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Discovery of a novel *Tymoviridae*-Like virus in mosquitoes from Mexico

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Abstract

A novel *Tymoviridae*-like virus, designated Ek Balam virus, was isolated from male *Culex quinquefasciatus* mosquitoes collected in Yucatan, Mexico. The genome was fully sequenced and shown to have no more than 69% nt sequence identity to its closest known relative. Mosquito cells were permissive to Ek Balam virus replication, but mammalian and avian cells were refractory, suggesting that vertebrates are not involved in the maintenance of the virus in nature.

Keywords

metagenomics; virus discovery; RNA-seq; mosquito; *Tymoviridae*; Mexico

All viruses currently classified into as members of the family *Tymoviridae* are plant viruses, and some are major agricultural pathogens (1, 2). The family includes three genera: *Maculavirus*, *Marafivirus* and *Tymovirus*. Viruses in the genera *Marafivirus* and *Tymovirus* are transmitted by leafhoppers and beetles, respectively, but no vectors have been identified for viruses in the genus *Maculavirus*. Family members contain a positive-sense, single-stranded RNA genome of 6.0 to 7.5 kb. Genomes are capped at the 5' end, often possess a tRNA structure at the 3' end and, except for viruses in the genus *Tymovirus*, are polyadenylated. The number of open reading frames (ORFs) varies according to genus and species. Viruses in the genus *Maculavirus* have four ORFs, and these encode a replicase, coat protein and two small proline-rich proteins, p31 and p16 (2, 3).

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The research reported here did not involve the use of human subjects or vertebrate animals.

Ethical statement

This study represents original work that has not been submitted to any other journal for publication. The authors have no conflict of interest to declare. No human or animal ethics approval was required for the completion of this study.

Mosquitoes are not recognized vectors of viruses in the family *Tymoviridae*, although a novel *Tymoviridae-like* virus, designated Culex-originated Tymoviridae-like virus (CuTLV), was recently isolated from *Culex* spp. mosquitoes in China (4). The genomic organization of CuTLV is similar to that of viruses in the genus *Maculavirus*, although notable differences were also described, including the absence of a polyadenylated tail and an ORF encoding p32. CuTLV has not yet been classified by the International Committee on Taxonomy of Viruses, and it remains to be seen whether it will be assigned to the genus *Maculavirus* or to a new genus or family. We report the discovery of a second novel *Tymoviridae-like* virus that naturally infects mosquitoes. The novel virus, designated Ek Balam virus (EkBV), was detected in male *Culex quinquefasciatus* collected in 2007 in the rural town of Tixkokob in Yucatan, Mexico. The virus was named after an archeological ruin in Yucatan. Descriptions of study sites and protocols for the collection, identification and homogenization of mosquitoes are provided elsewhere (5, 6). EkBV was isolated after two blind passages in *Aedes albopictus* (C6/36) cells, and its genome was fully sequenced by Ion Proton sequencing, reverse transcription polymerase chain reaction, Sanger sequencing, and 5' and 3' rapid amplification of cDNA ends as described previously (7). The genome of EkBV consists of 6516 nt (GenBank accession no. [MH822863](#)). The sequence is most closely related to the genome sequence of CuTLV (68.3% nt sequence identity with 29.6% coverage), followed by bat tymo-like virus (65.8% nt sequence identity with 41.5% coverage), an unclassified *Tymoviridae-like* virus detected in guano of *Myotis yumanensis* (GenBank accession nos. [NC_030844.1](#) and [KX580887.1](#); Alex Greninger, University of Washington, Seattle, WA, USA, personal communication). It is not known whether bat tymo-like virus replicates in bats or was acquired through the consumption of virus-infected material (i.e., insects), but the latter appears more likely because the virus is most closely related to EkBV and CuTLV. The next-closest relatives to EkBV are viruses in the genus *Maculavirus*. The genome of EkBV has a high cytosine and low guanine content (A, 23.0%; C, 31.7%, G, 18.6%; T, 26.7%), as has been reported for CuTLV and recognized members of the family *Tymoviridae* (1,3, 4, 8). The genome lacks a polyadenylate tail, and its 5' and 3' untranslated regions consist of 144 nt and 433 nt, respectively. We could not provide any convincing evidence of a tRNA-like structure in the 3' UTR (data not shown). The genome does not possess a classical “tymobox” or “marafibox”, conserved subgenomic promoter sequences characteristic of tymoviruses and marafiviruses, respectively (1, 2).

The genome of EkBV is predicted to contain at least two major ORFs (Fig. 1). The first ORF encodes a putative replicase of 1724 residues that is most closely related to the corresponding translation product of CuTLV (50.9% identity and 64.1% similarity, with 100% coverage). Predicted viral methyltransferase (pfam01660), tymovirus endopeptidase (pfam05381), helicase (pfam01443) and RdRp (pfam00978) domains are present at residues 38 to 318, 683 to 770, 870 to 1102 and 1400 to 1591, respectively. The second ORF encodes a putative coat protein of 243 residues that is most closely related to the corresponding translation product of CuTLV (61.3% identity and 75.4% similarity, with 100% coverage). A predicted tymovirus coat protein domain (pfam00983) is present at residues 113 to 196. The ORFs are separated by a 32-nt intergenic region.

A phylogenetic tree was constructed using Bayesian methods based on the deduced amino acid sequence of the replicase of EkBV and the corresponding regions of some related

sequences (Fig. 2). EkBV is most closely related phylogenetically to CuTLV, and the posterior support for this topological arrangement is 0.97. EkBV, CuTLV and bat tymo-like virus belong to a clade that is basal to recognized members of the family *Tymoviridae*. Viruses in this family separate into three distinct genus-specific clades.

The *in vitro* host range of EkBV was determined by assessing its replicative ability in cell lines of avian, mammalian and mosquito origin. Six cell lines were tested: African green monkey kidney (Vero), *Anopheles gambiae* (Sua 4.0), baby hamster kidney (BSR-T7/5), C6/36, *Culex tarsalis* (CT) and duck embryonic fibroblast (DEF) cells. A cell line was considered to support EkBV replication if viral RNA was detected by RT-PCR after five passages. All mosquito cell lines were permissive to replication, indicating that the virus has a broad mosquito host range. A cytopathic effect was not observed in any mosquito cell cultures. All vertebrate cell lines were refractory to virus infection, consistent with a vertebrate replication-incompetent phenotype. CuTLV is also assumed to have a vertebrate-replication-incompetent phenotype because it cannot replicate in Vero or baby hamster kidney (BHK-21) cells (4).

The inability of EkBV to replicate in vertebrate cells and its detection in male mosquitoes suggests that the virus is not maintained in nature in a mosquito-vertebrate transmission cycle. Our findings indicate that EkBV is more likely to be maintained by vertical (i.e., transovarial) or horizontal (i.e., venereal) transmission among insect hosts. Alternatively, the virus could be maintained by mechanical (i.e., *per os*) transmission. Experimental infections are needed to determine whether EkBV is maintained in nature by direct mosquito-to-mosquito transmission and whether it is capable of infecting plants. The detection of EkBV in field-collected mosquitoes and C6/36 cells after five passages suggests that the virus replicates in mosquitoes and is not a surface or gut contaminant acquired after coming in contact with or feeding upon infectious material.

In conclusion, we report the isolation of a novel *Tymoviridae*-like virus from mosquitoes in Mexico and add to the rapidly increasing number of newly discovered viruses. Based on the genetic distance between EkBV, its closest known relative (CuTLV) and recognized members of the family *Tymoviridae*, we propose that EkBV and CuTLV are members of two distinct species that should be assigned to the same, yet-to-be-established genus. It remains to be determined whether these viruses will be classified as members of the family *Tymoviridae* or of a new family.

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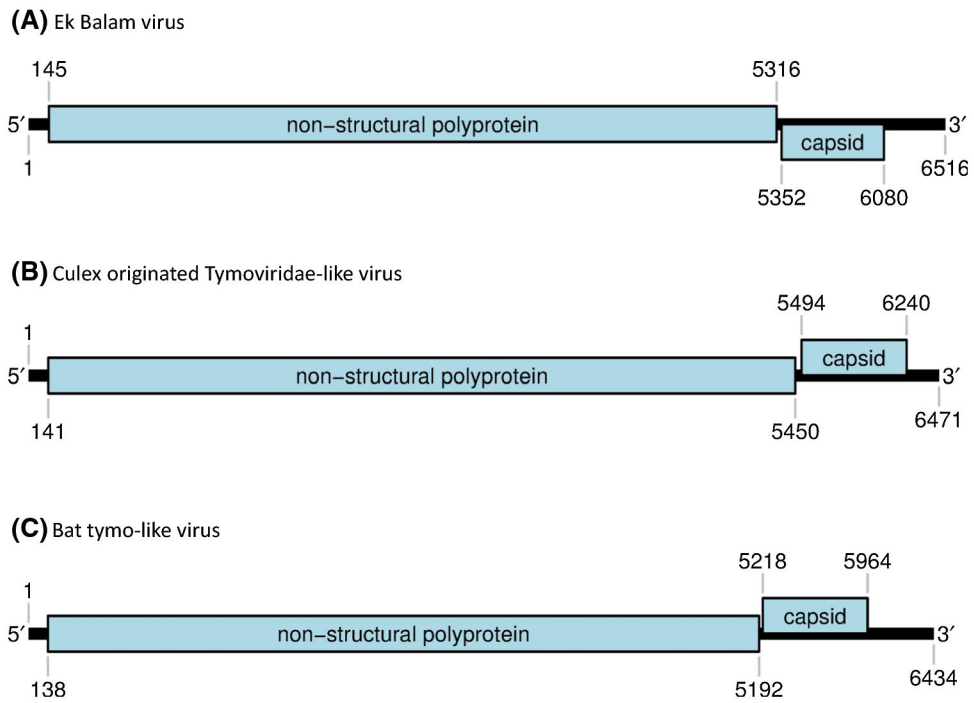


Fig. 1. Genomic organization of (A) Ek Balam virus, (B) Culex originated Tymoviridae-like virus and (C) bat tymo-like virus

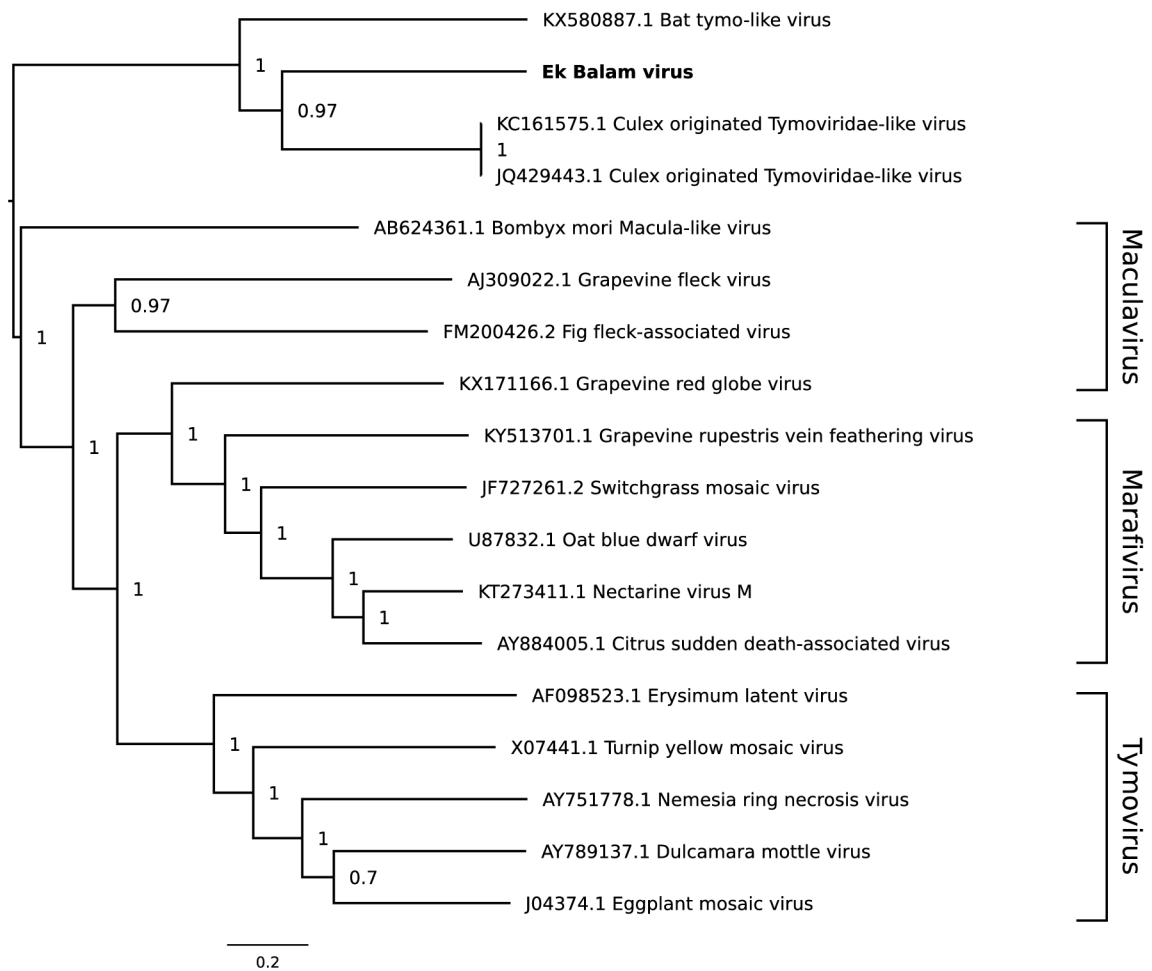


Fig. 2. Phylogenetic tree for EkBV and selected related reference and non-reference sequences. Amino acid sequences of the open reading frame encoding the replicase were aligned using MUSCLE (9). A maximum-likelihood phylogenetic tree was constructed using the Bayesian Markov chain Monte Carlo method implemented in MrBayes version 3.2.6 (10), sampling across the default set of fixed amino acid rate matrices with 1,000,000 generations, discarding the first 25% as burn-in. The figure was produced using FigTree (available at <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 shown next to virus names.