



HAL
open science

Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes

Yasutsugu Suzuki, Lionel A Frangeul, Laura B. Dickson, Hervé Blanc, Yann Verdier, Joelle Vinh, Louis Lambrechts, Maria-Carla P Saleh

► **To cite this version:**

Yasutsugu Suzuki, Lionel A Frangeul, Laura B. Dickson, Hervé Blanc, Yann Verdier, et al.. Uncovering the Repertoire of Endogenous Flaviviral Elements in Aedes Mosquito Genomes. *Journal of Virology*, 2017, 91 (15), pp.e00571. 10.1128/JVI.00571-17 . hal-01636504

HAL Id: hal-01636504

<https://hal.science/hal-01636504>

Submitted on 17 Nov 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.



L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution 4.0 International License



Uncovering the Repertoire of Endogenous Flaviviral Elements in *Aedes* Mosquito Genomes

Yasutsugu Suzuki,^a Lionel Frangeul,^a Laura B. Dickson,^b Hervé Blanc,^a Yann Verdier,^c Joelle Vinh,^c  Louis Lambrechts,^b  Maria-Carla Saleh^a

Institut Pasteur, Viruses and RNA Interference Unit, CNRS Unité Mixte de Recherche 3569, Paris, France^a; Institut Pasteur, Insect-Virus Interactions Group, Department of Genomes and Genetics, CNRS Unité de Recherche Associée 3012, Paris, France^b; Spectrométrie de Masse Biologique et Protéomique, CNRS Unité de Service et de Recherche 3149, École Supérieure de Physique et de Chimie Industrielles Paris, Paris, France^c

ABSTRACT Endogenous viral elements derived from nonretroviral RNA viruses have been described in various animal genomes. Whether they have a biological function, such as host immune protection against related viruses, is a field of intense study. Here, we investigated the repertoire of endogenous flaviviral elements (EFVEs) in *Aedes* mosquitoes, the vectors of arboviruses such as dengue and chikungunya viruses. Previous studies identified three EFVEs from *Aedes albopictus* cell lines and one from *Aedes aegypti* cell lines. However, an in-depth characterization of EFVEs in wild-type mosquito populations and individual mosquitoes *in vivo* has not been performed. We detected the full-length DNA sequence of the previously described EFVEs and their respective transcripts in several *A. albopictus* and *A. aegypti* populations from geographically distinct areas. However, EFVE-derived proteins were not detected by mass spectrometry. Using deep sequencing, we detected the production of PIWI-interacting RNA-like small RNAs, in an antisense orientation, targeting the EFVEs and their flanking regions *in vivo*. The EFVEs were integrated in repetitive regions of the mosquito genomes, and their flanking sequences varied among mosquito populations. We bioinformatically predicted several new EFVEs from a Vietnamese *A. albopictus* population and observed variation in the occurrence of those elements among mosquitoes. Phylogenetic analysis of an *A. aegypti* EFVE suggested that it integrated prior to the global expansion of the species and subsequently diverged among and within populations. The findings of this study together reveal the substantial structural and nucleotide diversity of flaviviral integrations in *Aedes* genomes. Unraveling this diversity will help to elucidate the potential biological function of these EFVEs.

IMPORTANCE Endogenous viral elements (EVEs) are whole or partial viral sequences integrated in host genomes. Interestingly, some EVEs have important functions for host fitness and antiviral defense. Because mosquitoes also have EVEs in their genomes, characterizing these EVEs is a prerequisite for their potential use to manipulate the mosquito antiviral response. In the study described here, we focused on EVEs related to the *Flavivirus* genus, to which dengue and Zika viruses belong, in individual *Aedes* mosquitoes from geographically distinct areas. We show the existence *in vivo* of flaviviral EVEs previously identified in mosquito cell lines, and we detected new ones. We show that EVEs have evolved differently in each mosquito population. They produce transcripts and small RNAs but not proteins, suggesting a function at the RNA level. Our study uncovers the diverse repertoire of flaviviral EVEs in *Aedes* mosquito populations and contributes to an understanding of their role in the host antiviral system.

Received 4 April 2017 Accepted 12 May 2017
Accepted manuscript posted online 24 May 2017

Citation Suzuki Y, Frangeul L, Dickson LB, Blanc H, Verdier Y, Vinh J, Lambrechts L, Saleh M-C. 2017. Uncovering the repertoire of endogenous flaviviral elements in *Aedes* mosquito genomes. *J Virol* 91:e00571-17. <https://doi.org/10.1128/JVI.00571-17>.

Editor Julie K. Pfeiffer, University of Texas Southwestern Medical Center

Copyright © 2017 Suzuki et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Maria-Carla Saleh, carla.saleh@pasteur.fr.

KEYWORDS ancient flaviviral infections, endogenous viral elements, mosquito-borne viruses, mosquito genome, viral infectious diseases

Endogenous viral elements (EVEs), also known as viral fossils, are whole or partial viral sequences integrated in host genomes (1). When viral DNA integration occurs in the germ line, it can be inherited and retained in the host genome as evidence of ancient viral infections. Retrovirus-derived EVEs are the best-known examples since retroviruses actively integrate their DNA into the host genome as part of their life cycle during infection. However, single-stranded DNA virus-derived elements have been detected in plants (2), and more recently, non-retrovirus-derived EVEs have been shown in various animal hosts (3–7). Indeed, recent advances in bioinformatics have dramatically changed the landscape of paleovirology. *In silico* surveys were used to screen for EVEs in various animal genomes and identified a number of nonretroviral EVE sequences belonging to several virus families (5–8). The integrated viral elements mostly accumulate random mutations that render them inactive. In several instances, however, EVEs have maintained open reading frames (ORFs) and produce functional proteins that can serve during infections by closely related viruses (9–12). For example, endogenous bornavirus-like nucleoproteins from the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus* (itEBLN), were the first nonretroviral EVEs demonstrated to serve as a negative regulator against infection by a related virus (10). Overexpression of itEBLN inhibits Borna disease virus (BDV) infection in mammalian cell lines, presumably by decreasing BDV polymerase activity. More recently, a virophage mavirus which is a parasite of the *Cafeteria roenbergensis* virus (CroV) was shown to be endogenized as an EVE in a marine flagellate, *Cafeteria roenbergensis* (9). CroV infection activates the endogenized mavirus genes in *C. roenbergensis* and produces infectious mavirus particles. These particles are secreted and protect surrounding flagellates from subsequent CroV infection. These studies demonstrate that EVEs can play critical roles in the host antiviral system.

Like most species examined, *Aedes* mosquitoes also have EVEs in their genomes. *Aedes aegypti* and *Aedes albopictus* mosquitoes are major vectors of arthropod-borne viruses (arboviruses), such as dengue virus (DENV) and Zika virus (ZIKV). DENV and ZIKV are members of the *Flavivirus* genus, which consists of enveloped viruses with a positive-sense, single-stranded RNA genome. In addition to these medically important mosquito-borne flaviviruses, *Aedes* mosquitoes in nature are infected by insect-specific flaviviruses (ISFs), such as cell-fusing agent virus (CFAV), Kamiti River virus (KRV), and *Aedes flavivirus* (AEFV) (13–15). Crochu et al. experimentally identified four endogenous flaviviral elements (EFVEs), three in *A. albopictus* cell lines and one in *A. aegypti* cell lines, which contain nonstructural (NS) genes related to ISFs (4). In the same study, a partial DNA sequence of these EFVEs was detected in *A. albopictus* and *A. aegypti* mosquitoes *in vivo* (4). Another study showed that it was possible to partially amplify, using degenerate primers, the NS5 region of flaviviruses in *A. albopictus* mosquitoes collected in northern Italy (16). In addition to these experimentally validated studies, *in silico* studies identified 28 and 24 genomic locations harboring EFVEs in *A. aegypti* and *A. albopictus* reference genomes, respectively (5, 8). All of the predicted EFVEs in *A. aegypti* mosquitoes were related to ISFs. For the 24 EFVEs predicted by Chen et al. in *A. albopictus* mosquitoes, they were mostly partial NS1 or NS5 sequences close to ISF and DENV sequences (8). However, no experimental validation of any of these bioinformatically predicted EFVEs has been performed either *in vitro* or *in vivo*.

To support our hypothesis that EFVEs could be used to manipulate the mosquito antiviral response to stop arbovirus transmission to the human host, we conducted a comprehensive characterization of EFVEs in *Aedes* mosquitoes representative of natural populations worldwide. We specifically focused on the only four EFVEs experimentally identified by Crochu et al. (4) and their known flanking regions. We investigated the EFVEs from several populations of each species sampled from geographically distinct locations. We show the presence of EFVE DNA and RNA transcripts *in vivo*. We

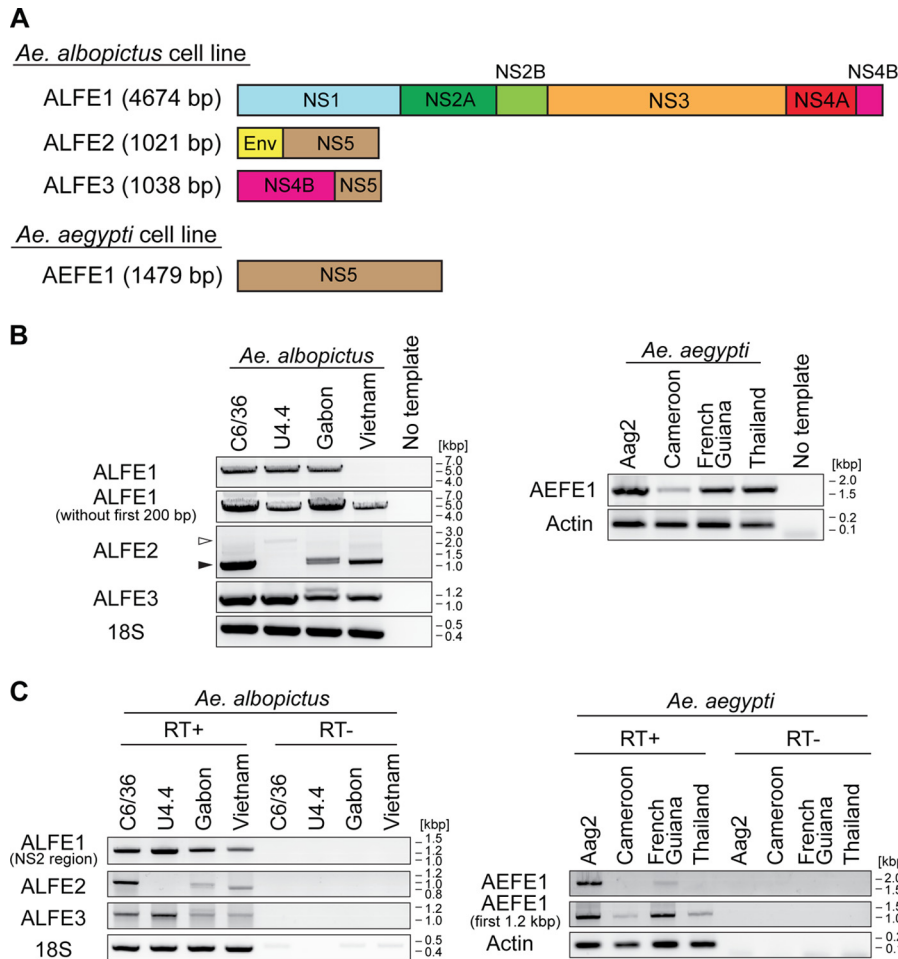


FIG 1 Detection of ALFE and AEFE DNA and RNA in *Aedes* mosquitoes. (A) Schematic representation of ALFE1 to ALFE3 and AEFE1. (B and C) Detection of ALFE1 to ALFE3 and AEFE1 DNA by PCR (B) and transcripts by RT-PCR (C) in C6/36 and U4.4 cell lines and the *A. albopictus* Gabon and Vietnam strains (left) and in the Aag2 cell line and the *A. aegypti* Cameroon, French Guiana, and Thailand strains (right). Black arrowheads in panel B, ALFE2-derived bands in the C6/36 cell line and in the Gabon and Vietnam strains; white arrowheads in panel B, ALFE2 fused with the host sequence in the U4.4 cell line. 18S rRNA and actin genes were used as controls for *A. albopictus* and *A. aegypti*, respectively.

confirmed the production of small RNAs derived from EFVE RNA *in vivo*. We further performed sequencing analysis of genomic DNA from *A. albopictus* mosquitoes and applied an *in silico* screening procedure that identified several new EFVEs in this species. Together, our results demonstrate the ubiquitous presence of diverse EFVEs *in vivo* and contribute to an understanding of the putative role of these elements in the antiviral defense system of mosquitoes during EFVE-related viral infection.

(This article was submitted to an online preprint archive [17].)

RESULTS

Detection of endogenous flaviviral elements in *Aedes* mosquitoes. Previous *in silico* studies predicted dozens of genomic locations harboring EFVEs in *Aedes* mosquito genomes (5, 8). However, only four annotated EFVEs with known flanking regions have been experimentally validated *in vitro* (by amplification of the full-length DNA sequence) and *in vivo* (by amplification of the partial DNA sequence) (4). We focused on these four annotated EFVEs that have been characterized for containing flaviviral nonstructural (NS) genes, three in *A. albopictus* (one named CSA1 and two unnamed) and one in *A. aegypti* (named CSA2) (4). For simplicity, we renamed these EFVEs *A. albopictus* flaviviral element (ALFE) 1 (ALFE1) to ALFE3 and *A. aegypti* flaviviral element (AEFE) 1 (AEFE1) (Fig. 1A). ALFE1 to ALFE3 were originally identified in the C6/36 cell

line (*A. albopictus*), and AEFE1 was originally identified in the A20 cell line (*A. aegypti*). Laboratory and field-collected *A. albopictus* and *A. aegypti* mosquitoes were positive by PCR for the NS3 and/or NS5 region of both ALFEs and AEFE (4). However, the full-length elements were not confirmed in individual mosquitoes. Because geographical origin is expected to be associated with genetic divergence due to selective pressures particular to each geographical region and/or genetic drift, we explored EFVEs in mosquitoes from different parts of the world. We used *A. albopictus* mosquitoes from Gabon and Vietnam and *A. aegypti* mosquitoes from Cameroon, French Guiana, and Thailand. First, we attempted to detect full-length DNA for ALFE1 to ALFE3 and AEFE1 *in vivo*. Cell lines corresponding to each mosquito species (the Aag2 cell line for *A. aegypti* mosquitoes and the C6/36 and U4.4 cell lines for *A. albopictus* mosquitoes), in addition to individual mosquitoes, were used. PCR with primer pairs whose sequences started and ended at the very first and the very last nucleotide of each EFVE showed amplicons with the expected sizes for ALFE1 to ALFE3 and AEFE1, except for ALFE1 in the *A. albopictus* Vietnam strain (for which no amplification band was present) and ALFE2 in the U4.4 cell line (for which a larger band was present) (Fig. 1B, white arrowhead). We further characterized ALFE1 in the Vietnam strain by PCR with different primer pairs. ALFE1 was amplified with a primer pair targeting the element without the first 200 bp at its 5' end (Fig. 1B). This result suggests that ALFE1 in *A. albopictus* Vietnam differs from ALFE1 in the *A. albopictus* Gabon strain and C6/36 and U4.4 cells in the first 200 bp of the element. For ALFE2, sequencing of the PCR product in U4.4 cells showed a partial ALFE2 sequence fused with unannotated mosquito sequences, suggesting that ALFE2 is recombined in U4.4 cells.

Next, we searched for the transcripts of ALFE1 to ALFE3 and AEFE1 by reverse transcriptase (RT) PCR. Previous work by Crochu et al. showed the ALFE1 transcript in C6/36 cells but not *in vivo*, while ALFE2, ALFE3, and AEFE1 mRNAs were not searched for *in vitro* or *in vivo* (4). The ALFE1 transcript was observed with a primer pair targeting the NS2 region but not a primer pair targeting the full-length element (Fig. 1C). ALFE2 and -3 transcripts were present in all samples, except for ALFE2 in U4.4 cells. The full-length AEFE1 transcript was observed in Aag2 cells and in the *A. aegypti* French Guiana strain but not in the *A. aegypti* Cameroon and Thailand strains. The first 1.2 kb of the AEFE1 transcripts was detected in all *A. aegypti* samples (Fig. 1C). The *A. aegypti* French Guiana strain was the only one to show the full-length transcript of AEFE1 among all *A. aegypti* strains tested. Altogether, our results indicate that ALFE1 to ALFE3 and AEFE1 were established by ancient viral infections in nature and not by artificial recombination in cell culture. In addition, ALFE1 to ALFE3 and AEFE1 are almost identical at the sequence level, and their transcripts are expressed among *Aedes* mosquito populations from different parts of the world.

ALFEs and AEFEs produce piRNA-like molecules in *Aedes* mosquitoes. We previously reported that *A. albopictus* and *A. aegypti* infected with chikungunya virus (CHIKV) produce viral cDNA through endogenous retrotransposon activity and that this viral cDNA generates small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNA) mediating viral persistence (18). CHIKV is an alphavirus from the *Togaviridae* family, which consists of enveloped viruses with a single-stranded, positive-sense RNA genome. CHIKV is also a mosquito-borne virus with a great impact on human health (19). To check if ALFE1 to ALFE3 and AEFE1 transcripts were also capable of generating small RNAs, such as CHIKV-derived viral cDNA, we reanalyzed small RNA libraries from *A. albopictus* and *A. aegypti* mosquitoes that were infected or uninfected with CHIKV and that were already available in the laboratory (Fig. 2). The majority of small RNAs that mapped to ALFE1 to ALFE3 and AEFE1 were 27 to 29 bases in length with an enrichment of uridine at the first position of the small RNA and were thus identified as primary piRNA-like molecules (Fig. 2A). We observed that piRNAs derived from these endogenous viral elements were only in the antisense orientation. CHIKV infection did not affect the size distribution and profiles of the small RNAs derived from ALFE1 to ALFE3 and AEFE1 (Fig. 2B). We examined the production of piRNAs targeting the

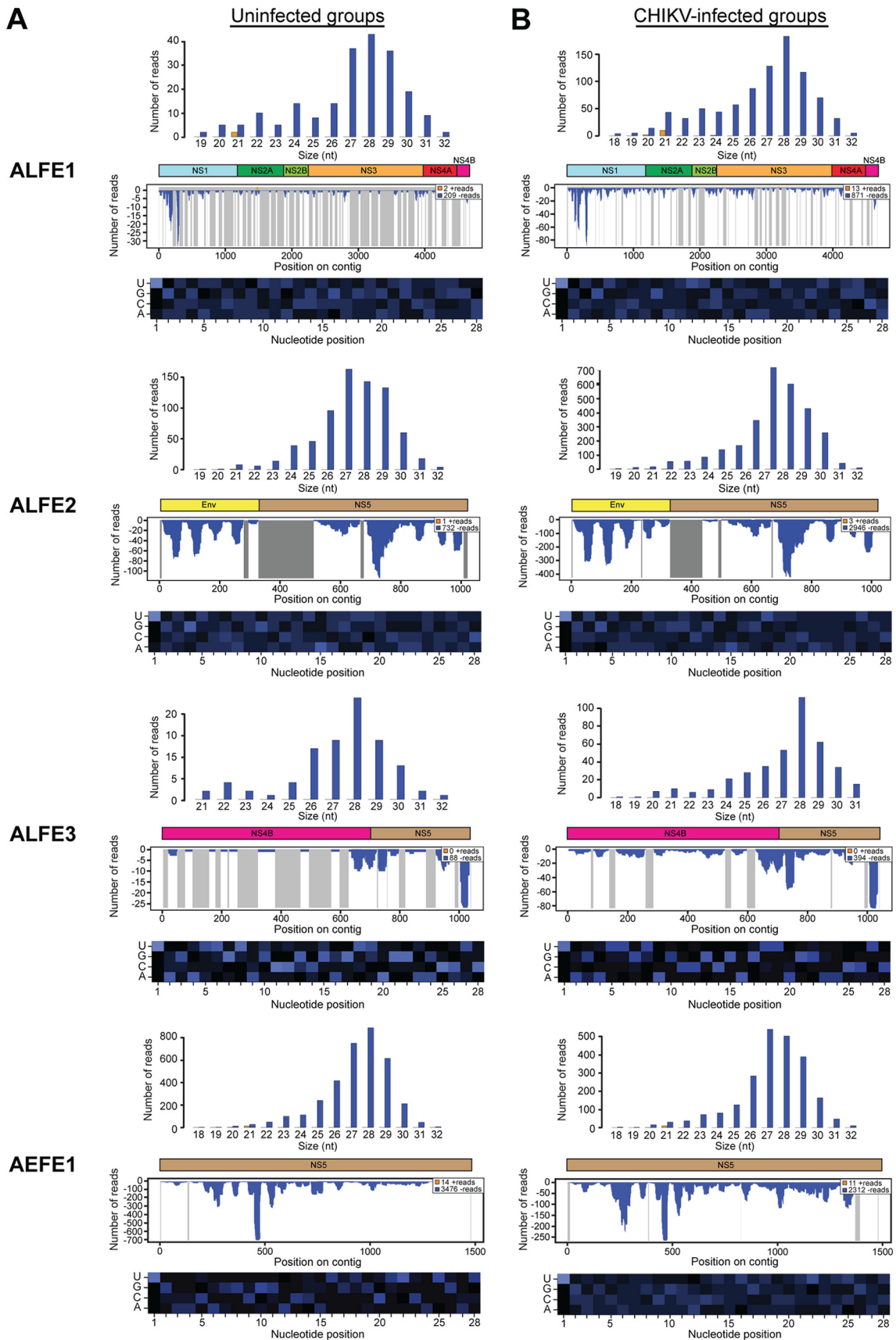


FIG 2 Primary piRNA-like small RNA production from ALFE1 to ALFE3 and AEFE1. The size distribution (top) and profiles (middle) of small RNAs mapped to ALFE1 to ALFE3 in *A. albopictus* mosquitoes and AEFE1 in *A. aegypti* mosquitoes not infected with CHIKV (A) or (Continued on next page)

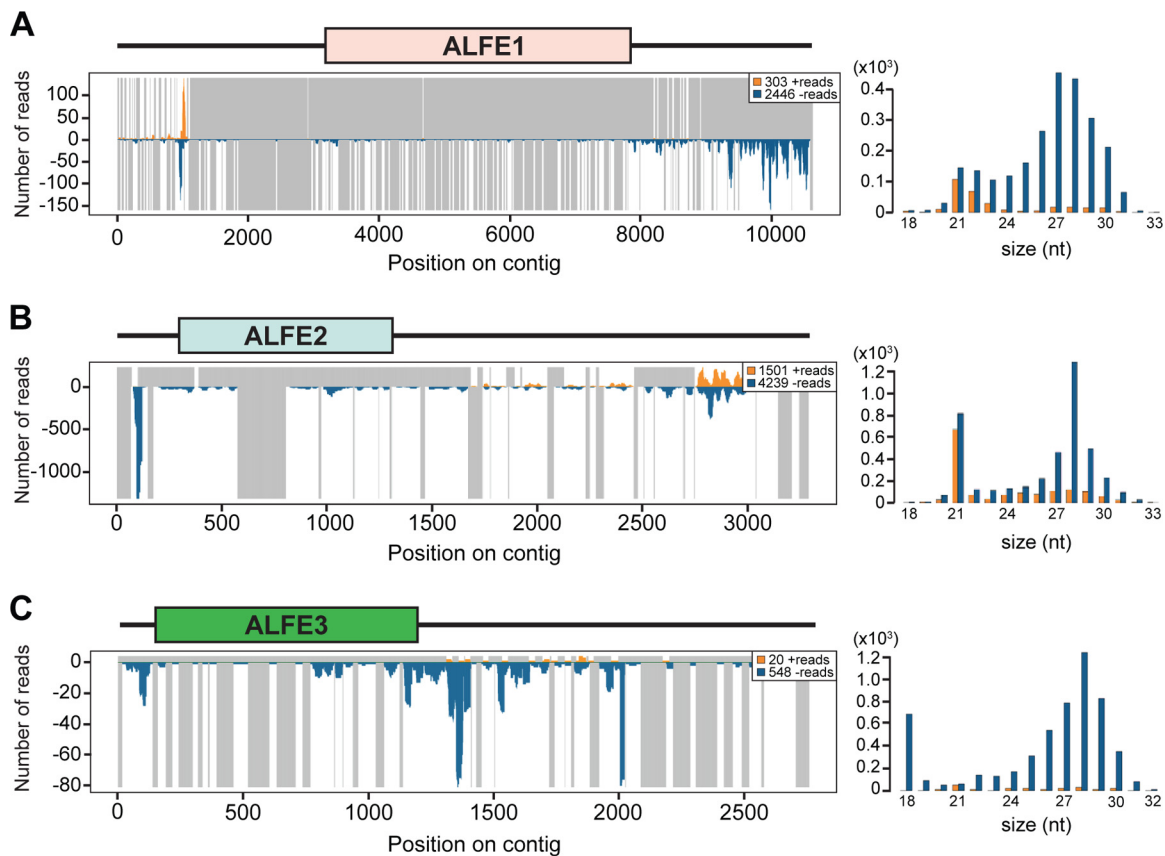


FIG 3 Small RNA production from ALFE1 to ALFE3 and their flanking regions in the *A. albopictus* genome. The small RNA profiles (left) and the size distributions (right) of small RNAs mapped to ALFE1 (A), ALFE2 (B), and ALFE3 (C) and their flanking sequences are shown. The schematic representation in each panel indicates the ALFE and its flanking region. The x axis represents the nucleotide position on the respective contig. The y axis shows the number of reads for each nucleotide position. Orange and blue bars, positive- and negative-stranded small RNAs, respectively; gray lines in the left panels, uncovered regions.

flanking regions of ALFE1 to ALFE3 determined by Crochu et al. (4). We detected the production of only antisense-strand piRNA-like molecules on these regions, similar to the findings for ALFE1 to ALFE3 (Fig. 3). In addition, the flanking regions of ALFE1 and -2 produced 21-nucleotide-long small RNAs corresponding to siRNAs. These results indicate that ALFE1 to ALFE3 and AEFE1 produce piRNAs only in the antisense orientation and also that these elements are located in specific regions of the genome that generate long transcripts for endogenous siRNA and piRNA production.

DENV infection does not affect AEFE1 transcript abundance in *A. aegypti* mosquitoes. Virus infections affect the expression of a number of host genes; for instance, DENV suppresses immune gene expression in the *A. aegypti* Aag2 cell line (20). The presence of transcripts from ALFEs and AEFEs in *Aedes* mosquitoes prompted us to check whether the expression level of their transcripts was altered during DENV infections *in vivo*. We utilized unpublished transcriptome data sets from female *A. aegypti* mosquitoes that were orally infected with either of two DENV serotypes (DENV serotype 1 [DENV1] and DENV3) or that were uninfected, which were available in the Lambrechts laboratory, to examine AEFE1 transcript expression. DENV1 infection did not affect AEFE1 transcript expression at 24 and 96 h postinfection (Fig. 4A). No

FIG 2 Legend (Continued)

infected with CHIKV (B) are shown. Orange and blue bars, positive- and negative-stranded reads, respectively. For the small RNA profiles, the x axis represents the nucleotide position on the ALFE1-, ALFE2-, ALFE3-, or AEFE1-containing contig; the y axis shows the number of reads for each nucleotide position; gray lines, uncovered regions. (Bottom) Relative nucleotide frequency per position of the 28-nucleotide (nt) ALFE1-, ALFE2-, ALFE3-, or AEFE1-derived small RNAs shown as a heat map. The intensity varies in correlation with frequency.

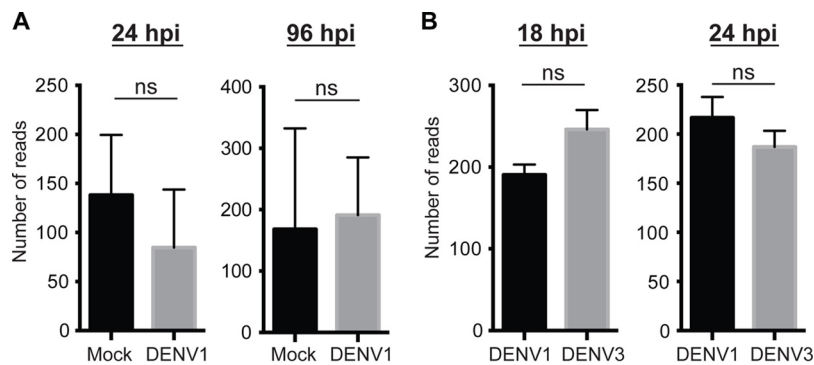


FIG 4 DENV1 and -3 infections have no detectable impact on AEFE1 transcript expression *in vivo*. The AEFE1 transcription level was analyzed by use of the DESeq2 package and a transcriptome data set from *A. aegypti* mosquitoes infected or not infected (Mock) with DENV1 (A) and *A. aegypti* mosquitoes infected with DENV1 or -3 (B) at the indicated time points. The average number of reads was calculated from several individual libraries: 7 and 17 libraries for mock- and DENV1-infected mosquitoes, respectively, at 24 h postinfection (hpi); 6 and 17 libraries for mock- and DENV1-infected mosquitoes infected, respectively, at 96 h postinfection; and 3 for each library for DENV1- or DENV3-infected mosquitoes at both time points. Statistical significance was determined by use of the DESeq2 package. ns, no significant difference (adjusted *P* value, >0.05).

significant difference in AEFE1 expression was observed between DENV1- and DENV3-infected *A. aegypti* mosquitoes at 18 and 24 h postinfection (Fig. 4B). The data suggest that AEFE1 is constitutively transcribed and not affected by DENV1 and -3 infections in adult female *A. aegypti* mosquitoes.

Searching for new flavivirus-like elements in *Aedes* mosquitoes. PCR for full-length ALFE1 DNA in the *A. albopictus* Vietnam strain did not result in amplification (Fig. 1B). We then performed PCR with primer pairs targeting ALFE1 and its flanking regions and amplified bands with unexpected sizes (data not shown). Because of the heterogeneity of the PCR products, we moved on to confirm the existence of ALFE1 and to search for new ALFEs in *A. albopictus*. We performed whole-genome DNA sequencing of the *A. albopictus* Vietnam strain and the C6/36 cell line. First, we mapped reads from the *A. albopictus* Vietnam strain and C6/36 cell DNA libraries to the ALFE1 to ALFE3 sequences and to the sequences of their respective flanking regions (Fig. 5). DNA coverage showed the presence of full-length ALFE1 in the genomes of the Vietnam strain and of C6/36 cells (Fig. 5A), despite the lack of PCR amplification. In addition, the relative coverage of the flanking regions of the ALFE1 to ALFE3 sequences was substantially higher, indicating that these elements are integrated in multicopy regions (Fig. 5A to C). We also observed a similar trend of insertion of ALFEs in multicopy sequences when analyzing the genome sequence of the *A. albopictus* Foshan strain, available in the VectorBase database (data not shown).

To identify new ALFEs, we performed an *in silico* screening based on an iterative mapping and assembly procedure using ALFE1 to ALFE3 sequences as scaffolds and the DNA library from the *A. albopictus* Vietnam strain as the query (details are provided in Materials and Methods). This *in silico* screening yielded eight contigs named ALFE4 to ALFE11 harboring partial sequences of ALFE1 to ALFE3 (Fig. 6A). For instance, ALFE4 was composed of a portion of ALFE1 followed by full-length ALFE3. We detected different genomic sequences flanking each ALFE, suggesting that multiple versions of each ALFE are present in the genome of the *A. albopictus* Vietnam strain. The sequences of the nearby regions are unique to *Aedes* mosquitoes, as a search for homology against the sequences in the GenBank and VectorBase databases did not show homology with any known organisms. Moreover, the six-phase translation of the flanking sequences did not show any similarity with known proteins from the UniProt Knowledge database.

To confirm the existence of the bioinformatically predicted ALFE4 to ALFE11, we used two different assessments: (i) the quality of the DNA mapping of the *A. albopictus* Vietnam strain and C6/36 cell reads to the new ALFE contigs and (ii) PCR with primer

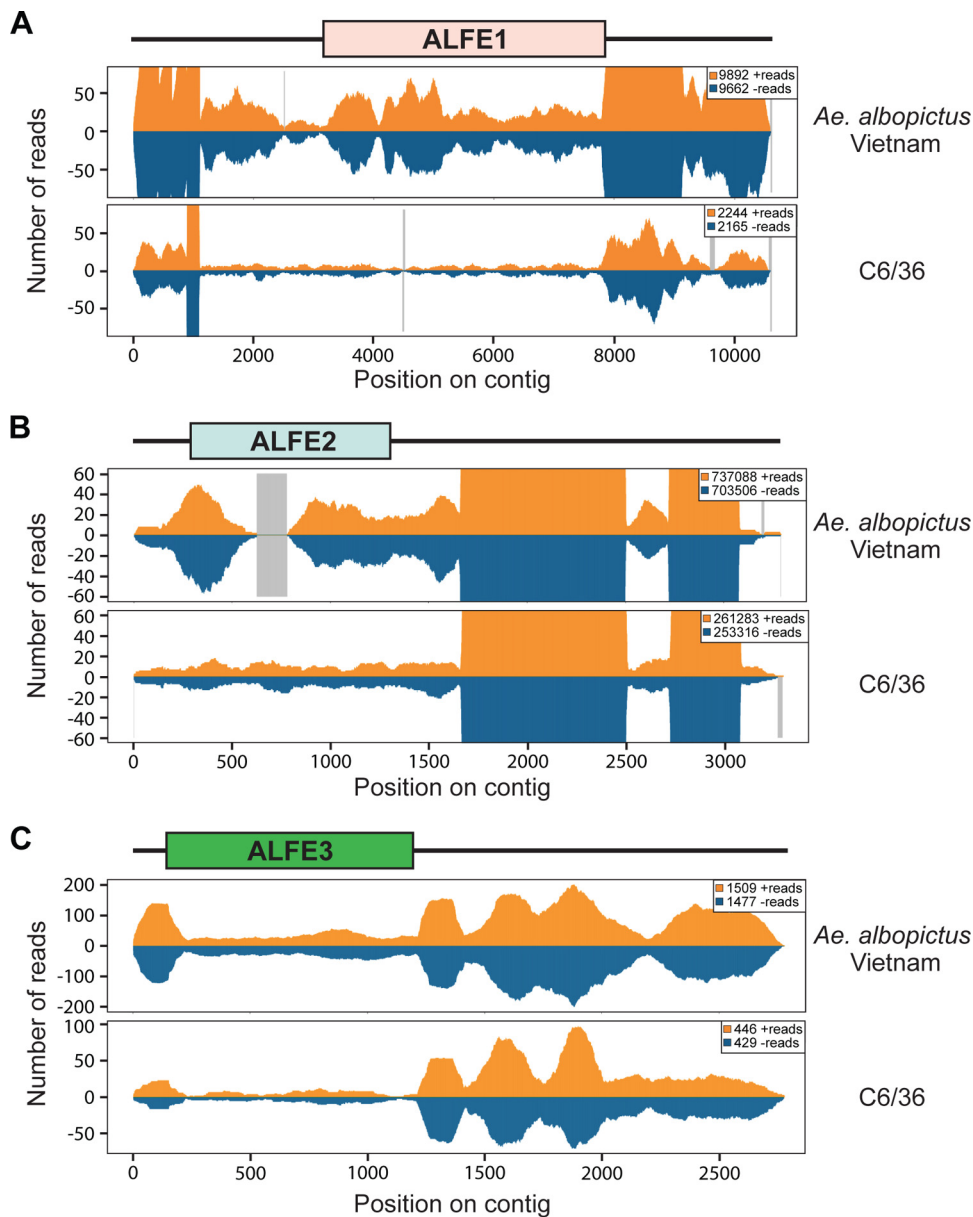


FIG 5 ALFEs are integrated into repetitive regions in *Aedes* mosquito genomes. DNA reads from the *A. albopictus* Vietnam strain and C6/36 cell DNA library were mapped to ALFE1 (A), ALFE2 (B), and ALFE3 (C) and their flanking regions. Orange and blue, positive- and negative-stranded reads, respectively. The box at the top of each panel indicates each ALFE, and the black bars represent the flanking regions. The x axis represents the nucleotide position on the ALFE-containing contig. The y axis shows the number of reads for each nucleotide position. Gray lines, uncovered regions.

pairs specific to the new ALFE contigs. For DNA mapping, the depth and continuity of coverage along the ALFE sequences were used to confirm the existence of ALFEs in the Vietnam strain. Figure 6B shows the DNA coverage and the continuity of the reads on ALFE7 as an example of the validation. In the *A. albopictus* Vietnam strain, the reads continuously covered the ALFE7 contig sequence, while in C6/36 cells, there were differences in the continuity of coverage (a gap was present). In addition, the read counts corresponding to the flanking regions of ALFE7 confirmed that this element is integrated in multicopy sequences in the *A. albopictus* genome. All the predicted ALFEs were confirmed by PCR amplification to be present in the Vietnam strain, except for ALFE5, which could not be distinguished from ALFE1 due to sequence similarity. Figure 6C shows an example of the PCR amplification products of the predicted ALFE4, -7,

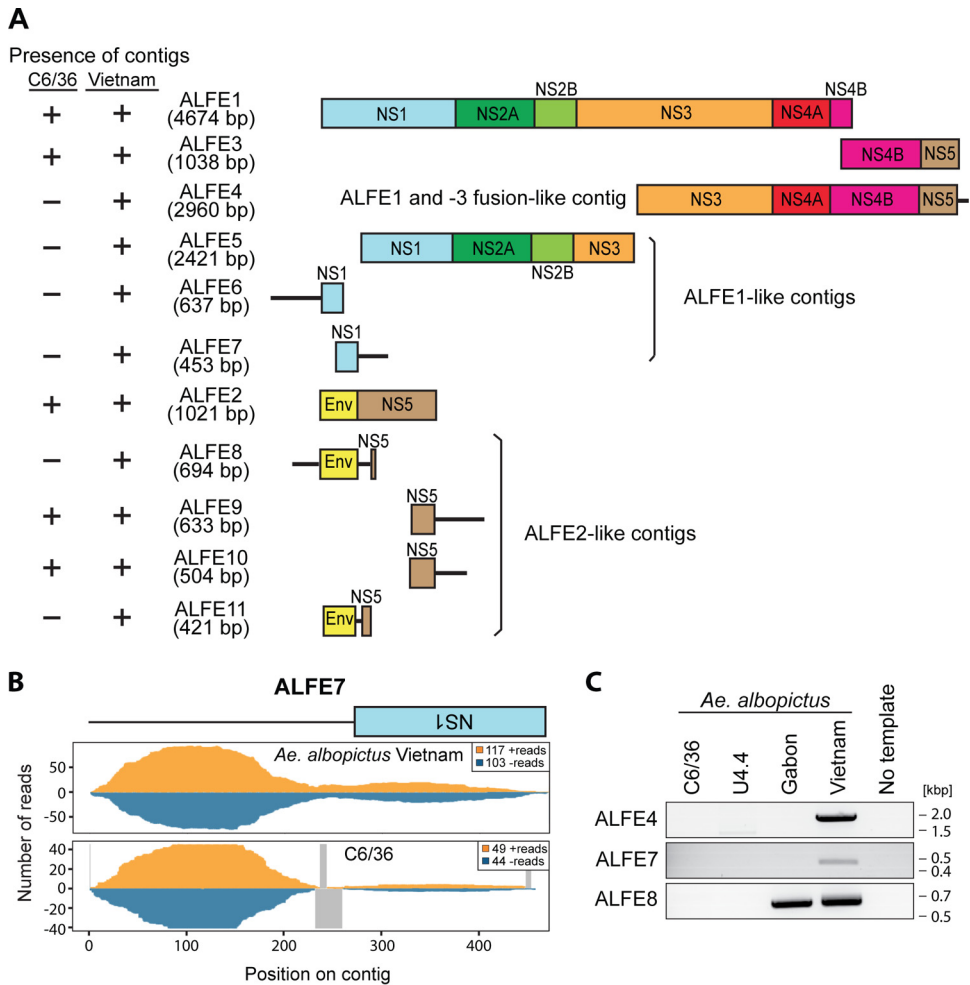


FIG 6 New ALFEs identified in the *A. albopictus* Vietnam strain. The *in silico* pipeline generated 8 new ALFE-like contigs. (A) Schematic representation of ALFE contigs. ALFE4 is a fusion element composed of ALFE1 and -3. ALFE5 to ALFE7 are partial sequences of ALFE1. ALFE8 to ALFE11 are ALFE2-like contigs. The boxes indicate ALFE-derived sequences, and the black lines indicate non-ALFE sequences. The presence (+) or absence (-) of ALFE-like contigs is summarized in the left column. (B) DNA coverage of ALFE7 with the *A. albopictus* Vietnam DNA library. Orange and blue, positive- and negative-stranded DNA, respectively; gray lines, uncovered regions. (C) ALFE4, -7, and -8 DNA detection in cells of the C6/36 and U4.4 cell lines and in the *A. albopictus* Gabon and Vietnam strains by PCR.

and -8. These three elements were detected in the *A. albopictus* Vietnam strain, whereas only ALFE8 was present in the Gabon strain. Neither C6/36 cell nor U4.4 cell genomes showed the presence of these elements. In addition, we checked for small RNA production from the newly described ALFEs. As observed with ALFE1 to ALFE3, the bioinformatically predicted ALFEs and their flanking sequences were covered only by antisense piRNA-like molecules with a U-1 bias (Fig. 7).

Lastly, we compared the ALFE contigs identified from C6/36 cells and the *A. albopictus* Vietnam strain with *A. albopictus* Foshan genomic DNA supercontigs. We observed considerable variation in the flanking regions between the ALFEs in the different strains. For example, the *in silico* pipeline predicted the existence of ALFE4 (a fusion between ALFE1 and ALFE3) in the Vietnam strain (Fig. 8). When the ALFE4 contig was compared to the ALFE4-like contig in C6/36 cells, a 1.3-kbp host sequence was present between ALFE1 and -3. This host sequence also exists in the Foshan genome but is much longer (11 kbp) and contains a repeat sequence at both extremities of a coding DNA sequence (CDS) with Gag-, reverse transcriptase-, and proteinase-like domains. The analysis suggests that ALFE1 and -3 were originally the same element in

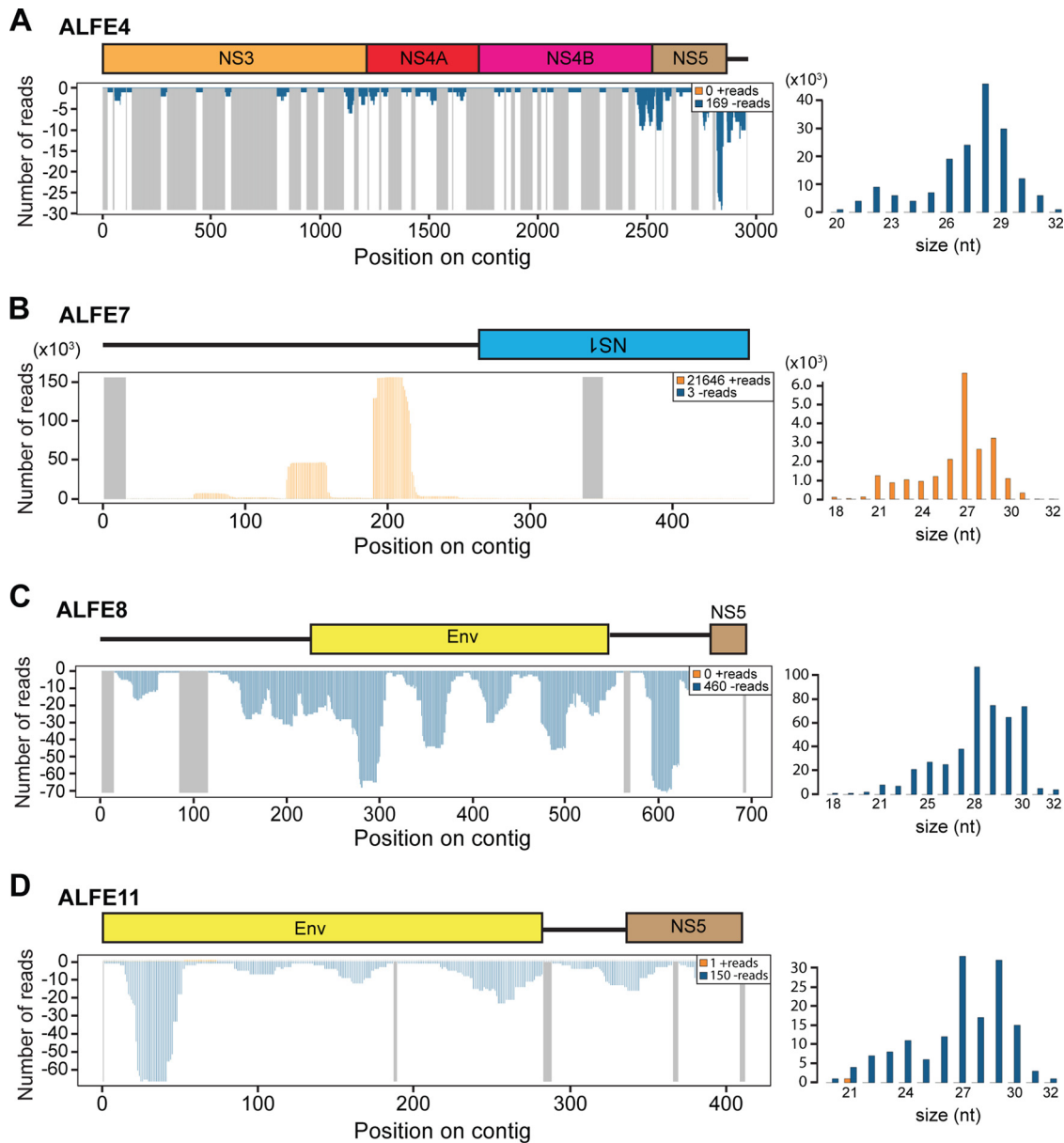


FIG 7 Small RNA production from ALFE4, -7, -8, and -11. The small RNA profiles (left) and size distribution (right) of small RNAs mapped to the ALFE4 (A), ALFE7 (B), ALFE8 (C), and ALFE11 (D) sequences are shown. The schematic representation in each panel indicates each ALFE and its flanking region. The x axis represents the nucleotide position on the contig. The y axis shows the number of reads for each nucleotide position. Orange and blue, positive- and negative-stranded small RNAs, respectively; gray lines in the left panels, uncovered regions.

the Vietnam strain (ALFE4) and were separated by insertion of a retrotransposon in the Foshan strain or vice versa.

ALFE- and AEFE-derived proteins are undetectable by MS. Because ALFE1 to ALFE3 and AEFE1 generated transcripts *in vitro* and *in vivo*, we checked whether they could produce detectable proteins. As antibodies against ALFEs, AEFEs, or similar flaviviruses are not available, we performed mass spectrometry (MS) analysis with the C6/36 and Aag2 cell lines to find ALFE- and AEFE-derived peptides. Proteins from C6/36 and Aag2 cells were purified and subjected to MS for bottom-up proteomics analysis. Although we identified Ago2, Dcr2, and Piwi5, as well as thousands of proteins from all the subcellular fractions ranging from 5.6 to 811 kDa in molecular mass (Table 1; see also Table S1 in the supplemental material), no ALFE- or AEFE-proteolytic peptides were identified in either C6/36 or Aag2 cells. Of note, it is not possible to use the expression

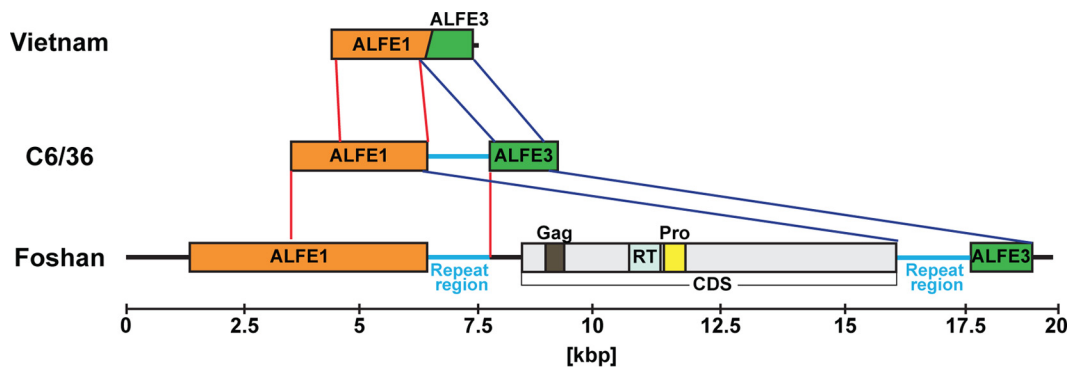


FIG 8 Comparison of the ALFE1/ALFE3 sequences in the *A. albopictus* Vietnam strain, Foshan strain, and C6/36 cells. Schematic representations of ALFE4 (from the Vietnam strain), an ALFE4-like element (from C6/36 cells), and Foshan strain ALFE1 and -3 with their flanking regions are shown. ALFE1/ALFE3 in the Foshan strain has a gap of approximately 11 kbp in length with a CDS harboring retrotransposon Gag, reverse transcriptase, and protease domains and repeat sequences at both the 5' and 3' extremities. Overlapped regions of ALFE1 and -3 are visualized with red and blue lines, respectively. Light blue bars, repeat sequences.

level of the identified proteins to estimate a maximum expression level of ALFEs and AEFs, because the ionization properties of proteotypic peptides can be very different. However, the number of identified proteins, their large molecular mass range, and their subcellular origin suggest that a large part of the proteome has been covered. Our result suggests that ALFE1 to ALFE3 and AEF1 are not translated at a high level, if they are translated at all, in both mosquito cell lines.

Phylogenetic analysis of AEF1 in *A. aegypti* mosquitoes. To study the evolutionary history of AEF1, we examined its genetic diversity in *A. aegypti* mosquitoes from Cameroon, French Guiana, and Thailand. The full-length sequence of AEF1 in individual mosquitoes from each country was amplified and sequenced. Some individual mosquitoes from the Cameroon and Thailand populations did not show amplification of AEF1. Next, we generated maximum likelihood phylogenetic trees of AEF1 consensus sequences from each individual and their homologous sequences in the genomes of ISFs closely related to AEF1, such as CFAV, KRV, and AEFV, as well as homologous sequences in related medically important flaviviruses. As expected, the AEF1 sequence was closely related to the ISF sequences (Fig. 9). The phylogenetic analysis showed that among the three ISFs considered, KRV is the closest to AEF1. To further examine the relationship among AEF1 sequences from different geographical locations, an additional tree was generated and rooted with the ISFs as the outgroup but is presented without the ISFs for visual clarity (Fig. 10). The phylogenetic relationships among AEF1 sequences mainly recapitulated the recent evolutionary history of *A. aegypti* populations. With two exceptions, the AEF1 sequences in mosquitoes from the non-African (Thailand and French Guiana) populations were derived from AEF1 sequences found in the African (Cameroon) population. A basal evolutionary position of African populations is typically observed for *A. aegypti* genes (21), consistent with the recent out-of-Africa geographical expansion of the species. Thus, the phylogenetic analysis suggests that AEF1 evolved consistently with other host genes at the species level.

TABLE 1 Proteomic characterization of C6/36 and Aag2 cell lines

Cell line	No. of times that proteolytic peptides were found ^a				No. of identified proteins ^b
	EFVEs	Piwi5	Dcr2	Ago2	
C6/36	0	3	0 ^c	2	1,942 ± 637
Aag2	0	3	2	2	2,054 ± 291

^aNumber of times that proteolytic peptides corresponding to EVE proteins and positive controls (out of 3 experiments) were found.

^bThe average total number of identified proteins is depicted.

^cC6/36 cells are Dcr2 deficient.

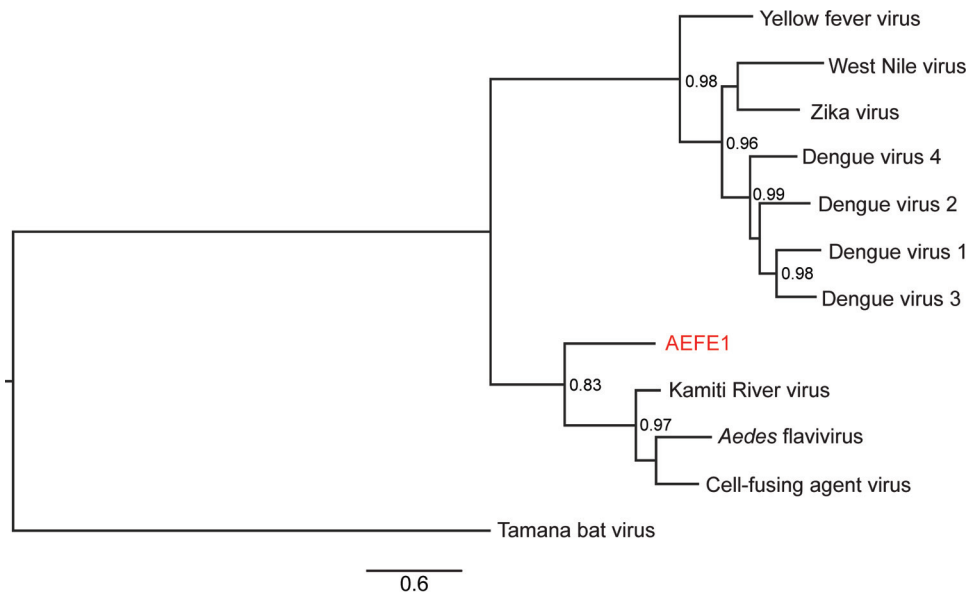


FIG 9 Phylogenetic relationships among ALFE1 and insect-specific flaviviruses. A maximum likelihood tree was generated using the Fast likelihood-based approach. ALFE1 DNA from individual mosquitoes of the *A. aegypti* Cameroon, French Guiana, and Thailand strains was sequenced. The tree was generated with *Aedes flavivirus* (GenBank accession no. [AB488408.1](#)), cell-fusing agent virus (GenBank accession no. [NC_001564.2](#)) and Kamiti River virus (GenBank accession no. [AY149905.1](#)) as ISFs and DENV1 (GenBank accession no. [KX225493.1](#)), DENV2 (GenBank accession no. [AY858035.2](#)), DENV3 (GenBank accession no. [AY858047.2](#)), DENV4 (GenBank accession no. [KU523872.1](#)), ZIKV (GenBank accession no. [KU955595.1](#)), West Nile virus (GenBank accession no. [M12294.2](#)), Yellow fever virus (GenBank accession no. [NC_002031.1](#)), and Tamana bat virus (GenBank accession no. [AF285080.1](#)) and rooted using Tamana bat virus. The scale bar indicates the number of nucleotide substitutions per site. Node values represent Shimodaira-Hasegawa (SH)-like branch support (only values of >0.7 are shown).

DISCUSSION

Recently, reactivation of EVEs due to related or unrelated viral infections has been reported, strongly suggesting a role of EVEs during the immune response of the host (9–11). Dozens of EVEs comprising flavivirus-, rhabdovirus-, and reovirus-related EVEs have been bioinformatically predicted for different mosquito strains, and some of them were confirmed in mosquito cell lines or strains (4, 5, 7, 8, 22). However, few studies have been conducted to characterize EVEs *in vivo*, a necessary step to further investigate their role during viral infection of mosquitoes. Due to the current disease outbreaks caused by mosquito-borne flaviviruses, such as Zika and dengue viruses, we decided to study and characterize *in vivo* endogenous flaviviral elements (EFVEs) that were previously identified in *A. albopictus* and *A. aegypti* mosquito cell lines. To improve our understanding of the forces shaping the evolution of these EFVEs, we assessed their presence, transcription, small RNA production, protein production, and phylogeny using African, Asian, and South American populations of *Aedes* mosquitoes. These wild-type populations are epidemiologically relevant because they occur in regions where they act as the main arbovirus vectors (23, 24). To simplify the nomenclature, we propose to name the EFVEs identified in *A. albopictus* mosquitoes ALFEx and the ones identified in *A. aegypti* mosquitoes AEFEx, where *x* is a number.

We first showed the presence of full-length ALFE1 to ALFE3 DNA and AEFEx DNA in several populations of individual *A. albopictus* and *A. aegypti* mosquitoes, respectively. This indicates that ALFE1 to ALFE3 and AEFEx are likely derived from ancient events of flavivirus DNA integration in nature that have persisted during the course of evolution. We also detected complete or partial RNA transcripts of ALFE1 to ALFE3 and AEFEx *in vivo*. The expression level of AEFEx remained unchanged following DENV1 and -3 infection of *A. aegypti* mosquitoes, suggesting a constitutive expression. However, experiments addressing AEFEx and ALFE transcriptional regulation during different virus

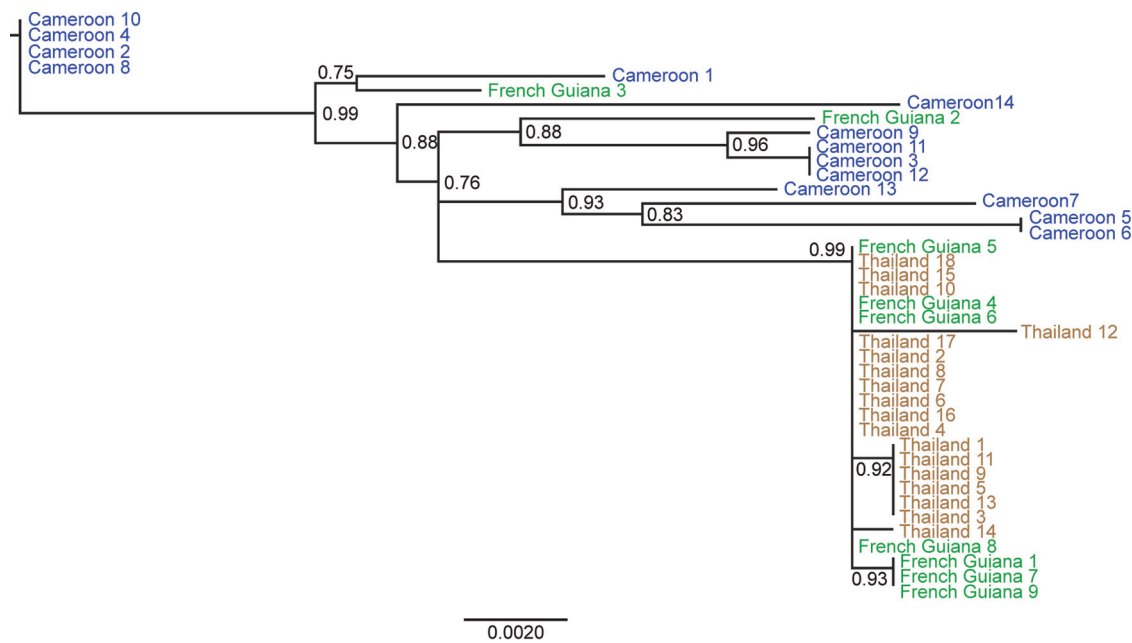


FIG 10 Phylogenetic relationships among AEFE1 sequences from different *A. aegypti* populations. A maximum likelihood tree was generated using the Fast likelihood-based approach. AEFE1 DNA from individual mosquitoes of the *A. aegypti* Cameroon, French Guiana, and Thailand strains was sequenced. The scale bar indicates the number of nucleotide substitutions per site, and node support values are shown for major nodes. The tree was rooted with ISF sequences (Fig. 9), but the outgroup was omitted for visual clarity. Node values represent Shimodaira-Hasegawa (SH)-like branch support (only values of >0.7 are shown).

infections or different abiotic or biotic stimuli should be designed and performed in the future.

In most documented cases, functional EVEs play a role at the protein level (9–12). We performed a powerful bottom-up proteomic approach to search for ALFE- and AEFE-proteolytic peptides in the C6/36 and Aag2 cell lines. The mass spectrometry analysis could not validate the expression of ALFE1 to ALFE3 and AEFE1 proteins in any of the subcellular fractions analyzed, while control peptides for each cell type were readily detected. Since ALFEs and AEFEs have been maintained in the cell lines and individual mosquitoes at the DNA and RNA levels, this result strongly suggests that ALFE and AEFE transcripts are not translated into proteins. However, EFVEs could need specific conditions to be translated. For instance, the mosquito cell lines that we used were established from larvae or embryos (25, 26). ALFEs and AEFEs could be translated only in specific tissues or stages of development *in vivo*. Another interesting possibility is that because the transcripts were observed by RT-PCR and RNA sequencing, ALFEs and AEFEs could have a function at the RNA level.

Small RNAs have critical roles in various aspects of host fitness and antiviral immunity in mosquitoes (18, 27–33). The piRNA pathway is one of the small RNA pathways and mainly controls nonself sequences, such as retrotransposable elements and DNA transposons (34–36). According to the PIWI proteins involved and available for piRNA biogenesis, they are classified into primary piRNAs or secondary piRNAs, with the latter being produced from an amplification cycle known as the ping-pong cycle using primary piRNAs as the template. Infections with arboviruses, such as CHIKV and DENV, have been shown to produce both primary and secondary piRNAs predominantly in the sense orientation in mosquitoes (18, 29, 37). We found that ALFE1 to ALFE3, AEFE1, and their flanking regions produced a population of primary piRNA-like molecules only in the antisense orientation and that these molecules were not affected during CHIKV infection. A recent study also found that some annotated genes, including EVEs, produce antisense primary piRNA-like small RNAs in *A. aegypti* mosquitoes and Aag2 cells (38, 39). Two nonexclusive hypotheses could account for the production of only primary piRNA-like molecules by ALFEs and AEFEs. First, the production of ALFE- and

AEFE-derived primary piRNAs may occur only in specific tissues lacking PIWI proteins, such as Piwi5 and/or -6, involved in ping-pong amplification. Second, the transcript that produces primary piRNAs of EFVEs is predominantly transcribed in the antisense orientation. Indeed, we observed a similar tendency in piRNA production from the flanking regions of ALFEs and AEFEs, suggesting that primary piRNAs from EFVEs are produced from the same precursor transcript as their flanking regions. Curiously, in vertebrates, some murine EBLNs and their flanking regions also generate antisense piRNA-like small RNAs (40). This suggests that EFVEs have common features in small RNA production across species and kingdoms. To elucidate how and why this is happening deserves further studies.

Regarding the function of these EFVE-derived piRNAs, it is tempting to propose that they could regulate infections by closely related viruses by targeting viral RNA in *Aedes* mosquitoes. ALFEs and AEFEs do not contain sequences of 24 to 30 nucleotides that perfectly span AEFV and KRV sequences, which are the most closely related known ISFs. However, piRNAs allow some mismatches with target sequences (41–43). If ALFE- and AEFE-derived piRNAs are loaded into the ping-pong amplification cycle during viral infection, a number of piRNAs that match the virus sequence could be produced and those piRNAs could contribute to the control of viral replication. We and others previously demonstrated that viral piRNAs (vpiRNAs) were detected in *Aedes* mosquitoes and in mosquito cell lines infected with mosquito-borne viruses, such as CHIKV and Rift Valley Fever virus (phlebovirus, *Bunyaviridae* family) (18, 30). Although the direct effects of vpiRNA on viral replication remain unclear, vpiRNAs could render the mosquitoes tolerant to infections with the viruses (18). By providing closely related piRNAs to the ping-pong cycle, ALFEs and AEFEs may contribute to the control of pathogenesis during exogenous viral infections.

Using a bioinformatics approach, we identified several new ALFEs composed of partial or complete ALFE1 to ALFE3 sequences in *A. albopictus* mosquitoes from Vietnam. Some of these new ALFEs were predicted only in our mosquito strain and were not found in the C6/36 cell line genome. PCR also showed variability in the detection of ALFE4 to ALFE11 among the mosquito strains, suggesting that the genome of each mosquito population has a different set of EFVEs. The recent genome sequencing of the *A. albopictus* Foshan strain by Chen et al. (8) found more than 20 EFVEs that were mostly related to NS1 or NS5 genes from ISFs. Likewise, we also found three elements containing NS1 and four containing NS5 out of eight identified ALFE-like contigs. Another study performed on field-collected *A. albopictus* mosquitoes in northern Italy detected flaviviral NS5 related to CFAV and KRV NS5 (16). Therefore, *Aedes* mosquitoes seem to have preferentially accumulated ancient flaviviral NS5 sequences in their genomes. There are two potential and not exclusive explanations for this: (i) NS5 could be preferably endogenized over other flaviviral sequences, and (ii) NS5 sequences could be positively maintained over generations. Although the mechanism of endogenization has not been elucidated, we and others have suggested that it happens in a retrotransposon-dependent manner (18, 44, 45). The genomes of *A. albopictus* and *A. aegypti* mosquitoes harbor 50% to 70% repetitive sequences, such as retrotransposons and long interspersed nuclear elements (LINEs) (8, 46). Accordingly, it is interesting to propose that the sequence of NS5 could be efficiently recognized by retrotransposons in *Aedes* mosquito genomes.

One interesting observation arose when we compared the newly identified ALFE4 from our Vietnam strain and ALFE4 from the Foshan strain of *A. albopictus*. In the Vietnam strain, ALFE4 appears as a fusion of ALFE1 and ALFE3. In the Foshan strain, a large CDS containing a retrotransposon RT-like domain with terminal repeat sequences exists between ALFE1 and ALFE3. Katzourakis and Gifford (5) observed in *A. aegypti* mosquitoes the presence of an almost entire flaviviral genome fragmented in several pieces across the mosquito genome. We propose that ALFE- and AEFE-like sequences are likely inserted in repetitive regions of the mosquito genome, where transposons can act as a trap for nonretroviral DNA. These intergenic flanking regions display substantial structural variation among mosquito populations, arguing for an active

rearrangement and continuous change of EFVE-containing regions. Nevertheless, EFVE sequences have been maintained in each mosquito population, suggesting that these captured viral sequences play a role during host evolution.

It is, however, important to stress that we faced significant challenges working with the genome sequence of the *A. albopictus* Vietnam strain. This was presumably due to the difficulties in assembling the different contigs, despite a comfortable depth of coverage, due to the high content of repetitive sequences. Indeed, the currently available genome assemblies of *A. albopictus* and *A. aegypti* in the VectorBase database consist of thousands of unassembled supercontigs. There is an urgent need to assemble these fragmented reference genome sequences into end-to-end chromosome maps. It is hoped that the advent of new tools, such as long-read sequencing technologies and chromosome confirmation capture, will produce a leap forward in the study of mosquito genomes.

Finally, a phylogenetic analysis of AEFE1 in *A. aegypti* mosquitoes from Cameroon, French Guiana, and Thailand showed that the evolutionary history of AEFE1 was similar to that of most nuclear genes. Note that we did not investigate the phylogenetic relationships of ALFEs because the current lack of population genomics studies of *A. albopictus* mosquitoes would have made any interpretation difficult. According to population genomics studies of *A. aegypti*, this species originated in Africa and colonized the rest of the world following a single migration event, probably by traveling in ships along trading routes in past centuries (21). AEFE1 was genetically more diverse in the Cameroon strain than the French Guiana and Thailand strains, which is also consistent with the patterns of genetic diversity for nuclear genes. Our phylogenetic analysis suggests that the AEFE1 integration events occurred before *A. aegypti* mosquitoes expanded out of the African continent. However, the findings for two individual mosquitoes from French Guiana were inconsistent with the out-of-Africa model. This could be due to introgression from African mosquitoes subsequently introduced into South America (47) or because the sequences were not orthologous. We also found some *A. aegypti* individuals from Cameroon and Thailand in which the full-length AEFE1 could not be amplified, suggesting that both structural and nucleotide variants of AEFE1 exist within the same population. It is likely that following the initial integration event, complex evolutionary forces have shaped the genetic diversity of EFVEs that is observed today. Unraveling this diversity will be necessary to elucidate their potential functional role.

MATERIALS AND METHODS

Ethics statement. The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with French and European regulations on the care and protection of laboratory animals. The rabbit blood draws performed during the course of this study were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur under protocol number 2015-0032, in accordance with European directive 2010/63/UE and French legislation.

Cell culture. Cells of the C6/36 (ATCC CRL-1660) and U4.4 cell lines (*A. albopictus*) (26) and the Aag2 cell line (*A. aegypti*) (48) (kindly provided by G. P. Pijlman, Wageningen University, the Netherlands) were maintained at 28°C in L-15 Leibovitz medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% nonessential amino acids (Gibco), 2% tryptose phosphate broth (Sigma), and 1% penicillin-streptomycin (Gibco).

Mosquitoes. Laboratory colonies of *A. aegypti* mosquitoes were established from field collections in Cameroon (2014), French Guiana (2015), and Thailand (2013). Laboratory colonies of *A. albopictus* were established from field collections in Gabon (2014) and Vietnam (2011). All the experiments were performed within 16 generations of laboratory colonization. The insectary conditions for mosquito maintenance were 28°C, 70% relative humidity, and a 12-h light and 12-h dark cycle. Adults were maintained with permanent access to 10% sucrose solution. Adult females were offered commercial rabbit blood (BCL) twice a week through a membrane feeding system (Hemotek Ltd.).

Experimental DENV infections *in vivo*. Wild-type, low-passage-number DENV isolates (DENV1 KDH0030A [49], DENV3 GabonMDA2010 [50]) were originally obtained in 2010 from the serum of dengue patients. Informed consent of the patients was not necessary because the viruses isolated in cell culture are no longer considered human samples. KDH0030A was isolated at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. GabonMDA2010 was isolated at the Centre International de Recherches Médicales de Franceville, Gabon. Virus stocks were prepared and experimental mosquito infections were conducted as previously described (51). Briefly, 4- to 7-day-old female mosquitoes were

TABLE 2 Primer pairs for PCR and RT-PCR

Target	Name of primer	Sequence
ALFE1 flanking region	ALFE1_-100F	CCCTGTCACACAAGCTTGGAG
	ALFE1_ + 99R	CCTACCATGGAGGGTCTCTGTTT
Full-length ALFE1	ALFE1_F	ATGGTGGTCGTCTTCACTATGTACATAC
	ALFE1_R	CTAATTCCTTGCTGCAAGCTGAGTTCTG
ALFE1 without first 200 bp	ALFE1_200	GCACAAATAAACCATGCCTAATCTGCG
	ALFE1_R	CTAATTCCTTGCTGCAAGCTGAGTTCTG
ALFE1 NS2 region	ALFE1_NS2_F	AGATCGGATTTTCAACCAGCGAAC
	ALFE1_NS2_R	TCTACAATGCTCGTTAAGTTTAAAGCG
Full-length ALFE2	ALFE2_F	ACGGCAGTAATGACCTGTGCTG
	ALFE2_R	CCCCAATACTTTATCTCCGTGCCG
Full-length ALFE3	ALFE3_F	CGAGATCACCGCGGAATCC
	ALFE3_R	GTGGCTAGATTGTAGCCGCGAG
Full-length AEFE1	AEFE1_F	TCTGCCAGGGACGTGTACATG
	AEFE1_R	GTTTTCGTTGTTGGTGTGATGGATGG
First 1.2 kbp of AEFE1	AEFE1_F	TCTGCCAGGGACGTGTACATG
	AEFE1_1200_R	GAAAGTGAAGGGCTACTCGAAGCTG
18S rRNA	18S_F	GGTCGGCGGGTCGTAGTGTGG
	18S_R	TCCTGGT GGTGCCCTTCCGTCAAT
Actin	Actin_F	AAGGCTAACCGYGAGAAGATGAC
	Actin_R	GATTGGGACAGTGTGGGAGAC
ALFE4	ALFE_NS4_F	TTGTCCATTGAGATGTATATCAATACCCG
	ALFE3_R	GTGGCTAGATTGTAGCCGCGAG
ALFE6	ALFE6_F	AAAGCTTTACTACTCGAAACTCCC
	ALFE7_R	TCCTTTATGTACAGATTTGTGAATGCG
ALFE7	ALFE7_F	TCACTCCGTGGAGCTGGATG
	ALFE7_R	TCTTCTTAAAATTGATACCTTCCATTGATC
ALFE8	ALFE8_F	CTAAAACATTATGAGTGAAAATGGCAG
	ALFE8_R	GCTCTTCCTCATCTTGAAGTCG
ALFE9	ALFE9_F	AGATGAGAACCTTGAAGACACCCTTAG
	ALFE9_R	CAGTCTGTGGTAATGTTGTTGGCG
ALFE10	ALFE10_F	AGATGAGAACCTTGAAGACACCC
	ALFE10_R	CAAATCACCAGCGAAGGCTTTC

deprived of sucrose for 24 h and transferred to a biosafety level 3 insectary. Washed rabbit erythrocytes resuspended in phosphate-buffered saline were mixed 2:1 with prediluted viral stock and supplemented with 10 mM ATP (Sigma-Aldrich). The viral stock was prediluted in Leibovitz L-15 medium with 10% FBS, 0.1% penicillin-streptomycin, and 1% sodium bicarbonate (Gibco) to reach an infectious titer ranging from 5×10^6 to 1.1×10^7 focus-forming units per ml of blood using a standard focus-forming assay in C6/36 cells (51). A control blood meal was prepared identically except that the supernatant of mock-inoculated cells replaced the viral suspension. Mosquitoes were offered the infectious or control blood meal for 30 min through a membrane feeding system (Hemotek Ltd.) set at 37°C with a piece of desalted pig intestine as the membrane. Following the blood meal, fully engorged females were selected and incubated at 28°C and 70% relative humidity under a 12-h light and 12-h dark cycle with permanent access to 10% sucrose.

PCR and RT-PCR. Total DNA was extracted from the mosquito cell lines or individual mosquitoes with a NucleoSpin tissue kit (Macherey-Nagel). Total RNA was extracted from the mosquito samples with the TRIzol reagent (Invitrogen). Following DNase I (Promega) treatment, cDNA was synthesized with Maxima H Minus reverse transcriptase and oligo(dT) primers according to the manufacturer's instructions (Thermo Fisher Scientific). PCR and nested PCR were performed with DreamTaq DNA polymerase (Thermo Fisher Scientific). The sequences of the primers are provided in Table 2.

Transcriptome data analysis. Individual midgut libraries of *A. aegypti* mosquitoes infected with DENV1 or DENV3 were prepared from total RNA extracts from individual midguts after quality control

with a Bioanalyzer RNA 6000 kit (Agilent). Purification and fragmentation of mRNA, cDNA synthesis, end repair, A tailing, Illumina index ligation, and PCR amplification were performed using a TruSeq RNA sample preparation (v2; Illumina), followed by a cDNA quality check by use of a Bioanalyzer DNA 1000 kit (Agilent). Libraries were diluted to 10 pM after Qubit quantification (Thermo Fisher Scientific), loaded onto a flow cell, and clustered with TruSeq SR Cluster kit v3-HS on a cBot system (Illumina). Single-end reads of 51 nucleotides in length were generated on a HiSeq2000 sequencing platform (Illumina). Sequencing reads with a quality score of <30 were trimmed using the Cutadapt program (<https://cutadapt.readthedocs.io/en/stable/>). Passing-filter reads were mapped to *A. aegypti* transcripts (AaegL3.1; <http://vectorbase.org>) using the Bowtie 2 tool and then processed with the SAMtools suite to create a matrix of raw counts used for gene expression analysis by the DESeq2 package.

Small RNA and genome DNA libraries. To analyze small RNA production from ALFEs and AEFEs, we used small RNA libraries of *A. albopictus* and *A. aegypti* mosquitoes infected or not infected with CHIKV that are publically available in the Sequence Read Archive under accession number SRP062828. For genomic DNA libraries, total DNA was extracted from C6/36 cells and the *A. albopictus* Vietnam strain using a NucleoSpin tissue kit (Macherey-Nagel). Genomic DNA was then sheared into 200-bp fragments using a Covaris S220 device with the following parameters: peak incident power, 175; duty factor, 10; cycle burst, 200; and duration, 180 s. Genomic DNA libraries were prepared using a Kapa LTP library preparation kit Illumina Platforms (Kapa Biosystems). The library was amplified with 10 PCR cycles, and 2×151 paired-end reads were sequenced on a NextSeq 500 sequencer.

For bioinformatics analysis, the quality of the fastq files was assessed with FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality bases and adaptors were trimmed from each read by use of the Cutadapt program. Only reads with an acceptable quality (Phred score, 20) were retained. A second set of graphics was generated by the FastQC software using the fastq files trimmed by the Cutadapt program. Reads were mapped to target sequences using the Bowtie 1 tool with the $-v$ 1 (one mismatch between the read and its target) or the Bowtie 2 tool with default options for the small RNA or DNA library, respectively. The Bowtie 1 tool (small RNA library) and the Bowtie 2 tool (DNA library) generate results in sequence alignment/map (SAM) format. All SAM files were analyzed by the SAMtools package to produce bam indexed files. Homemade R scripts with Rsamtools and Shortreads in Bioconductor software were used for analysis of the bam files.

In silico screening for new EFVEs. To identify all versions of EFVEs in *A. albopictus* mosquito DNA contigs, we developed an iterative bioinformatics pipeline. Each iteration is composed of 4 steps: (i) BLASTN analysis using raw reads from the DNA library as the database and an E value threshold of $1E-20$, (ii) read extraction according to the BLASTN result, (iii) assembly of these reads using the SPAdes (v3) genome assembler, and (iv) extraction of contigs larger than 400 bases.

For the first iteration, the already known ALFE sequences were used as queries. For the following iterations, the contigs selected from the previous iteration were used as queries. Due to the very large number of matches detected by BLASTN analysis after some iterations (repetitive regions from the flanking region of EFVEs), only 5 iterations were used in order to analyze the contigs obtained.

Mass spectrometry. C6/36 and Aag2 cells (10^7) were lysed by sonication (twice for 20 s each time using an ultrasonic probe) in lysis buffer (urea, 6 M; Tris HCl, 150 mM, pH 8.8; β -octyl, 1%; dithiothreitol [DTT], 10 mM). In addition, in order to have an indication of the subcellular location of the proteins of interest, subcellular fractionation of the cell extracts was performed using a commercial kit (subcellular protein fractionation kit for cultured cells; Thermo Fisher Scientific) according to the manufacturer's instructions, resulting in 6 fractions: the cytoplasmic, membrane, soluble nuclear, chromatin-bound, cytoskeletal extract, and insoluble pellet fractions. Proteolysis was performed using a filter-assisted sample preparation strategy. Briefly, proteins were transferred over a filter with a 10-kDa-molecular-mass cutoff (Microcon, Amicon Merck), reduced, and alkylated (for DTT, 10 mM final concentration, 2 h at 37°C ; for iodoacetamide, 50 mM final concentration, 30 min in the dark at room temperature). After the proteins were washed 3 times with ammonium bicarbonate (50 mM), the proteins were proteolysed with trypsin (10 ng modified sequencing-grade trypsin [Roche], 37°C , overnight). The resulting proteolytic peptides were recovered by centrifugation (15 min at $10,000 \times g$), acidified with 0.1% aqueous trifluoroacetic acid, and desalted using C_{18} sample preparation pipette tips (Ziptip C_{18} ; Millipore). The peptides were purified on a capillary reversed-phase column (C_{18} Acclaim PepMap; inside diameter, 75 μm ; length, 50 cm; Thermo Fisher Scientific) at a constant flow rate of 220 nl/min with a gradient of 2% to 40% buffer B in buffer A over 170 min (buffer A, H_2O , acetonitrile [ACN], and formic acid [FA] [98:2:0.1, vol/vol/vol]; buffer B, H_2O , ACN, and FA [10:90:0.1, vol/vol/vol]). The MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) with a top 10 acquisition method (MS resolution, 70,000; mass range, 400 to 2,000 Da), followed by 1 MS/MS run on each of the 10 most intense peaks at a resolution of 17,500 with a dynamic exclusion for 90 s. Raw data were processed using Proteome Discoverer (v2.1) software (Thermo Fisher Scientific). The database search was done with the Mascot search engine (Matrix Science Mascot, v2.2.04) on a homemade protein data bank containing the putative proteins for endogenous viral elements as well as *Aedes* proteins (17,756 sequences). The following parameters were used: MS tolerance, 10 ppm; MS/MS tolerance, 0.02 Da; semitryptic peptides; two miscleavages allowed; partial modifications, carbamidomethylation (on cysteine), oxidation (on methionine), and deamidation (on asparagine and glutamine).

Phylogenetic analysis of AEF1. DNA was extracted from individual *A. aegypti* mosquitoes. PCR was performed for the full-length AEF1 element, and the amplicons were sequenced by the Sanger technique. Forward and reverse sequences were trimmed on the basis of the chromatogram quality and aligned to generate a consensus sequence using the program Geneious (v7) (52). Sequences from all successfully sequenced individuals, closely related ISFs, and medically important flaviviruses were aligned

using the ClustalW program and trimmed to the same length. The program PHYML (53) was used to generate two phylogenetic trees using the PhyML best Akaike information criterion tree and the Fast likelihood-based method. The first tree contains closely related ISFs, medically relevant flaviviruses, and a representative AEFE1 sequence. The second tree was constructed using only the ISF sequences and the AEFE1 sequences from *A. aegypti*. The best nucleotide substitution method was general time reverse (GTR) +G for both trees.

Accession number(s). Sequences are available under NCBI BioProject numbers [PRJNA386455](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA386455) (*Aedes aegypti* transcriptome DENV1 versus the control at 24 and 96 hpi) and [PRJNA386453](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA386453) (*Aedes aegypti* transcriptome DENV1 versus DENV3).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00571-17>.

SUPPLEMENTAL FILE 1, XLSX file, 0.8 MB.

ACKNOWLEDGMENTS

We thank all members of the M.-C. Saleh and L. Lambrechts labs for fruitful discussions, Catherine Lallemand for assistance with mosquito rearing, and Valerie Dorey for help with preparation of the DNA libraries. We are grateful to Christophe Paupy, Diego Ayala, Davy Jiolle, Elisée Nchoutpouen, Karima Zouache, Alongkot Ponlawat, Thanyalak Fansiri, and Isabelle Dusfour for the original mosquito colonies.

This work was supported by the European Research Council (FP7/2013-2019 ERC CoG 615220 to M.-C.S.), the French Government's Investissement d'Avenir Program, Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-10-LABX-62-IBEID to L.L. and M.-C.S.), the City of Paris Emergence(s) Program in Biomedical Research (to L.L.), the European Union's Horizon 2020 Research and Innovation Program under ZikaPLAN grant agreement no. 734584 (to L.L.), and the Conseil Régional Ile de France (Sesame 2010 grant agreement 10022268 to J.V.). Y.S. is supported by Roux-Cantarini postdoctoral fellowships and Japan Society for the Promotion of Science postdoctoral fellowships for research abroad.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

- Holmes EC. 2011. The evolution of endogenous viral elements. *Cell Host Microbe* 10:368–377. <https://doi.org/10.1016/j.chom.2011.09.002>.
- Bejarano ER, Khashoggi A, Witty M, Lichtenstein C. 1996. Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. *Proc Natl Acad Sci U S A* 93:759–764. <https://doi.org/10.1073/pnas.93.2.759>.
- Horie M, Honda T, Suzuki Y, Kobayashi Y, Daito T, Oshida T, Ikuta K, Jern P, Gojobori T, Coffin JM, Tomonaga K. 2010. Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature* 463:84–87. <https://doi.org/10.1038/nature08695>.
- Crochu S, Cook S, Attoui H, Charrel RN, De Chesse R, Belhouchet M, Lemasson JJ, de Micco P, de Lamballerie X. 2004. Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. *J Gen Virol* 85:1971–1980. <https://doi.org/10.1099/vir.0.79850-0>.
- Katzourakis A, Gifford RJ. 2010. Endogenous viral elements in animal genomes. *PLoS Genet* 6:e1001191. <https://doi.org/10.1371/journal.pgen.1001191>.
- Belyi VA, Levine AJ, Skalka AM. 2010. Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. *PLoS Pathog* 6:e1001030. <https://doi.org/10.1371/journal.ppat.1001030>.
- Fort P, Albertini A, Van-Hua A, Berthomieu A, Roche S, Delsuc F, Pasteur N, Capy P, Gaudin Y, Weill M. 2012. Fossil rhabdoviral sequences integrated into arthropod genomes: ontogeny, evolution, and potential functionality. *Mol Biol Evol* 29:381–390. <https://doi.org/10.1093/molbev/msr226>.
- Chen XG, Jiang X, Gu J, Xu M, Wu Y, Deng Y, Zhang C, Bonizzoni M, Dermauw W, Vontas J, Armbruster P, Huang X, Yang Y, Zhang H, He W, Peng H, Liu Y, Wu K, Chen J, Lirakis M, Topalis P, Van Leeuwen T, Hall AB, Jiang X, Thorpe C, Mueller RL, Sun C, Waterhouse RM, Yan G, Tu ZJ, Fang X, James AA. 2015. Genome sequence of the Asian tiger mosquito, *Aedes albopictus*, reveals insights into its biology, genetics, and evolution. *Proc Natl Acad Sci U S A* 112:E5907–E5915. <https://doi.org/10.1073/pnas.1516410112>.
- Fischer MG, Hackl T. 2016. Host genome integration and giant virus-induced reactivation of the viroplasm mavirus. *Nature* 540:288–291. <https://doi.org/10.1038/nature20593>.
- Fujino K, Horie M, Honda T, Merriman DK, Tomonaga K. 2014. Inhibition of Borna disease virus replication by an endogenous bornavirus-like element in the ground squirrel genome. *Proc Natl Acad Sci U S A* 111:13175–13180. <https://doi.org/10.1073/pnas.1407046111>.
- Best S, Le Tissier P, Towers G, Stoye JP. 1996. Positional cloning of the mouse retrovirus restriction gene Fv1. *Nature* 382:826–829. <https://doi.org/10.1038/382826a0>.
- Anderson MM, Lauring AS, Burns CC, Overbaugh J. 2000. Identification of a cellular cofactor required for infection by feline leukemia virus. *Science* 287:1828–1830. <https://doi.org/10.1126/science.287.5459.1828>.
- Cammsa-Parks H, Cisar LA, Kane A, Stollar V. 1992. The complete nucleotide sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. *Virology* 189:511–524. [https://doi.org/10.1016/0042-6822\(92\)90575-A](https://doi.org/10.1016/0042-6822(92)90575-A).
- Hoshino K, Isawa H, Tsuda Y, Sawabe K, Kobayashi M. 2009. Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. *Virology* 391:119–129. <https://doi.org/10.1016/j.virol.2009.06.025>.
- Crabtree MB, Sang RC, Stollar V, Dunster LM, Miller BR. 2003. Genetic and phenotypic characterization of the newly described insect flavivirus,

- Kamiti River virus. Arch Virol 148:1095–1118. <https://doi.org/10.1007/s00705-003-0019-7>.
16. Roiz D, Vazquez A, Seco MP, Tenorio A, Rizzoli A. 2009. Detection of novel insect flavivirus sequences integrated in *Aedes albopictus* (Diptera: Culicidae) in northern Italy. Virol J 6:93. <https://doi.org/10.1186/1743-422X-6-93>.
 17. Suzuki Y, Frangeul L, Dickson LB, Blanc H, Verdier Y, Vinh J, Lambrechts L, Saleh M-C. 2017. Uncovering the repertoire of endogenous flaviviral elements in *Aedes* mosquito genomes. bioRxiv <https://doi.org/10.1101/124032>.
 18. Goic B, Stapleford KA, Frangeul L, Doucet AJ, Gausson V, Blanc H, Schemmel-Jofre N, Cristofari G, Lambrechts L, Vignuzzi M, Saleh MC. 2016. Virus-derived DNA drives mosquito vector tolerance to arboviral infection. Nat Commun 7:12410. <https://doi.org/10.1038/ncomms12410>.
 19. Burt FJ, Chen W, Miner JJ, Lenschow DJ, Merits A, Schnettler E, Kohl A, Rudd PA, Taylor A, Herrero LJ, Zaid A, Ng LF, Mahalingam S. 2017. Chikungunya virus: an update on the biology and pathogenesis of this emerging pathogen. Lancet Infect Dis 17:e107–e117. [https://doi.org/10.1016/s1473-3099\(16\)30385-1](https://doi.org/10.1016/s1473-3099(16)30385-1).
 20. Sim S, Dimopoulos G. 2010. Dengue virus inhibits immune responses in *Aedes aegypti* cells. PLoS One 5:e10678. <https://doi.org/10.1371/journal.pone.0010678>.
 21. Brown JE, Evans BR, Zheng W, Obas V, Barrera-Martinez L, Egizi A, Zhao H, Caccone A, Powell JR. 2014. Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the dengue and yellow fever mosquito. Evolution 68:514–525. <https://doi.org/10.1111/evo.12281>.
 22. Lequime S, Lambrechts L. 2017. Discovery of flavivirus-derived endogenous viral elements in *Anopheles* mosquito genomes supports the existence of *Anopheles*-associated insect-specific flaviviruses. Virus Evol 3:vev035. <https://doi.org/10.1093/ve/vev035>.
 23. Forum on Microbial Threats, Board on Global Health, Health and Medicine Division. 2016. Global health impacts of vector-borne diseases: workshop summary. National Academies Press, Washington, DC.
 24. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, Moore CG, Carvalho RG, Coelho GE, Van Bortel W, Hendrickx G, Schaffner F, Elyazar IR, Teng HJ, Brady OJ, Messina JP, Pigott DM, Scott TW, Smith DL, Wint GR, Golding N, Hay SI. 2015. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. eLife 4:e08347. <https://doi.org/10.7554/eLife.08347>.
 25. Walker T, Jeffries CL, Mansfield KL, Johnson N. 2014. Mosquito cell lines: history, isolation, availability and application to assess the threat of arboviral transmission in the United Kingdom. Parasit Vectors 7:382. <https://doi.org/10.1186/1756-3305-7-382>.
 26. Condreay LD, Brown DT. 1986. Exclusion of superinfecting homologous virus by Sindbis virus-infected *Aedes albopictus* (mosquito) cells. J Virol 58:81–86.
 27. Waldock J, Olson KE, Christophides GK. 2012. *Anopheles gambiae* antiviral immune response to systemic O'nyong-nyong infection. PLoS Negl Trop Dis 6:e1565. <https://doi.org/10.1371/journal.pntd.0001565>.
 28. Sanchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, Wilusz J, Olson KE, Blair CD. 2009. Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway. PLoS Pathog 5:e1000299. <https://doi.org/10.1371/journal.ppat.1000299>.
 29. Morazzani EM, Wiley MR, Murreddu MG, Adelman ZN, Myles KM. 2012. Production of virus-derived ping-pong-dependent piRNA-like small RNAs in the mosquito soma. PLoS Pathog 8:e1002470. <https://doi.org/10.1371/journal.ppat.1002470>.
 30. Leger P, Lara E, Jagla B, Sismeiro O, Mansuroglu Z, Coppee JY, Bonnefoy E, Bouloy M. 2013. Dicer-2- and Piwi-mediated RNA interference in Rift Valley fever virus-infected mosquito cells. J Virol 87:1631–1648. <https://doi.org/10.1128/JVI.02795-12>.
 31. Bryant B, Macdonald W, Raikhel AS. 2010. microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito *Aedes aegypti*. Proc Natl Acad Sci U S A 107:22391–22398. <https://doi.org/10.1073/pnas.1016230107>.
 32. Lucas KJ, Roy S, Ha J, Gervaise AL, Kokoza VA, Raikhel AS. 2015. MicroRNA-8 targets the Wingless signaling pathway in the female mosquito fat body to regulate reproductive processes. Proc Natl Acad Sci U S A 112:1440–1445. <https://doi.org/10.1073/pnas.1424408112>.
 33. Liu S, Lucas KJ, Roy S, Ha J, Raikhel AS. 2014. Mosquito-specific microRNA-1174 targets serine hydroxymethyltransferase to control key functions in the gut. Proc Natl Acad Sci U S A 111:14460–14465. <https://doi.org/10.1073/pnas.1416278111>.
 34. Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD. 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313:320–324. <https://doi.org/10.1126/science.1129333>.
 35. Luteijn MJ, Ketting RF. 2013. PIWI-interacting RNAs: from generation to transgenerational epigenetics. Nat Rev Genet 14:523–534. <https://doi.org/10.1038/nrg3495>.
 36. Miesen P, Joosten J, van Rij RP. 2016. PIWIs go viral: arbovirus-derived piRNAs in vector mosquitoes. PLoS Pathog 12:e1006017. <https://doi.org/10.1371/journal.ppat.1006017>.
 37. Miesen P, Ivens A, Buck AH, van Rij RP. 2016. Small RNA profiling in dengue virus 2-infected *Aedes* mosquito cells reveals viral piRNAs and novel host miRNAs. PLoS Negl Trop Dis 10:e0004452. <https://doi.org/10.1371/journal.pntd.0004452>.
 38. Girardi E, Miesen P, Pennings B, Frangeul L, Saleh MC, van Rij RP. 2017. Histone-derived piRNA biogenesis depends on the ping-pong partners Piwi5 and Ago3 in *Aedes aegypti*. Nucleic Acids Res 45:4881–4892. <https://doi.org/10.1093/nar/gkw1368>.
 39. Arensburger P, Hice RH, Wright JA, Craig NL, Atkinson PW. 2011. The mosquito *Aedes aegypti* has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs. BMC Genomics 12:606. <https://doi.org/10.1186/1471-2164-12-606>.
 40. Parrish NF, Fujino K, Shimoto Y, Iwasaki YW, Ha H, Xing J, Makino A, Kuramochi-Miyagawa S, Nakano T, Siomi H, Honda T, Tomonaga K. 2015. piRNAs derived from ancient viral processed pseudogenes as transgenerational sequence-specific immune memory in mammals. RNA 21:1691–1703. <https://doi.org/10.1261/rna.052092.115>.
 41. Zhang P, Kang JY, Gou LT, Wang J, Xue Y, Skogerboe G, Dai P, Huang DW, Chen R, Fu XD, Liu MF, He S. 2015. MIWI and piRNA-mediated cleavage of messenger RNAs in mouse testes. Cell Res 25:193–207. <https://doi.org/10.1038/cr.2015.4>.
 42. Goh WS, Falcatori I, Tam OH, Burgess R, Meikar O, Kotaja N, Hammell M, Hannon GJ. 2015. piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis. Genes Dev 29:1032–1044. <https://doi.org/10.1101/gad.260455.115>.
 43. Post C, Clark JP, Sytnikova YA, Chirn GW, Lau NC. 2014. The capacity of target silencing by *Drosophila* PIWI and piRNAs. RNA 20:1977–1986. <https://doi.org/10.1261/rna.046300.114>.
 44. Goic B, Vodovar N, Mondotte JA, Monot C, Frangeul L, Blanc H, Gausson V, Vera-Otarola J, Cristofari G, Saleh MC. 2013. RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model *Drosophila*. Nat Immunol 14:396–403. <https://doi.org/10.1038/ni.2542>.
 45. Chiba S, Kondo H, Tani A, Saisho D, Sakamoto W, Kanematsu S, Suzuki N. 2011. Widespread endogenization of genome sequences of non-retroviral RNA viruses into plant genomes. PLoS Pathog 7:e1002146. <https://doi.org/10.1371/journal.ppat.1002146>.
 46. Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, Debruyjn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, et al. 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 316:1718–1723. <https://doi.org/10.1126/science.1138878>.
 47. Powell JR. 2016. Mosquitoes on the move. Science 354:971–972. <https://doi.org/10.1126/science.aal1717>.
 48. Lan Q, Fallon AM. 1990. Small heat shock proteins distinguish between two mosquito species and confirm identity of their cell lines. Am J Trop Med Hyg 43:669–676. <https://doi.org/10.4269/ajtmh.1990.43.669>.
 49. Fansiri T, Fontaine A, Diancourt L, Caro V, Thaisomboonsuk B, Richardson JH, Jarman RG, Ponlawat A, Lambrechts L. 2013. Genetic mapping of specific interactions between *Aedes aegypti* mosquitoes and dengue viruses. PLoS Genet 9:e1003621. <https://doi.org/10.1371/journal.pgen.1003621>.
 50. Caron M, Grard G, Paupy C, Mombo IM, Bikie Bi Nso B, Kassa Kassa FR, Nkoghe D, Leroy EM. 2013. First evidence of simultaneous circulation of three different dengue virus serotypes in Africa. PLoS One 8:e78030. <https://doi.org/10.1371/journal.pone.0078030>.
 51. Fontaine A, Jiolle D, Moltini-Conclois I, Lequime S, Lambrechts L. 2016. Excretion of dengue virus RNA by *Aedes aegypti* allows non-destructive

- monitoring of viral dissemination in individual mosquitoes. *Sci Rep* 6:24885. <https://doi.org/10.1038/srep24885>.
52. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>.
53. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59:307–321. <https://doi.org/10.1093/sysbio/syq010>.