RNA virome of sylvatic mosquitoes from northeast Brazil reveals a divergent and diverse insect-specific viral community

Alexandre Freitas da Silva[1,2*], Laís Ceschini Machado[1], Luisa Maria Inácio da Silva[1], Filipe Zimmer Dezordi[1,2] and Gabriel Luz Wallau[1,2,3*]

[1] *Departamento de Entomologia, Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil.*
[2] *Núcleo de Bioinformática, Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil.*
[3] *Department of Arbovirology, Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Center for Arbovirus and Hemorrhagic Fever Reference and Research. National Reference Center for Tropical Infectious Diseases. Bernhard-Nocht-Straße 74, 20359 Hamburg - Germany.*
*Corresponding author

Abstract

Mosquitoes can transmit several pathogenic viruses to humans, but their natural viral community is also composed of many insect-specific viruses (ISV) that modulate arthropod-borne virus (arbovirus) transmission and mosquito fitness. Besides a growing number of studies investigating the mosquito virome the majority are focused on few urban species and relatively little is known about the virome of sylvatic mosquitoes. Here, we characterized the RNA virome of 10 sylvatic mosquitos species from Atlantic forest remains at a sylvatic-urban interface in Northeast Brazil employing a metatranscriptomic approach. We detected a total of 21 viral families. The phylogenetic reconstructions of 13 viral families revealed that the majority of the sequences are putative ISVs. The phylogenetic positioning and, in most cases, the association with a high RdRp amino acid divergence from other known viruses suggests that the viruses characterized here represent 60 new viral species. Therefore, the sylvatic mosquitoes viral community revealed a predominance of highly divergent viruses highlighting the limited knowledge we still have about the natural virome that infect these blood feeding insects. Moreover, we found a strong species-virome association as none of the viruses recovered were shared between the species investigated. These background findings will help to understand the interactions and coevolution between mosquito and viruses in nature.

Keywords: Metatranscriptome sequencing; virus discovery; viromics; vectors; symbionts

1. Introduction

Mosquitoes are blood-sucking insects that transmit several pathogens to humans and animals (Cox, 2010; Manrique-Saide et al., 2010; Wilson and Schlagenhauf, 2016). In the last few years an increasing number of studies are showing that mosquitoes carry a much larger diversity of viruses (Atoni et al., 2019; Pettersson et al., 2019; Shi et al., 2016; Xia et al., 2018) including the most abundant and prevalent insect-specific viruses (ISVs) when compared to the most studied arthropod-borne viruses (arboviruses) (Bolling et al., 2015; de Almeida et al., 2021). Arboviruses infect vertebrates including humans while ISVs are unable to do so. But there is mounting evidence showing the importance of ISVs interference on arboviruses replication, impacting mosquito vector competence (Bolling et al., 2015; Laureti et al., 2020; Patterson et al., 2020). Some example of ISVs and arbovirus replication interference are: Palm Creek Virus - West Nile virus (Hall-Mendelin et al., 2016); Nhumirim virus - Zika virus and Dengue virus 2 (Romo et al., 2018) and Eilat virus - Venezuelan equine encephalitis, Eastern equine encephalitis virus, Chikungunya virus and West equine encephalitis virus (Nasar et al., 2015).

The discovery of viruses including mosquito-borne viruses is historically a laborious process requiring cell isolation and classical virology analysis (Mokili et al., 2012; Shi et al., 2018; Zhang et al., 2019). Moreover, most viruses are not amenable to cell isolation in laboratory conditions creating a substantial bias in our understanding of the natural viral communities (Shi et al., 2018). In the last three decades the revolution in nucleic acid sequencing has opened new possibilities to more comprehensively characterize the viral communities. Among several strategies available, bulk metatrancriptome sequencing is one of the less biased approaches for RNA virus genome sequencing (Zhang et al., 2018). Coupled with large scale metagenomic sequencing, new bioinformatic tools are accelerating virus discovery and characterization (Ibañez-Lligoña et al., 2023; Nooij et al., 2018). Still, continued development and integration of new tools is required both because of the increasing data volume and the ever increasing difficulty to characterize highly divergent virus genomes (virome dark matter) which compose a large fraction of every virome (Mokili et al., 2012).

The majority of mosquito viruses were identified in species from *Culex, Anopheles* and *Mansonia* genera due to a focus on epidemiological important genera (Atoni et al., 2019; de Almeida et al., 2021; Moonen et al., 2023). Hence, the large majority of the mosquito species diversity which likely maintain viral pathogens transmission cycle in the sylvatic environment have not been accessed regarding its virome composition. Yet, due to our narrow view of the virosphere and particularly the mosquito viral communities, every new mosquito virome study has revealed many novel viruses. For instance, a recent review of mosquito virome has shown that 14 mosquito genera were positive for viruses which were assigned into 102 viral families (Moonen et al., 2023).

In Brazil, there are some scattered studies focusing on characterizing the virome of sylvatic mosquitoes covering different genera such as *Anopheles, Aedes, Culex, Psorophora, Sabethes, Coquillettidia* and *Mansonia* sampled at different biomes such as Amazon, Cerrado, Pantanal and Atlantic forest (da Silva et al., 2021; da Silva Ferreira et al., 2020a; Maia et al., 2019a; Pinto et al., 2017, 2017; Scarpassa et al., 2019a). However, some biomes cover extensive territory and more comprehensive spatiotemporal sampling of mosquitoes and viromes are necessary to characterize these diverse mosquito symbionts.

Here, we sequenced the metatranscriptome of ten different mosquito species sampled at a sylvatic-urban interface of the Atlantic forest in Northeast Brazil aiming to characterize its RNA virome composition and potential viral threats to humans. We demonstrated that these viromes harbor a total of 21 different viral families. The newly discovered viruses exhibit high divergence from known viruses, while the mosquito genera and species demonstrate virome profiles. Although the majority of viruses in our analysis clustered with previously identified mosquito viruses, the low RdRp amino acid identity suggests that they belong to new viral species.

Material & Methods

Sample collection and species identification

Collected mosquito samples were taxonomically identified using available literature for neotropical Culicidae (Forattini, 2002). All specimens were processed in a cooled bench (~15-20ºC) to restrict RNA degradation. After morphological identification, each specimen had its abdomen dissected and processed to DNA extraction using the protocol from (Ayres et al., 2003). We amplified the Cytochrome c oxidase subunit I (COI) for each abdomen DNA using the GoTaq (Promega) protocol, and carried out sequencing using the Sanger method on an ABI 3500xL (Applied Biosystems), following the manufacturer's instruction. Electropherograms were analyzed on Geneious Prime® 2020.0.5 to extract the consensus fasta sequence representing the barcodes (COI) that were identified through BLASTN (Altschul et al., 1990) analysis against a custom database composed by NCBI and BOLD mosquito COI sequences retrieved on March 4th 2020 and a database of mitochondrial genomes from local mosquitoes available from da Silva et al., (2020). The reconstructed phylogenetic tree of the COI sequences corroborated with the previous similarity searches (**Supplementary fig. 1**).

RNA extraction and sequencing

Based on previous mosquitoes identification, specimens from the same taxon were pooled and macerated using 40 µL of ultrapure water per specimen. One hundred microliter (µl) of macerated samples were used to extract the total RNA following the Trizol protocol (Invitrogen, Carlsbad, CA, USA). After the RNA elution in a final volume of 30 ul, the samples were processed for DNAse reaction using TURBO™ DNase (2u/ul - Ambion)

protocol following the manufacturer's instructions. RNA samples were quantified and quality checked through Qubit RNA HS kit and Bioanalyzer respectively. The RNA samples were processed for the ribosomal RNA depletion with the RiboMinus™ Eukaryote System v2 kit following manufacturer's instructions and the sequencing library was prepared using the TruSeq Stranded Total RNA library kit and sequenced on a NextSeq 500 Illumina platform using a paired-end approach of 75 bp.

Virome characterization and taxonomic classification

The viromes were characterized following the study workflow presented in **Supplementary fig. 2**. The sequenced reads were firstly quality checked using FastQC tool followed by trimming of low quality reads using Trimmomatic (Bolger et al., 2014) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36 and TruSeq2-PE as the adapters file to be removed. Metatranscriptome assemblies were generated using three different assembly tools: Trinity v.2.11 (Grabherr et al., 2011), rnaSpades (Bushmanova et al., 2019) and metaSpades (Nurk et al., 2017) in default mode. All contig sequences obtained from the three different assembly tools described above were clustered using the CD-HIT-EST tool with the following parameters: -c 0.98 -G 0 -aS 0.9 -g 1 -n 9, in order to exclude redundant sequences having >= 98% of identity. In order to identify and recover the viral sequences from the assembled metatranscriptomes, we firstly download protein sequences assigned with the viral taxonomy tag (txid10239) from the National Center for Biotechnology Information (NCBI) on December 21th, 2020. The sequences were clustered using the CD-HIT tool (Li and Godzik, 2006) with the following parameters: -G 0 -aS 0.95 -g 1 -M 100000 -n 5 aiming to exclude the redundant sequences. The sequenced viral contigs were identified using DIAMOND (Buchfink et al., 2015) performing a blastx search against the retrieved viral proteins. False positive viral hits were removed through a second DIAMOND blastx search against the Non-redundant protein database (NR) from NCBI. Only contigs where the first 5 hits were of viral origin were kept for further analysis.

In order to evaluate viral genome/segment completeness of the identified viral contigs we used the ViralComplete module from Metaviralspades tool (Antipov et al., 2020). Briefly, the ViralComplete analysis predicts the genes and the viral proteins from the contigs and it performs a BLAST analysis against the viral RefSeq databases. Moreover, the tool compares if the length of analyzed contig is similar to the viral hit and then classifies them into full-length or partial sequences. Additional similarity searches were performed to assign the best hit for each contig and predicted proteins using BLASTN and DIAMOND blastp program respectively using the databases with the viral assignment (txid10239). All BLASTN and DIAMOND analysis were run using the E-Value of $1E^{-3}$. All DIAMOND analyses were run using the more-sensitive set up.

To further in depth characterize more distant related viral contigs that might had been missed from the DIAMOND search, we performed additional searches using Hidden Markov models (HMM) profiles from Reference Viral Database (RVDB-prot-HMM) using HMMER3 (http://hmmer.org/). Briefly, we predicted the proteins from the metatranscriptomes with the

Prodigal tool (Hyatt et al., 2010) using the flag meta. The predicted protein sequences were used in the hmmsearch analysis against the RVDB HMM database. Hits showing E-Value of $1E^{-3}$ were selected for further analysis.

The best hit from DIAMOND blastp analysis for each query and the best hit for HMM analysis were used to obtain the viral taxonomy information of contigs. To assign taxonomic information to the viral contigs we only considered those with annotation for the hallmark RdRp. Since RdRp naming in both NCBI sequences or HMM profiles can vary, we performed searches for different terms aiming to filter the RdRp sequences (**Supplementary table 1**). To obtain the virome profile, we utilized the set of sequences with RdRp hallmark, for sequences identified by both methodologies, we used the taxonomic classification obtained by DIAMOND. For sequences that showed similarity with unclassified viruses we performed phylogenetic analysis and reclassified them based on annotation transfer from the closest homologs from high supported clades. After viral identification the reads were mapped against identified viral contigs and were submitted to European Bioinformatic Institute under the project number: PRJEB63303 under the Accessions: ERR11583309-ERR11583318.

Phylogenetic analysis

Phylogenetic trees were reconstructed for each viral family that showed RdRp contigs larger than 1,000 bp and amino acid sequences larger than 100 aa. The protein sequences used in the phylogenetic analysis were recovered based on the best fifty hits shown in a DIAMOND analysis for each identified RdRp viral protein. The viral proteins recovered from the same viral family were clustered using CD-HIT with the parameters: -G 0 -aS 0.95 -g 1 -n 5 and the remaining sequences were aligned together with the identified viral protein sequences using MAFFT v.7 (Katoh and Standley, 2013) using default parameters. The non-aligned blocks were removed from the alignment using the Trimal tool (Capella-Gutiérrez et al., 2009). The phylogenetic trees were reconstructed using the IQ-TREE 2.0 (Nguyen et al., 2015) and the best evolutionary model for each alignment was selected by the ModelFinder (Kalyaanamoorthy et al., 2017) implemented in the same tool. The node supports were evaluated with 1,000 replicates of ultrafast bootstrapping (Hoang et al., 2018). The consensus trees were visualized and annotated with the ggtree package from R programming language (Yu et al., 2017).

The phylogenies of mosquitoes were reconstructed using the same approach cited above. In brief, draft mitochondrial genomes for each mosquito species were assembled using the metatranscriptome data on MITObim 1.9 (Hahn et al., 2013). The mitogenomes were aligned using MAFFT v.7 and and trimmed using Trimal. The phylogenetic tree was obtained and plotted using IQ-TREE and ggtree as previously cited. The COI phylogeny was reconstructed using the same approach.

Results

A total of 194 specimens were processed and identified at species level applying both morphological and barcode sequencing approaches with exception for *Ae. albopictus* that was identified only by morphological characters. The mosquito species were splitted into ten pools representing the following species: *Mansonia titillans, Mansonia wilsoni, Ae. albopictus, Psorophora ferox, Aedes scapularis, Coquillettidia chrysonotum, Coquillettidia. venezuelensis, Limatus durhamii, Coquillettidia hermanoi, Coquillettidia albicosta* (**Supplementary table 2**).

The Illumina sequencing generated a total of 94.29 Gbp representing 593.3 million of paired end reads distributed along the ten mosquito pools (**Table 1)**. The generated reads per pools ranged from 47.1 million of paired reads for *Ae. scapularis* to 69.1 million of paired reads for *Cq. chrysonotum* (**Table 1)**. A total of 1,971,030 contigs were generated from the three assembly approaches (Trinity, metaSpades and rnaSpades), while 1,285,976 were retained after redundancy removal (**Table 1)**.

A total of 33,919 viral contigs were identified using the two approaches (DIAMOND and HMM analysis) (**Figure 1A-I**). Thirty-one thousand were identified only using the HMM searches. While 998 contigs were identified using DIAMOND analysis. One thousand one hundred sixty eighth contigs were identified for both approaches (**Figure 1A-I**). However, only 421 out of ~30 thousand viral contigs contained RdRp annotation that were used for the virome characterization (**Figure 1A-II**). One hundred twelve contigs were only identified by DIAMOND, while only 39 were identified by HMM analysis and 270 combining sequences identified from both approaches respectively (**Figure 1A-II)**. Here, we identified 21 different viral families using the two approaches for viral identification based on analysis of sequences containing RdRp markers (**Figure 1A-III**). Six viral families (Chuviridae, Dicistroviridae, Endornaviridae, Iflaviridae, Luteoviridae, Nodaviridae) were identified using only DIAMOND and only one (Caliciviridae) using exclusively HMMs (**Figure 1A-III**). Thirteen viral families (Aliusviridae, Chrysoviridae, Flaviviridae, Narnaviridae, Orthomyxoviridae, Partitiviridae, Phasmaviridae, Phenuiviridae, Rhabdoviridae, Solemoviridae, Totiviridae, Virgaviridae, Xinmoviridae) and unclassified viral sequences were identified by both approaches (**Figure 1A-III**). The majority of the RdRp containing viral contigs were classified into known viral families (**Figure 1B**). Moreover, 4 genomic architectures were found: the majority belonged to ssRNA(+), followed by ssRNA(-) and few viral contigs were annotated as dsRNA or RNA viruses only (**Figure 1C**). From 421 contigs representing the viral sequences analyzed on ViralComplete, we identified 20 sequences from 9 viral families (Orthomyxoviridae, Partitiviridae, Phasmaviridae, Phenuiviridae, Rhabdoviridae, Solemoviridae, Totiviridae, Virgaviridae and Xinmoviridae) classified as full-length representing segments for segmented viruses or linear complete genome (**Table 1** and **Supplementary fig. 3**), while the remainder sequences were classified as partial. The full-length sequences with RdRp annotation ranged from 1.75 kb to 13.08kb representing a

Partitiviridae segment and a complete genome from the Xinmoviridae family, respectively (**Supplementary fig. 4**).

Table 1. General sequencing and viral identification statistics.

Species name	Library name	Million of Raw paired reads	Million of trimmed paired reads*	Total contigs**	Total clustered contigs	Total identified as viral***	Full-length RdRp seqs****
Ma. wilsoni	P8	50.20	47.1	204,403	129,918	90	5
Cq. venezuelensis	P2	65.40	61.8	161,589	99,069	62	4
Li. durhamii	P5	51.40	48.5	139,978	80,031	55	2
Cq. chrysonotum	P6	69.50	65.6	175,237	115,351	50	1
Ma. titillans	P10	68.40	64.4	210,612	132,164	49	3
Ae. albopictus	P7	69.10	64.5	260,363	178,599	46	2
Cq. hermanoi	P9	58.80	54.7	289,556	192,588	32	0
Cq. albicosta	P1	61.30	57.1	263,504	178,387	23	1
Ae. scapularis	P3	47.10	44.3	139,261	90,437	8	2
Ps. ferox	P4	52.10	48.5	126,527	89,432	6	0
Total	-	593.30	556.5	1,971,030	1,285,976	421	20

*Number considering the paired reads plus the unpaired. ** Considering Trinity, rnaSpades and metaSpades assemblies .*** Considering only those viral contigs with RdRp markers.**** Analysis from ViralComplete.

Regarding the mosquito species, the *Ma. wilsoni, Cq. venezuelensis* and *Li. durhamii* showed the highest viral content, respectively (**Table 1**). While, *Ps. ferox* and *Ae. scapularis* showed the smallest number of viral contigs (**Table 1**). The viral contig sequences showed a wide length distribution ranging from 104 bp up to 13,088 kb with a median value of 454 bp (**Supplementary fig. 5**). We were able to recover 5 full-length viral sequences for *Ma. wilsoni* assigned in Xinmoviridae (N=1), Orthomyxoviridae (N=3) and Phenuiviridae (N=1) families, while only one full-length sequence for each *Cq. albicosta* and *Cq, chrysonotum* species, representing viruses from Rhabdoviridae and Solemoviridae respectively (**Table 1, Supplementary fig. 4** and **Supplementary table 3**). We did not recover full-length sequences for *Ps. ferox* and *Cq. hermanoi* species.

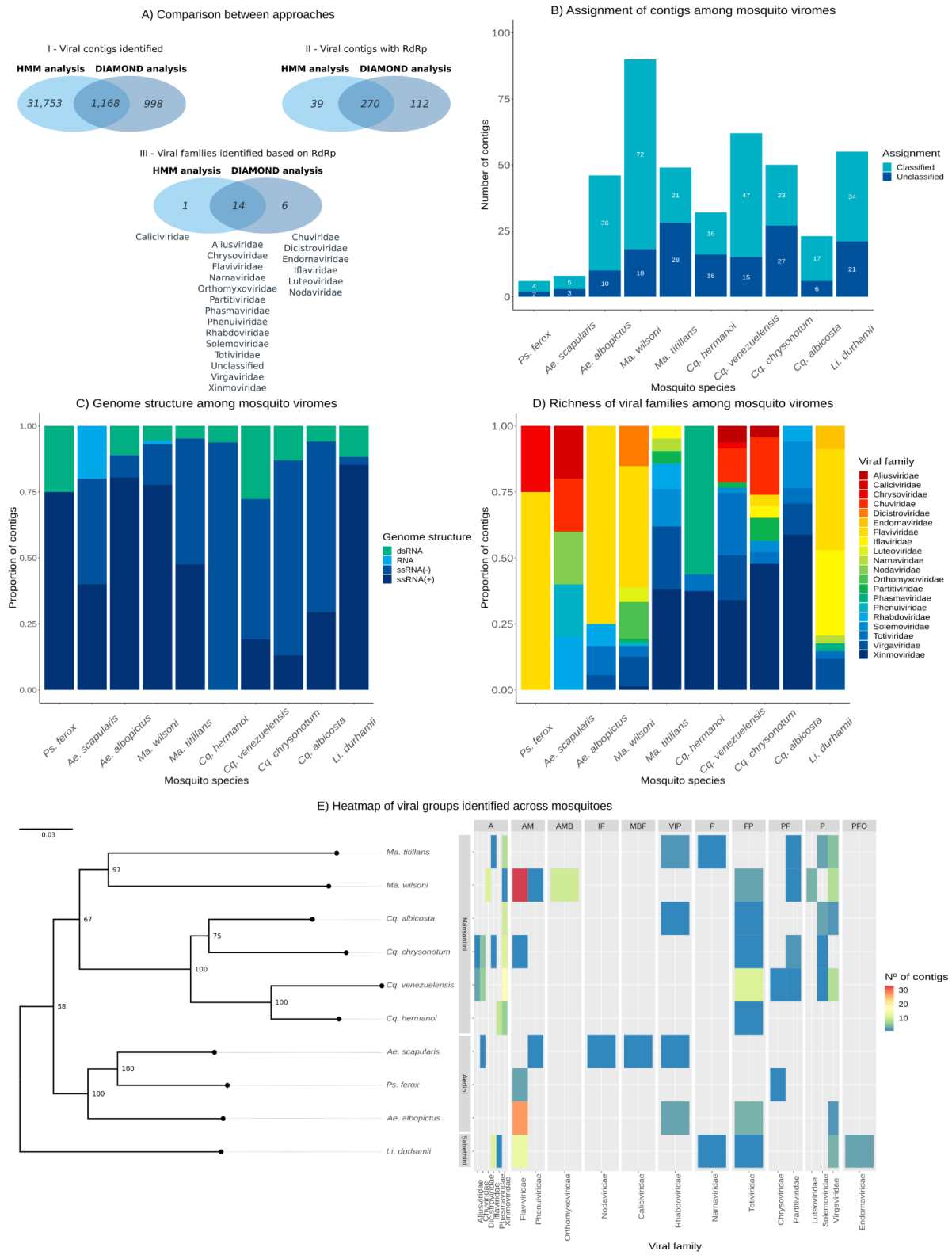

Figure 1. General information on viral identification. A) Venn diagrams illustrating the overlap of viral contigs and families identified using different viral discovery approaches. B) Stacked plot presenting the number of viral contigs assigned to known viral families or not assigned across mosquito viromes. C) Stacked plot showing the proportion of different viral genome structures among mosquito viromes. D) Stacked plot displaying the proportion of viral families identified among mosquitoes. Genome structure information for each family

was obtained from the NCBI Virus database. E) Phylogenetic tree of mosquito species and heatmap of viral families identified. The phylogenetic tree was reconstructed based on the mitogenomes assembled from sequenced data using MITObim 1.9 and analyzed on IQ-TREE2.0 performing the ultrafast bootstraping with 1,000 replicates and the evolutionary model GTR+F+G4 selected by the ModelFinder. The root tree was set in an outgroup (*Drosophila melanogaster* - U37541.1 that was pruned from the tree). The Heatmap was splitted in different grids based on different groups of hosts infected by each viral family according to ICTV (www.talk.ictvonline.org/) and ViralZone (www.viralzone.expasy.org/). A - Arthropoda, IF - Insects or Fishes, AM - Arthropoda and mammals, AMB - Arthropods, mammals and birds, MBF - Mammals, birds and fishes, VIP - Vertebrates, invertebrates and plants, F - Fungi, FP - Fungi and protozoa, P - Plants, PF - Plants and Fungi, PFO - Plants, fungi and oomycetes.

The virome profile of the mosquito species investigated here showed a host phylogenetic distinct pattern (**Figure 1D** and **Figure 1E**). Among Aedini mosquitoes we detected highly distinct virome composition: viral sequences belonging to Flaviviridae and Chrysoviridae families were identified in *Ps. ferox*. For *Ae. scapularis* and *Ae. albopictus* we identified between five and four viral families with a patchy distribution of specific viral families (**Figure 1D** and **Figure 1E**). *Ae. scapularis* showed a higher number of viral families in comparison with the remaining Aedini species analyzed in this study. The virome profile of mosquitoes from the Aedini tribe showed known viral families that infect a range of hosts such as Chuviridae, Phenuiviridae, Nodaviridae and Caliciviridae that infect arthropods and vertebrates and Rhabdoviridae which encompass more generalist viruses (**Figure 1E**).

Regarding Mansoniini viromes, a more similar pattern was observed among species within the same tribe compared to those within Aedini species (**Figure 1C-E**). In general Mansoniini mosquitoes showed viral sequences belonging to viral families known to infect Arthropods, Vertebrates, Birds, Fungi and Plants (**Figure 1E**). The species *Ma. wilsoni* was found to have the highest number of viral families from Mansonini species studied here. Nine viral families (Dicistroviridae, Xinmoviridae, Flaviviridae, Phenuiviridae, Totiviridae, Partitiviridae, Luteoviridae and Virgaviridae) were identified in *Ma. wilsoni*. While seven viral families (Iflaviridae, Xinmoviridae, Rhabdoviridae, Narnaviridae, Partitiviridae, Solemoviridae and Virgaviridae) were identified in *Ma. titillans*. The virome of mosquitoes from *Coquillettidia* genus showed a similar viral pattern (**Figure 1C-E**) with a larger amount of contigs from Xinmoviridae family. The viromes of *Cq. venezuelensis* and *Cq. chrysonotum* shared 4 viral families (Xinmoviridae, Totiviridae, Solemoviridae, Partitiviridae). While *Cq. hermanoi* and *Cq. albicosta* presented a lower number of viral families and shared only two viral families (Xinmoviridae and Totiviridae). Different from other *Coquillettidia* species, *Cq. hermanoi* showed the majority of contigs classified into Phasmaviridae. Futhermore, *Cq. albicosta* was the single species of the *Coquillettidia* genus showing viral sequences from Rhabdoviridae family.

Regarding the *Limatus* genus, we identified seven viral families (Iflaviridae, Phasmaviridae, Flaviviridae, Narnaviridae, Totiviridae, Virgaviridae and Endornaviridae) in

Li. durhamii species. The majority of contigs belong to Flaviviridae and Iflaviridae. Furthermore, only this species showed sequences from Endornaviridae (**Figure 1C-E**).

RNA-dependent RNA polymerase evolutionary history

Based on the 21 viral families identified in the mosquito viromes we reconstructed a total of 14 phylogenetic trees representing 13 viral families (Aliusviridae, Chrysoviridae, Flaviviridae, Narnaviridae, Orthomyxoviridae, Partitiviridae, Phasmaviridae, Phenuiviridae, Rhabdoviridae, Solemoviridae, Totiviridae, Virgaviridae, Xinmoviridae) and a phylogenetic tree for viral sequences with unclassified assignment (**Supplementary fig. 6-19**). From now on we will present the results of viral families known to infect arthropods (Aliusviridae, Phasmaviridae and Xinmoviridae) and/or vertebrates (Flaviviridae, Phenuiviridae, Orthomyxoviridae and Rhabdoviridae) (**Figure 2** and **Figure 3**).

Arthropod viruses

Aliusviridae family

For the Aliusviridae family we analyzed phylogenetically six viral sequences identified in *Cq. venezuelensis* (N=4) and *Cq. chrysonotum* (N=2) into a phylogenetic tree representing the Jingchuvirales order (**Figure 2 and Supplementary fig. 6**). The sequences were classified as members of Ollusvirus according to phylogenetic positioning and grouped in a major clade grouping other sequences identified in mosquitoes. All sequences grouped into a subclade together with Atrato Chu−like virus 5 (QHA33675.1), a virus originally identified in *Ps. albipes* with the RdRp identity varying from 55% to 85%.

Xinmoviridae family

For the Xinmoviridae family we reconstructed a tree including sixteen sequences identified in *Cq. albicosta* (N=5), *Cq. venezuelensis* (N=5), *Cq. chrysonotum* (N=1), *Ma. wilsoni* (N=1), *Cq. hermanoi* (N=3) and *Ma. titillans* (N=1) (**Figure 2)**. In general our sequences were placed into two distinct clades and (**Supplementary fig. 7**). The clade I grouping sequences from *Cq. albicosta, Cq. chrysonotum* and *Cq. venezuelensis* together with Aedes anphevirus (AWW01093.1), Xincheng Mosquito Virus (YP_009302387.1) and Culex mononega like virus 1 (QGA70931.1), viruses identified in *Ae. albopictus* cell line RML-12, *Anopheles sinensis* and *Culex spp.* respectively (**Supplementary fig. 7**). However the positioning inside the clade was not supported due the low UFboot value for most of the nodes. The clade II grouped sequences from *Cq. albicosta, Cq. venezuelensis, Cq. hermanoi, Ma. wilsoni* and *Ma. titillans* together with a high UFboot support of 100 (**Supplementary fig. 7**), while the basal sequence from this clade was Culex tritaeniorhynchus Anphevirus (BBQ04822.1), a virus identified in Japan. The identities of our sequences with Culex tritaeniorhynchus Anphevirus ranged from 27% to 47% suggesting a high divergence of

those sequences in relation with sequences from literature. On the other hand, the virus sequences identified in species from the same mosquito genus were more similar and were clustered together as can be seen in the sequences from *Cq. hermanoi* clustered with sequences identified in *Cq. venezuelensis and Cq. albicosta* (**Figure 2** and **Supplementary fig. 7**). The viral sequences identified in *Mansonia* (*Ma. wilsoni* and *Ma. titillans*) mosquitoes were more divergent and clustered together representing a sister branch in relation to *Coquillettidia* viral sequences (**Figure 2** and **Supplementary fig. 7**).

Phasmaviridae family

For the Phasmaviridae family we positioned two sequences representing viruses from *Cq. hermanoi* and *Li. durhamii* (**Figure 2** and **Supplementary fig. 8**). Our sequences were assigned into orthophasmavirus genus and clustered in different clades. The partial sequence from *Cq. hermanoi* was closely with Wuhan Mosquito Virus 2 (YP_009305135.1) and Anjon virus (QGA70910.1), viruses identified in *Culex spp.* and Coredo virus (QHA33845.1) identified in *Ma. titillans* that showed identities with them ranging from 58% to 63% respectively. While the full-length sequence from *Li. durhamii* showed to be more divergent and was placed as basal of a clade grouping with Flen bunya−like virus (QGA87322.1), Aedes phasmavirus (QOI91423.1), Yongsan bunyavirus 1 (YP_009553313.1), viruses identified in *Ae. cantans, Ae. albopictus* and *Ae. vexans nipponii*, respectively. The identities of the sequence from *Li. durhamii* with the other viruses varied from 45% to 46%.

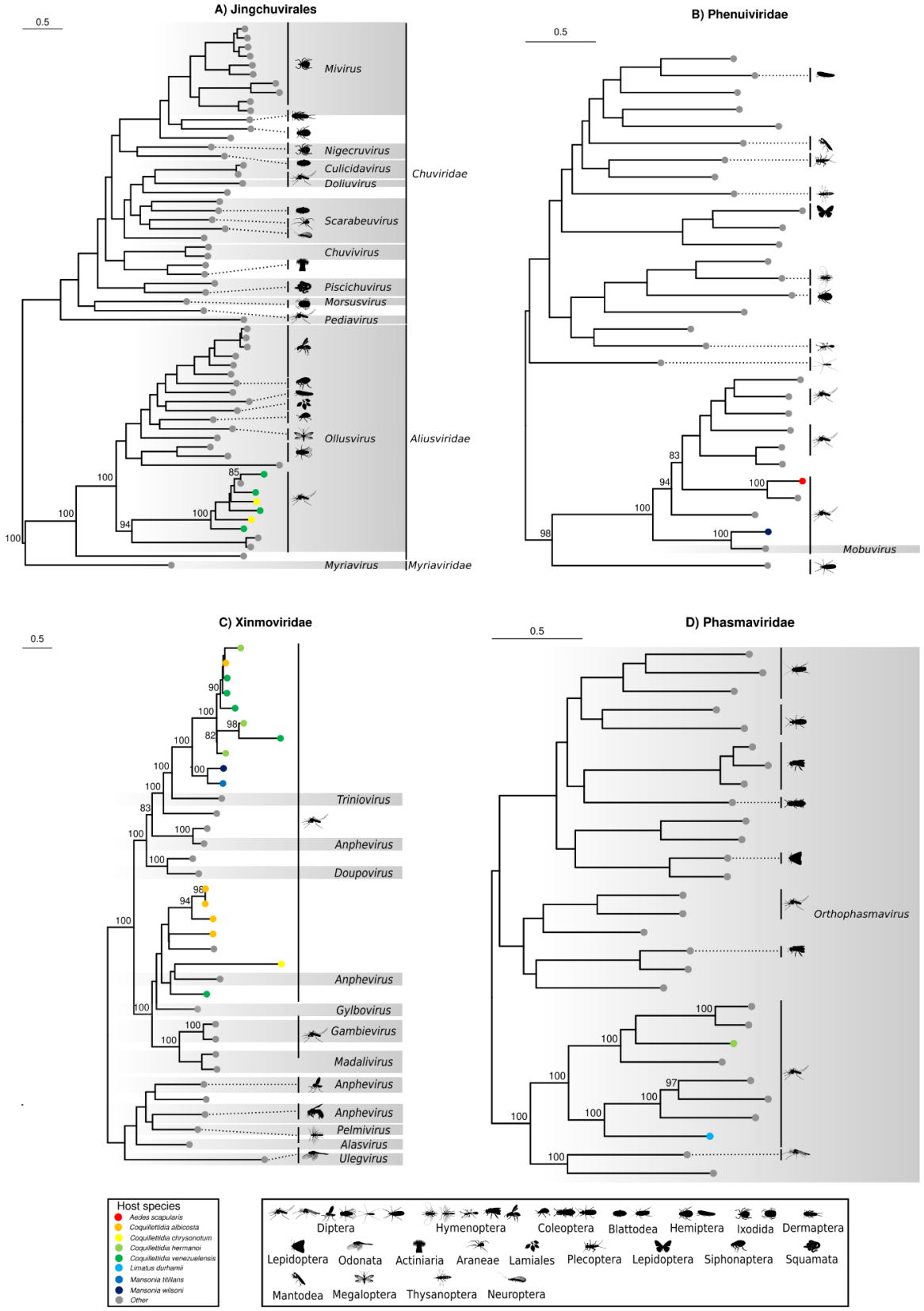

Figure 2. Phylogenetic trees of the Jingchuvirales order, Phenuiviridae, Xinmoviridae and Phasmaviridae families. The phylogenetic trees were reconstructed based on aligned sequences representing the RdRp identified for each viral family and analyzed on

IQ-TREE2.0 performing the ultrafast bootstraping with 1,000 replicates. Full phylogenetic trees can be seen in **Supplementary fig 6-9**. The trees were set as the midpoint root. The tip colors represent the different mosquito host species from this study, while silhouettes represent the host species from the databases. Grey boxes indicate the subgenus of viruses according to information from NCBI and ICTV.

Arthropod and vertebrate viruses

Phenuiviridae family

For the Phenuiviridae family we positioned two sequences representing complete sequences identified in *Ae. scapularis* and *Ma. wilsoni* (**Figure 2** and **Supplementary fig. 9**). The Phenuiviridae viral sequences were grouped in two distinct clades. The *Ma. wilsoni* Phenuiviridae sequence grouped together with the Narangue virus (QHA33858.1) with an amino acid identity of 66%, a virus identified in *Ma. titillans* from Colombia. While the Phenuiviridae sequence from *Ae. scapularis* was placed together with Salarivirus Mos8CM0 (API61884.1) showing an amino acid identity of 67%, a virus identified firstly in non-identified mosquito samples from the United States of America.

Rhabdoviridae family

For the Rhabdoviridae family we included five sequences representing complete genomes detected in *Cq. albicosta, Ma. titillans, Ae. albopictus* and *Ae. scapularis.* In general our sequences were clustered into 3 distinct clades. The Rhabdoviidae sequence identified in *Ae. scapularis* grouped into a clade composed by Atrato Rhabdo−like_virus 3 (QHA33680.1) with an amino acid identity of 51% and Primus virus (QIS62334.1) was placed as a basal branch (**Figure 3 and Supplementary fig. 10**). Those viruses were identified from *Cx. spp.* in Colombia and *Ae. vexans* from Senegal, respectively. The two Rhabdoviridae sequences identified in *Ae. albopictus* clustered into a clade composed by Puerto Almendras virus (YP_009094394.1), Arboretum almendravirus (YP_009094383.1) and Menghai rhabdovirus (YP_009552125.1) where the sequences were more closely with the first one and the last sequences from the literature, respectively. These sequences from literature were identified in *Ae. fulvus* and *Ae. albopictus* from Peru and China, respectively. The high identity of the RdRp sequence (98%) from the Aedes albopictus Rhabdoviridae virus 1 identified in *Ae. albopictus* suggests that it is the same virus identified in *Ae. fulvus* (YP_009094394.1), while the other sequence grouped with Menghai rhabdovirus represents a novel lineage showing 46% of identity based on RdRp sequences. The last Rhabdoviridae clade grouped two *Mansoniini* sequences characterized here, where the sequence for *Ma. titillans* grouped together with the sequence identified in *Cq. albicosta* that showed an amino acid identity between them of 50%. The basal sequence for this grouping was a branch represented by Culex rhabdovirus (QNJ99582.1) that was identified in *Culex spp.* in Switzerland.

Orthomyxoviridae family

For the Orthomyxoviridae family we reconstructed a tree comprising the three subunits that compose the RdRp: PB1, PB2 and PA (**Supplementary fig. 11**). The phylogenetic tree was based on the full-length sequences for the three complete segments: PB1, PB2 and PA of an Orthomyxoviridae virus identified in *Ma. wilsoni.* The PB1 sequence identified here grouped into a high supported clade together with Whidbey virus (AQU42764.1), Guadeloupe mosquito quaranja−like virus 1 (QEM39322.1), Aedes alboannulatus orthomyxo−like virus (ASA47420.1), Wuhan Mosquito Virus 5 (AJG39093.1) and Wuhan Mosquito Virus 3 (AJG39091.1) (**Figure 3** and **Supplementary fig. 11**). However, the positioning of this segment was close to the last one and showed a high divergence with an amino acid identity of 63%.

Flaviviridae family

For the Flaviviridae tree we analyzed ten sequences, however only one clade grouping five sequences containing NS5 domains that clustered the *Ma. wilsoni* Flaviviridae sequences together Mansonia flavivirus (BCI56826.1), a viral sequence identified in *Mansonia spp*. from Bolivia (**Figure 3** and **Supplementary fig. 12**). Based on the amino acid identities, the sequences from *Ma. wilsoni* have suggested a new lineages once the values ranged from 42% to 78%.

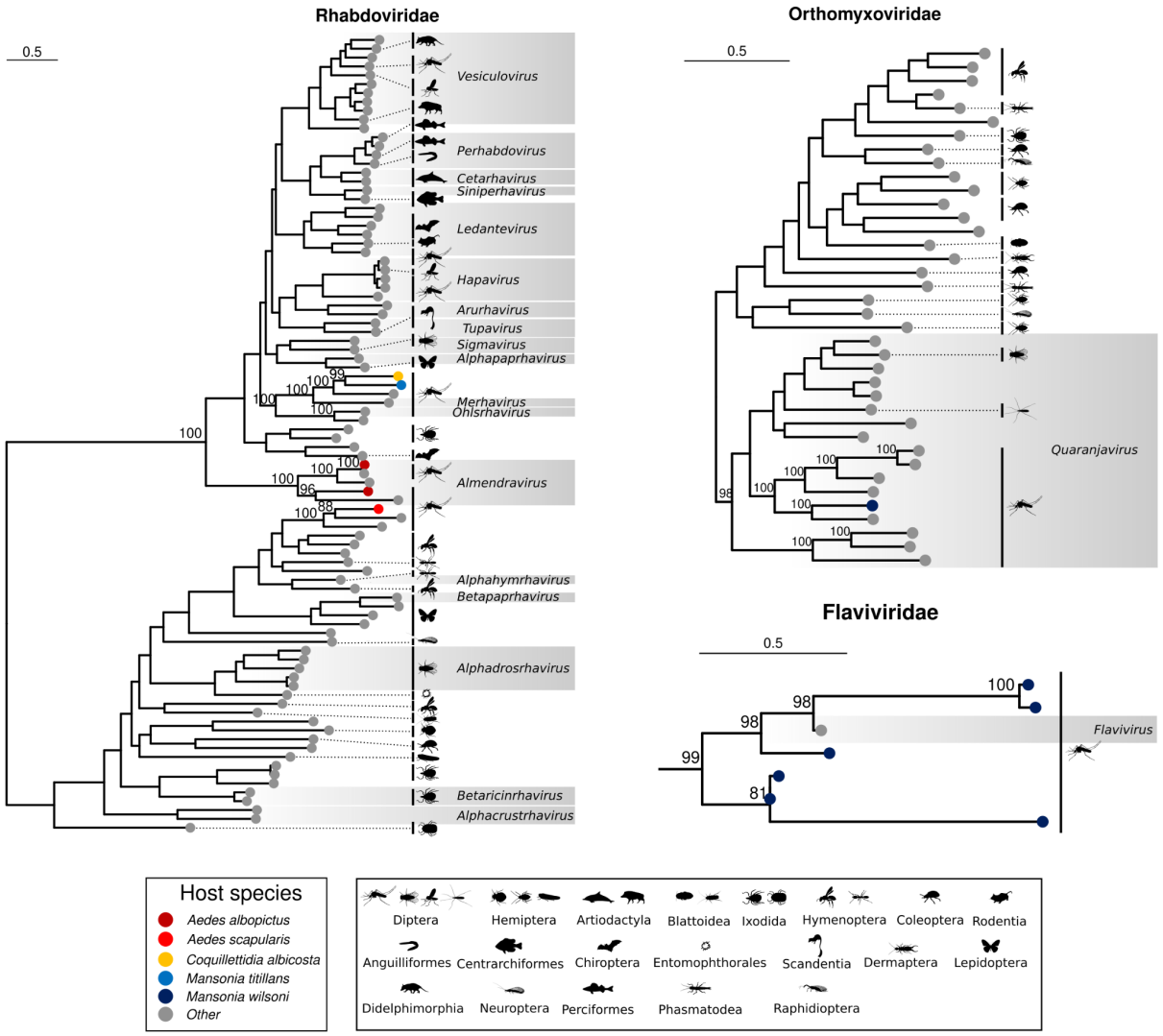

Figure 3. Phylogenetic trees of Rhabdoviridae, Orthomyxoviridae and Flaviviridae families. The phylogenetic trees were reconstructed based on aligned sequences representing or the RdRp for Rhabdoviridae or polyprotein (NS5 region) sequences for Flaviviridae and PB1 subunit for Orthomyxoviridae family and analyzed on IQ-TREE2.0 performing the ultrafast bootstrapping with 1,000 replicates. Full phylogenetic trees can be seen in **Supplementary fig. 10-12.** The trees were set as the midpoint root. The tip colors represent the different mosquito host species from this study, while the silhouettes represent the host species from the databases. Grey boxes indicate the subgenus of viruses according to information from NCBI and ICTV.

Discussion

Mosquitoes carry an abundant and diverse viral community. A recent review has shown that viruses were investigated and detected in at least 14 genera and 128 mosquito species around the globe (Moonen et al., 2023). Most viruses identified in mosquitoes have

been associated with species of the *Culex, Aedes, Anopheles*, and *Mansonia* genera, which have been the focus of extensive research efforts (Atoni et al., 2019; de Almeida et al., 2021; Moonen et al., 2023). Comprehensive mosquito virome data is only available for species of medical importance such as *Ae. aegypti* and *Ae. albopictus* (Moonen et al., 2023; Parry et al., 2021). However, it is crucial to study the virome composition from other mosquito genera and species, mainly those from sylvatic and urban-sylvatic interfaces once the majority of arthropod-borne viruses have emerged and are maintained in the sylvatic environment and can be transmitted to humans through bridge vectors (Weaver, 2005). Hence, the study of sylvatic mosquito viromes could help us to identify novel viruses that are maintained in nature and pinpoint potentially human pathogenic viruses. Some studies characterized the virome for sylvatic mosquitoes from *Haemagogus, Sabethes, Coquillettidia* genera and some species from the *Ochlerotatus* subgenus (Ali et al., 2021; da Silva et al., 2021; Maia et al., 2019b; Öhlund et al., 2019; Thongsripong et al., 2021; Truong Nguyen et al., 2022). In Brazil there have been few studies focusing on the virome of mosquitoes from different biomes such as Cerrado, Pantanal, Amazon and Atlantic forest from Southeast and Northeast Brazil (da Silva et al., 2021; da Silva Ferreira et al., 2020b; da Silva Neves et al., 2021; Maia et al., 2019b; Pinto et al., 2017; Scarpassa et al., 2019b). Thus, in the present study we applied a deep metatranscriptome sequencing and complementary bioinformatic approaches to uncover the virome of ten sylvatic mosquito species collected from Atlantic forest remains in Northeast Brazil.

Our research group was the first to investigate the virome of mosquitoes from the northeast region of Brazil. The virome of two Mansoniini species (*Ma, wilsoni* and *Cq. hermanoi*) showed diverse viral sequences (da Silva et al., 2021) from 8 viral families (Orthomyxoviridae, Xinmoviridae, Phenuiviridae, Rhabdoviridae, Chuviridae, Flaviviridae, Partitiviridae and Virgaviridae). In the present study we could reassess the virome composition of those two mosquito species (*Ma. wilsoni* and *Cq. hermanoi*) and we expanded the knowledge including another eight sylvatic species, some of them studied for the first time regarding their virome composition. The sequenced data allowed us to identify a total of 421 viral sequences bearing RdRp domains representing 21 viral families (**Figure 1**). Our findings have shown the presence of abundant highly divergent insect specific viruses in the virome profiles of mosquitoes studied here and that these viromes are substantially different inside the same mosquito tribe and genus and from the viruses detected around the world.

Virome of *Mansoniini* tribe

For the already studied species (*Ma. wilsoni* and *Cq. hermanoi*) from the *Mansoniini* tribe, we recovered seven viral families already identified in past studies (Orthomyxoviridae, Xinmoviridae, Phenuiviridae, Rhabdoviridae, Flaviviridae and Virgaviridae). Moreover, we characterized another three which were reported for the first time: Dicistroviridae, Totiviridae and Luteoviridae. These viral families are known to infect Arthropods, Fungi/Protozoa and Plants, respectively. However, we have not identified Chuviridae viral sequences in those species as previously identified (da Silva et al., 2021). Our analysis revealed the presence of

two families, Chuviridae and Aliusviridae, from the Jingchuvirales order in the studied mosquito species. These families were exclusively identified in *Cq. venezuelensis* and *Cq. chrysonotum*. Recently, the Chuviridae family was splitted into five viral families within the Jingchuvirales order, including four newly characterized families in addition to Chuviridae (Di Paola et al., 2021). In general, our results for *Mansoniini* were similar as observed in a previous study (Thongsripong et al., 2021) wherein the majority of sequences from *Ma. uniformis* classified into the Xinmoviridae family with exception of *Cq. hermanoi* and *Ma. wilsoni* where the majority of sequences belonged to Phasmaviridae and Flaviviridae families respectively. The high number of Flaviviridae sequences was also found in *Mansonia* mosquitoes (Cholleti et al., 2016). Another study showed the presence of distinct insect-specific flavivirus in different pools of *Mansonia* (*Ma. uniformis* and *Ma. africana*) and *Cq. metallica* mosquitoes (Abílio et al., 2020) that corroborates our finds on the virome profiles suggesting the constant presence of flaviviruses infecting different Mansonini species around the world. In Brazil, the only study assessing viruses from *Ma. wilsoni* has identified a few sequences classified in Totiviridae, Partitiviridae and Chuviridae (Pinto et al., 2017). However those authors used a different mosquito tissue (salivary glands) and sequencing approach, which may explain the differences found (Pinto et al., 2017).

Virome of *Aedini* tribe

Regarding Aedini mosquitoes, some studies have assessed the virome of *Ae. albopictus* and *Ae. aegypti* (He et al., 2021), *Psorophora* species (Charles et al., 2018), *Ochlerotatus* (Truong Nguyen et al., 2022) and *Armigeres* (Thongsripong et al., 2021). Here we analyzed the virome of three Aedini species, our results showed a low diversity of viral families detected (**Figure 1**). The *Ae. scapularis* showed the highest diversity withing the tribe, but with only five viral families, while the *Ae. albopictus* and *Ps. ferox* showed four and two respectively. The Aedini species showed the lowest diversity of viral families in relation to other genera also assessed in the current study such as *Limatus, Mansonia* and *Coquillettidia* mosquitoes (**Figure 1D-E**). Another study has also shown the virome of *Ae. albopictus* with lower number of viral sequences in relation to *Ae. aegypti* mosquitoes in Colombia (Calle-Tobón et al., 2022). On the other hand, a study on the virome of *Ae. albopictus* from China revealed a higher number of identified viral families, with at least 50 families specifically associated with vertebrates, invertebrates, plants, fungi, bacteria, and protozoa hosts (He et al., 2021). Although each study was conducted using distinct mosquito sampling, sequencing and bioinformatics approaches, more studies focusing on the virome of Aedini species in different geographical regions are needed to uncover the main differences among Adini around the world and the viral diversity that these species harbor. In this way, other studies focusing on mosquitoes from Northeast Brazil from different seasons and time points could reveals if Aedini mosquitoes from this region harbor a lower viral diversity in relation to others tribes or if our approach was not able to detect the natural virus diversity fluctuation.

Virome of *Sabethini* tribe

Regarding Sabethini viromes, few studies have been conducted only on species of the *Sabethes* genus (Maia et al., 2019b; Pinto et al., 2017). Maia et al. (2019) identified viruses from Flaviviridae, Chuviridae, Reoviridae, Phenuiviridae and Partitiviridae from *Sabethes gymonothorax* salivary glands sampled in Brazil. The two studies investigating the virome from salivary glands of the *Wyeomyia* genus in Brazil were not able to find viral sequences (Maia et al., 2019b; Pinto et al., 2017). The current study represents the first analysis of a virome of a mosquito from the *Limatus* genus revealing the presence of six viral families (Iflaviridae, Phasmaviridae, Flaviviridae, Narnaviridae, Virgairidae and Endornaviridae) in which Flaviviridae and Iflaviridae families were the most abundant. Although human pathogenic Flaviviridae sequences were identified in this mosquito species (Barrio-Nuevo et al., 2020), the phylogenetically studied sequences were grouped with other ISVs (**Supplementary fig. 12**).

Arthropods and/or vertebrates viruses

We identified some viral contigs belonging to families (Phenuiviridae, Orthomyxoviridae, Rhabdoviridae, Flaviviridae, Nodaviridae and Caliciviridae) known to harbor viruses that infect arthropods and vertebrates (**Figure 1E**). The Phenuiviridae family encompasses arboviruses such as the Rift Valley Virus which is transmitted mainly by *Aedes* and *Culex* mosquitoes (Kwaśnik et al., 2021; Sun et al., 2022). In our study we positioned two Phenuiviridae sequences, one from *Ae. scapularis* that clustered with Salarivirus Mos8CM0 (API61884.1) and one from *Ma. wilsoni* grouping with Narangue virus (QHA33858.1) (**Supplementary fig. 9**). Due to the limited knowledge about these viruses further studies are required to evaluate their effect in different cell lines and their infection capacity in invertebrate and vertebrate cells.

The Orthomyxoviridae also encompass known arboviruses that can infect both arthropods and vertebrates such as Quaranjarviruses (Quaranfil virus) and Thogotovirus (Thogotovirus Thogoto) (Hubálek and Rudolf, 2012; Presti et al., 2009). Here we recovered the Orthomyxovirus previously detected in *Ma. wilsoni* (da Silva et al., 2021). The *Ma. wilsoni* Othomyxoviridae virus 1 was assigned into the quaranjavirus genus. There is evidence of vertebrate and humans cell infection for viruses of this genus highlighting its medical significance (Allison et al., 2015; Taylor et al., 1966).

Viruses from the Rhabdoviridae are generalist and were identified into a wide range of hosts (Dietzgen et al., 2017). The sequences generated in this study clustered with other Rhabdoviridae viruses found in mosquitoes. Interestingly, one clade that clustered the sequences identified in *Ae. albopictus* revealed to be a basal clade encompassing viruses known to infect a wide range of vertebrates and arthropods (**Figure 3**). The sequences of this basal clade have been assigned in Almendravirus genus, which includes mosquito Rhabdoviruses. One sequence identified in *Ae. albopictus* (*Ae. albopitus* Rhabdoviridae virus 1) showed a high amino acid identity with Puerto Almendras virus that was previously

identified in *Aedes fulvus* in Peru (Vasilakis et al., 2014). Although this virus cause no cytopathic effect in vertebrates cells it could be detected in the cell culture supernatant suggesting active replication in vertebrate cells (Vasilakis et al., 2014).

Our phylogenetic analysis revealed that the sequences from Flaviviridae, Rhabdoviridae, Phenuiviridae and Orthomyxoviridae clustered with other ISVs that have been previously characterized although with substantial amino acid divergence. Based on known hosts of these clades, our findings suggest we characterized several mosquito-specific viruses (MSV) (**Figure 2** and **Figure 3**). Our results are consistent with previous studies that have also found a low proportion of pathogenic arboviruses among the discovered viruses (de Almeida et al., 2021; Moonen et al., 2023; Shi et al., 2017, 2016). The MSV capacity to infect vertebrate hosts remains unclear, and further research is needed to assess the infection potential of these novel viruses.

Arthropod viruses

Several viral contigs identified in this study were classified within known viral families bearing members that interfere with arboviruses replication such as Xinmoviridae (Anphevirus genus), Phasmaviridae and Phenuiviridae families. ISVs from those families such as Phasi Charoen virus (PCV) from Phenuiviridae family together Cell fuging agent virus from the Flaviviridae have been shown to interfer with replication of arboviruses such as DENV, ZIKV and La Crosse Virus from Flaviviridae and Peribunyaviridae respectively (Schultz et al., 2018). Furthermore Olmo et al's study showed a wide distribution of Phasi Charoen Like Virus on *Aedes* mosquitoes in different locations and its influence in DENV and ZIKV transmission *in vivo* (Olmo et al., 2023). No viral contig with similarity with this virus was found in our dataset. One study has described the capacity of Aedes anphevirus from Xinmoviridae family to interfering with Dengue virus replication in cell lines (Parry and Asgari, 2018). We found several contigs from both *Mansonia* and *Coquillettidia* genera that were clustered in a major clade with Aedes anphevirus.

In summary, we uncovered the virome of sylvatic mosquitoes from Northeast Brazil. We detected a diverse and species-specific virome largely composed of highly divergent ISVs and particularly MSVs.

Limitation of the study

The analysis of virome composition of eukaryotic species including mosquitoes has revealed a substantial dynamism through time linked with some specific factors that affected the viral richness and abundance (Atoni et al., 2018; Pettersson et al., 2019; Shi et al., 2017; Xia et al., 2018). Factors such as sampling collection in different seasons (Feng et al., 2022; He et al., 2021) or host feeding pattern (Shi et al., 2022) have shown to shape the virome profile. In this study, we did not evaluate the virome composition through time and limited our sampling to a narrow species range distribution, therefore our data is a snapshot of the

viral diversity carried by sylvatic mosquitoes. Moreover, due to the different mosquito tissues, viral enrichment, sequencing and bioinformatic analysis performed in other studies from Brazil and the world we could not directly compare the virome abundance and diversity with our findings. Anyhow, the data presented here is in line with a broad picture found in other studies in the sense that mosquito viromes are mainly composed of highly divergent ISV. Therefore, a more intense sampling and virome characterization using less biased wet and drylab protocols is needed to fully uncover sylvatic mosquitoes viromes in Brazil.

Another factor that can bias the virome findings is the presence of endogenous viral elements (EVEs), which are viral remnants endogenized into host genomes that can be transcribed (de Almeida et al., 2021; Nouri et al., 2018). Insects, including mosquitoes, have been shown to harbor several RNA virus EVEs into their genomes (Wallau, 2022). In order to differentiate EVEs from bonafide circulating viruses one could compare the identified viruses genomes against the host genomes, as performed in previously studies (da Silva et al., 2021; Zakrzewski et al., 2018). However, due to the lack of host genomic seqeunces and small RNA datasets for the mosquitoes investigated we were unable to perform this analysis. Therefore, further assessment of bonafide viruses found here is important to validate the findings. Although some of viral contigs identified warrant further in-depth analysis to discriminate them from EVEs, we identified several complete viral sequences that likely represent true bona fide viruses once the large majority of EVEs are composed of short fragments of viral genomes (Katzourakis and Gifford, 2010; Ter Horst et al., 2019).

Conclusion

In this study we conducted a virome profiling of 10 mosquito species from Northeast Brazil and identified viral sequences from 21 different families, including a total of 20 full-length sequences of viral segments or complete linear genomes. Our phylogenetic analysis revealed that the majority of the newly characterized viruses represent 60 new lineages based on amino acid identities of RdRp. Our study provides important basic knowledge about the virome composition of mosquitoes in Northeast Brazil and highlights several viral families that require further investigation to evaluate their potential to infect vertebrates or affect arbovirus replication. These findings enhanced our understanding of how these viruses are maintained in nature, their interactions with the sylvatic mosquito fauna and the natural viral community that infect those species.

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Author Contributions

Alexandre Freitas da Silva performed mosquito sampling, mosquito identification, data analysis and wrote the original draft. Laís Ceschini Machado performed the mosquito sampling, molecular experiments, contributed to drafting and review of the manuscript. Filipe Zimmer Dezordi performed the mosquito sampling, supported the sequencing data analysis and contributed to drafting and review of the manuscript. Luisa Maria Inácio da Silva performed the mosquito sampling, mosquito identification, molecular experiments and contributed to drafting and review of the manuscript. Gabriel Luz Wallau conceptualized, supervised and administered the project, designed the methodology and wrote, reviewed and edited the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Viral reads that mapped against the viral contigs assembled are available through the European Bioinformatic Institute under the project number: PRJEB63303. Supplementary files are available in Figshare (https://doi.org/10.6084/m9.figshare.23584827.v1).

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