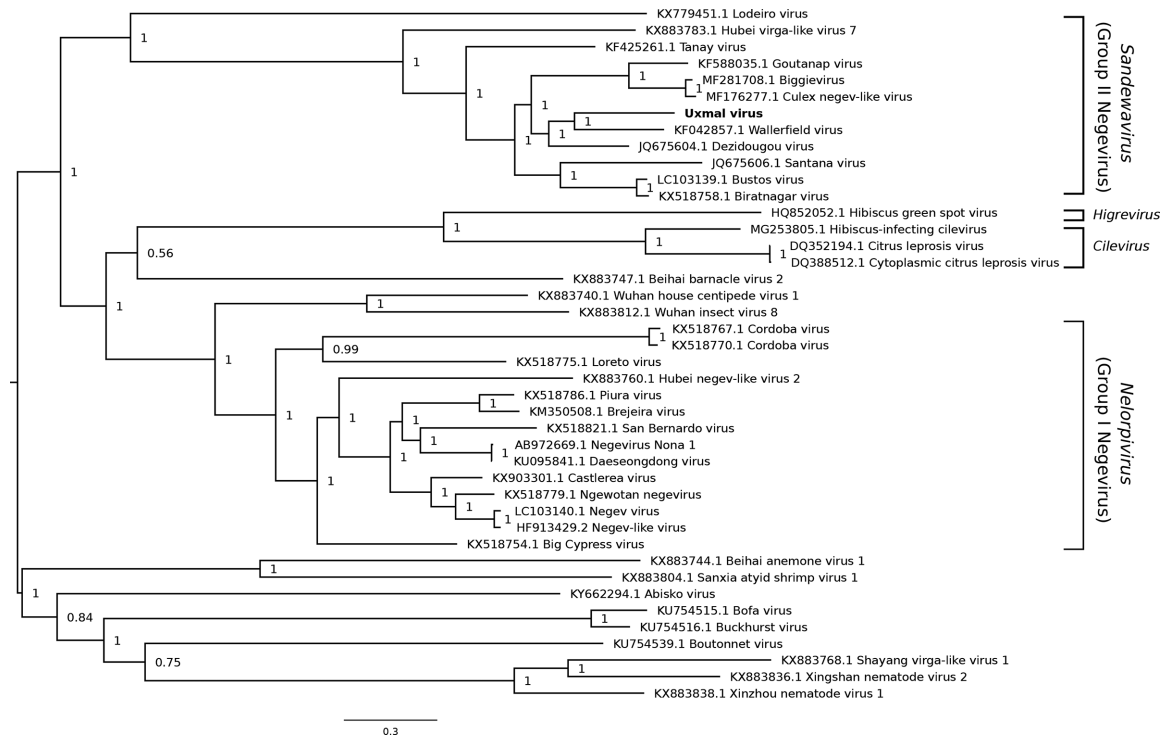


Fig. 3. Phylogenetic tree for Uxmal virus and selected related reference and non-reference sequences. The amino acid sequences of the ORF encoding the RdRp were aligned using MUSCLE [60]. A maximum-likelihood phylogenetic tree was estimated using the Bayesian Markov chain Monte Carlo method implemented in MrBayes version 3.2.6 [61], sampling across the default set of fixed amino acid rate matrices with one million generations and discarding the first 25% as burn-in. The figure was produced using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). The tree is midpoint-rooted and selected nodes are labelled with posterior probability values. GenBank accession numbers are indicated next to virus names.

characteristic of RdRps (cd01699) and dephospho-coenzyme A kinases (cd02022) are present at residues 469 to 685 and 283 to 349, respectively. A short ORF (nt 2591 to 2932) overlapping the 3' end of the RdRp ORF is also conserved in Hubei noda-like virus 5 and might encode a B2 protein of 113 aa (Figs 1b and 2b).

RNA2 of MYPV consists of 1446 nt and is most closely related to the corresponding region of Hubei noda-like virus 11 (69.2% identity and 12.1% coverage), an unclassified *Nodaviridae*-like virus detected in spiders in China [23]. The sequence is predicted to encode a single ORF flanked by 5' and 3' UTRs of 71 and 142 nt, respectively. The putative protein consists of 410 residues and is most closely related to a corresponding translation product of Hubei noda-like virus 11 (44.5% identity and 58.8% similarity, with 89.6% coverage). A domain homologous to the alphanoda-like capsid protein is present at residues 55 to 364.

Phylogenetic trees were constructed with Bayesian methods using the deduced amino acid sequences of the RdRp and capsid protein of MYPV and the corresponding regions of related viruses (Fig. 4). In the analysis performed using RdRp sequences, it was demonstrated that MYPV is most closely related to Hubei noda-like virus 5. The posterior support for this topological arrangement is 1.0. MYPV and

Hubei noda-like virus 5 belong to a clade that includes selected other unclassified *Nodaviridae*-like viruses. A second major clade consists of additional unclassified *Nodaviridae*-like viruses, along with viruses in the genus *Betanodavirus*. Notably, the RdRp of MYPV appeared to be more closely related to RdRps of betanodaviruses than to RdRps of alphanodaviruses such as Flock House, black beetle and Nodamura viruses. Alphanodaviruses were not included in the tree because their RdRps have limited (about 25%) amino acid identity (with 70–80% coverage) to that of MYPV. In contrast, in the analysis performed using capsid protein sequences, MYPV was positioned basally to viruses in the genus *Alphanodavirus*. The alphanodavirus capsid protein is structurally very different from the betanodavirus capsid protein [30], which is why betanodaviruses do not appear in the capsid protein tree.

Recognized viruses

CxFV was isolated from a pool of male *Cx. quinquefasciatus* collected in Tixkokob 2008 (Table 2). C6/36 cells were permissive to CxFV replication, but the virus was unable to replicate in any other cell line (Table 3). The genome of the CxFV isolate (designated CxFV-T1123) was fully sequenced (GenBank accession no. MH719098) and shown to consist of 10 837 nt. BLAST analysis revealed that CxFV-T1123 has

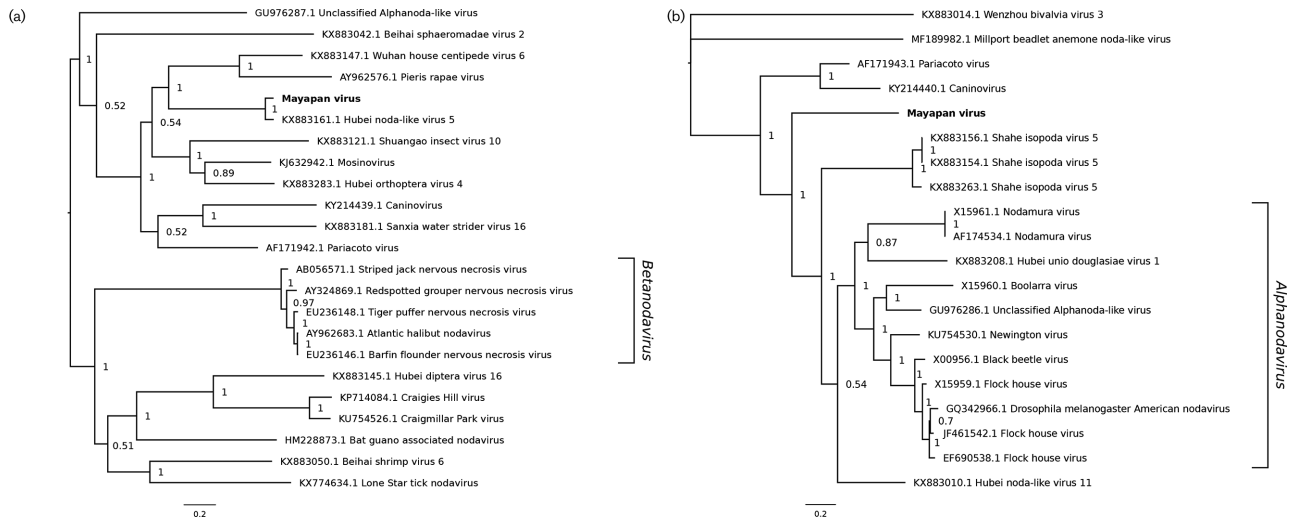


Fig. 4. Phylogenetic tree for Mayapan virus and selected related reference and non-reference sequences. Amino acid sequences of the ORF encoding the (a) RdRp and (b) capsid protein were aligned using MUSCLE [60]. A maximum-likelihood phylogenetic tree was estimated using the Bayesian Markov chain Monte Carlo method implemented in MrBayes version 3.2.6 [61], sampling across the default set of fixed amino acid rate matrices with one million generations and discarding the first 25 % as burn-in. The figure was produced using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). The tree is midpoint-rooted and selected nodes are labelled with posterior probability values. GenBank accession numbers are indicated next to virus names.

the greatest (99.8 %) nucleotide identity with CxFV-Mex07, an isolate of CxFV recovered from *Cx. quinquefasciatus* in Tixkokob in 2007 [31]. HOUV was isolated from two pools of female mosquitoes collected in Merida (Table 2). One isolate was recovered from *Cx. quinquefasciatus* and the other from *Ae. taeniorhynchus*. HOUV replicated in C6/36 and CT cells, with CPE only being observed in C6/36 cells (Table 3). The entire genome of one isolate (designated HOUV-M742) was fully sequenced and shown to consist of 20 129 nt, excluding the polyadenylate tail (GenBank accession number MH719099). BLAST analysis revealed that HOUV-M742 has the greatest (99.2 %) nucleotide identity with V3872, an isolate of HOUV recovered from *Cx. quinquefasciatus* in Houston in 2004 [32].

DISCUSSION

We report the isolation and identification of two novel viruses and two recognized viruses in mosquitoes from the Yucatan Peninsula of Mexico. Sequences corresponding to a putative novel rhabdovirus (Merida virus) were identified in mosquitoes in the same area in an earlier metagenomics study, but an isolate was not recovered by virus isolation in C6/36 cells, even though the cell line supports the replication of numerous mosquito-associated viruses [33]. Other research groups have also discovered novel virus genomes in mosquitoes by NGS but could not recover isolates from inoculated C6/36 cells [13, 34]. The inability to culture some mosquito-associated viruses in C6/36 cells could be because these viruses are species-specific or have only adapted to a few mosquito species and lack the capacity to replicate in *Ae. albopictus* cells. The experimental approach used in this

study was designed to avoid the situation in which novel virus-like sequences were identified but isolates were not recovered. To this end, the metagenomics analysis was preceded by virus isolation experiments and therefore an isolate was already on hand for every virus that was identified. Our experimental approach also allowed for the detection of both cytopathic and non-cytopathic viruses because NGS was not restricted to cultures that exhibited CPE.

Our data demonstrate that UXMV should be classified in the taxon *Negevirus*. This newly proposed monophyletic taxon is composed of viruses detected exclusively in mosquitoes and sandflies, with most being discovered through the use of metagenomics [8, 12, 14–20]. Negevirus are assumed to have vertebrate-incompetent replication phenotypes because they cannot replicate in suckling mice or any vertebrate cell lines that have been tested. The most comprehensive *in vitro* host range experiments were performed by Vasilakis and colleagues, who assessed the replicative abilities of six negevirus in three vertebrate cell lines [baby hamster kidney (BHK-21), human embryonic kidney (HEK-293) and Vero cells] and five mosquito cell lines [*Ae. albopictus* (C6/36 and C7/10), *Anopheles albimanus*, *An. gambiae* and *Cx. tarsalis* cells], in addition to *Drosophila melanogaster* and *Phlebotomus papatasi* cells [12]. All viruses replicated in every mosquito cell line, but none replicated in any other cell line. We provide additional evidence that negevirus lack the capacity to replicate in vertebrate cells because UXMV could not replicate in any of the six vertebrate cell lines tested. A notable difference between the two studies is that all previously tested negevirus replicated in *Anopheles* cells, while UXMV could not, suggesting

that the mosquito host range of UXMV is not as broad as that of other negevirus.

Negevirus have a ubiquitous geographical distribution, having been reported in every continent with the exception of Antarctica [8, 12, 14–21]. The first negevirus to be identified in Mexico was Piura virus after its isolation from *Aedes*, *Culex*, *Mansonia*, *Psorophora* and *Wyeomyia* spp. mosquitoes in the southern state of Chiapas [20]. Piura virus belongs to the *Nelorpivirus* clade. UXMV is the only *Sandewavirus* clade virus known to occur in Mexico.

MYPV is a *Nodaviridae*-like virus that was recovered from *Ps. ferox*. Two pools of female *Ps. ferox* were tested and both yielded isolates, indicating that MYPV commonly infects this species in the study area. However, the sample size was small and additional females need to be tested to accurately determine the prevalence of MYPV in *Ps. ferox*. Adult males and immatures should also be tested to provide some indication of whether MYPV is maintained in nature by vertical transmission, but none are available in our archived collections. All vertebrate cell lines were refractory to virus replication, indicating that MYPV has a vertebrate-incompetent replication phenotype. The ability of *Aedes*, but not *Anopheles* or *Culex*, cells to support MYPV replication indicates that the virus has a narrow mosquito host range. C6/36 cells have a dysfunctional RNAi response and it remains to be determined whether MYPV can establish an infection in *Aedes* cells that possess a functional RNAi pathway [35, 36]. Recent studies have also revealed that CT cells possess a dysfunctional RNAi response [37].

The isolation of CxFV from *Cx. quinquefasciatus* was not unexpected because we have previously reported a high prevalence of CxFV in mosquitoes of this species in the Yucatan Peninsula of Mexico [31, 38, 39]. CxFV is assumed to have an insect-restricted host range because it has been isolated exclusively from mosquitoes and cannot replicate in any vertebrate cell lines that have been tested [40–42]. Our study provides additional evidence that the virus is insect-specific, with several previously untested vertebrate cell lines being refractory to infection. The inability of CT cells to support CxFV infection was not unexpected because the cell line is persistently infected with Calbertado virus, a closely related insect-specific flavivirus, which is potentially suppressing the replication of CxFV by superinfection exclusion [43].

HOUV was originally isolated from *Ae. albopictus* and *Cx. quinquefasciatus* in the United States and was then isolated from *Cx. quinquefasciatus* in Xkaladzonot, a rural town in the Yucatan Peninsula of Mexico [32, 44]. HOUV is a variant of the species Alphamesonivirus-1, which also includes Cavally virus from Cote d'Ivoire, Nam Dinh virus from Vietnam and China, and Ngewotan virus from Indonesia and Australia [45–49]. Alphamesonivirus-1 belongs to the recently established family *Mesoniviridae*, which consists exclusively of viruses assumed to have insect-restricted host ranges [32, 50]. The findings from our *in vitro* host range

experiments support this assumption and indicate that the mosquito host range of HOUV is restricted to *Culicinae* mosquitoes because it replicated in *Aedes* and *Culex*, but not *Anopheles*, cell cultures.

In summary, we report the isolation and sequence characterization of both novel and recognized RNA viruses from mosquitoes collected in the Yucatan Peninsula of Mexico. None of the viruses could replicate in any of the vertebrate cell lines tested, suggesting that they all possess insect-restricted host ranges. Despite their apparent vertebrate-incompetent replication phenotypes, further investigation into these viruses is warranted because coinfection experiments have revealed that insect-specific viruses can alter the replication and transmission of pathogenic viruses in mosquitoes [51–54]. Viral sequences were not recovered from some C6/36 cell cultures that exhibited CPE, indicating that the metagenomics analysis failed to detect some viruses. One explanation for this observation is that these viruses are highly divergent from all known viruses and their genomes have no significant identity with any sequences in the GenBank database.

METHODS

Cell culture

The cell lines used in this study were as follows: *Aedes albopictus* (C6/36), African green monkey kidney (Vero), *Anopheles gambiae* (Sua 4.0), baby hamster kidney (BSR-T7/5), *Culex tarsalis* (CT), duck embryonic fibroblast (DEF), human epithelial (HeLa), murine microglia, rhesus macaque (LLC-MK2), *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High Five) cells. Cells were cultured in Dulbecco's modified Eagle's medium (all mammalian cells), Eagle's minimum essential medium (DEF cells), Express Five medium (High Five cells), Liebovitz L15 medium (C6/36 cells), Schneider's *Drosophila* medium (CT and Sua 4.0 cells) and SF-900 medium (Sf9 cells) (all cell culture media were obtained from Thermo Fisher Scientific, Massachusetts, USA). All media were supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 units ml⁻¹ of penicillin and 100 µg ml⁻¹ of streptomycin, with the exception of the Express Five and SF-900 media, which were serum-free. Vertebrate cells were cultured at 37 °C with 5% CO₂ and invertebrate cells were cultured at 28 °C.

Mosquitoes

Mosquitoes were collected at study sites in Merida, Tixkokob and Tzucacab in the Yucatan Peninsula of Mexico. Detailed descriptions of the study sites and the protocols used for the collection, identification and homogenization of mosquitoes have been provided elsewhere [31, 38, 55]. Briefly, collections were made in 2007 and 2008 using Mosquito Magnets Pro-Liberty (American Biophysics Corp., Rhode Island, USA) baited with propane and octenol. Mosquitoes were transported alive to the laboratory, frozen at –80 °C and sorted into pools of up to 50 according to species, sex, date of collection and study site. Mosquitoes were

placed in polypropylene, round-bottom 5 ml tubes with 1.8 ml of CO₂-independent cell culture medium (Thermo Fisher Scientific) and four 4.5mm diameter copper-clad steel beads (BB-calibre airgun shot) and then homogenized by vortexing for 30 s. The homogenates were centrifuged (12 000 g, 10 min, 4 °C) and supernatants were collected.

Virus isolation in C6/36 cell culture

An aliquot (100 µl) of each supernatant was added to 2 ml of Liebovitz L15 medium supplemented with 2% foetal bovine serum, L-glutamine, penicillin and streptomycin. Samples were filtered and inoculated onto subconfluent monolayers of C6/36 cells in 25 cm² flasks. Another 4 ml of the aforementioned medium was added to each flask and the cells were incubated at 28 °C and monitored regularly for CPE. Supernatants were collected when 40–60% of the cell monolayer displayed CPE. If CPE was not observed, supernatants were collected at 7 days post-inoculation. A second blind passage was performed and then the cell monolayers and supernatants were harvested.

Library preparation

Total RNA was extracted from all second-passage C6/36 cell cultures using Trizol Reagent (Thermo Fisher Scientific), fragmented using RNase III (Thermo Fisher Scientific) and assessed for quality using an Agilent 2100 Bioanalyser (Agilent, CA, USA). Libraries were constructed using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) and bar-coded using the Ion Xpress RNA-Seq Barcode 1–16 kit (Thermo Fisher Scientific). Libraries were assessed for quality and analysed at the Genomic Technologies Facility at Iowa State University using an Ion Proton Sequencer (Thermo Fisher Scientific).

Bioinformatics

Ion-proton reads were analysed using the FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) to remove bar-codes and low-quality ends (Phred quality score ≥ 33). Duplicate reads were identified and removed using Cdhit-454 (<http://weizhongli-lab.org/cd-hit/>). Host sequences were depleted by mapping the remaining reads to *Ae. aegypti*, *Ae. albopictus*, *An. gambiae* and/or *Cx. quinquefasciatus* genomes using Bowtie 2 [56]. Unmapped reads were analysed using the sortMeRNA program to remove ribosomal RNA-related reads [57]. The remaining reads were subjected to *de novo* SPAdes assembly (version 3.5.0) [58]. Contigs were aligned by BLASTN, BLASTX and TBLASTX to the NCBI nucleotide database (downloaded August 2017) using an e-value of $<10^{-5}$. Unaligned contigs were translated into all six reading frames and aligned to the HMM-FRAME, pfam, CDD and TIGRFAM databases (downloaded August 2017). The data were transformed by Python programming (<https://www.python.org/>).

RT-PCR and Sanger sequencing

RT-PCR and Sanger sequencing were performed to verify the virus sequences identified by NGS and to close gaps between contigs. RT-PCR was also used to test cell cultures

for the presence of viral RNA in the host range experiments. Complementary DNAs were generated using Superscript III reverse transcriptase (Thermo Fisher Scientific) and PCRs were performed using high-fidelity *Taq* polymerase (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Primers were designed from the sequences generated by NGS and are available upon request. RT-PCR products were purified using the PureLink gel extraction kit (Thermo Fisher Scientific) and sequenced using a 3730×1 DNA sequencer (Applied Biosystems, CA, USA).

5' and 3' rapid amplification of cDNA ends

The 5' and 3' ends of virus genomes were identified using 5' and 3' rapid amplification of cDNA ends, respectively. Briefly, a DNA adaptor (5'-rApp/TGGAATTCTCGGG TGCCAAGGT/ddC-3') was ligated to the viral genomic and anti-genomic RNAs using T4 RNA ligase (New England BioLabs, MA, USA). Complementary cDNAs were created using SuperScript III (Thermo Fisher Scientific) and an adapter-specific primer. PCRs were performed using adapter- and gene-specific primers, and amplicons were purified and subjected to Sanger sequencing.

Sequence alignments and identification of conserved domains

The nucleotide and deduced amino acid sequences of each virus were compared to other sequences in the GenBank database by the application of BLASTN and BLASTP, respectively [59]. Percentage amino acid identities and similarities were calculated using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Conserved domains were identified using BLASTP, InterProScan (<http://www.ebi.ac.uk/interpro/search/sequence-search>) and HHpred (PMID 15980461). Potential N-linked glycosylation sites were identified using the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) with the consensus sequence defined as Asn-X-Ser/Thr (where X is not Pro) in the context of specific surrounding sequences.

In vitro host range experiments

The *in vitro* host ranges of all novel and recognized viruses were assessed using 11 cell lines. Briefly, cell monolayers approaching confluency in 25 cm² flasks were inoculated with 50 µl of virus-containing supernatant and incubated for 7 days or until 40–60% of the cell monolayer displayed CPE. A total of five passages were performed, with supernatants being collected after each passage and tested by RT-PCR for viral RNA. A cell line was considered to support virus replication if RT-PCR products were detected after every passage.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

No human subjects or vertebrate animals were used in this study

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