Metagenomic shotgun sequencing reveals host species as an important driver of virome composition in mosquitoes

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High-throughput nucleic acid sequencing has greatly accelerated the discovery of viruses in the environment. Mosquitoes, because of their public health importance, are among those organisms whose viromes are being intensively characterized. Despite the deluge of sequence information, our understanding of the major drivers influencing the ecology of mosquito viromes remains limited. Using methods to increase the relative proportion of microbial RNA coupled with RNA-seq we characterize RNA viruses and other symbionts of three mosquito species collected along a rural to urban habitat gradient in Thailand. The full factorial study design allows us to explicitly investigate the relative importance of host species and habitat in structuring viral communities. We found that the pattern of virus presence was defined primarily by host species rather than by geographic locations or habitats. Our result suggests that insect-associated viruses display relatively narrow host ranges but are capable of spreading through a mosquito population at the geographical scale of our study. We also detected various single-celled and multicellular microorganisms such as bacteria, alveolates, fungi, and nematodes. Our study emphasizes the importance of including ecological information in viromic studies in order to gain further insights into viral ecology in systems where host specificity is driving both viral ecology and evolution.

Viruses are critically important to human and environmental health, and their diversity is predicted to be vast1,2. Next Generation Sequencing (NGS) technology has precipitated the discovery of many viruses, and expanded our knowledge of virus diversity, taxonomy, and evolution3–5. The RNA viruses of arthropod species, including mosquitoes, have been intensively characterized in the past several years, and stand out in their unparalleled diversity6–19.

Disease vectors such as mosquitoes pose a significant threat to public health. One sixth of the illness and disability suffered worldwide is due to vector-borne diseases, many of which are caused by mosquito-borne RNA viruses20. Examples of these viruses are dengue virus of the family Flaviviridae, chikungunya virus of the family Togaviridae, Rift Valley fever virus of the family Phenuiviridae, and Lassa virus of the family Peribunyaviridae. In addition to pathogenic viruses, myriad other mosquito-associated RNA viruses belonging to at least 15 other families do not pose direct public health concerns21.

Most virome studies apply NGS tools to identify new viruses and describe their diversity. This type of study may lead to the discovery of insect-specific viruses that have the ability to interfere with arbovirus transmission22–26. To maximize throughput while minimizing the sequencing cost, discovery-based studies have benefited from combining a large number of insects into a few pooled samples. Often, individuals from different species, or multiple locations, are pooled together hence information on their host-specificity and geographical location is lost27,28. The paucity of ecological information in virome studies limits our understanding of the host

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ranges of these viruses relative to their geographical distribution. As a result, we have little information on the relative importance of ecological drivers determining viral community structure.

In this study, we characterized RNA viruses and other microorganisms in pools of individuals of three common but overlooked vector species in Thailand: Armigeres subalbatus, Culex fuscocephala, and Mansonia uniformis, collected from three habitat types along a previously characterized rural to urban gradient. Our full factorial design (all three mosquito species were collected at all three sites) allows us to explicitly determine the relative importance of host species and habitat in structuring viral communities. As obligate intracellular parasites, viruses rely on specific molecular interactions with hosts to perform their biological functions. Thus, we hypothesize that the pattern of virus presence in our mosquito samples is defined primarily by host species rather than by the host geographical locations or habitats. Finally, to maximize the sensitivity of NGS, we tested a laboratory method to deplete host ribosomal RNA, and increase the relative proportion of microbial genetic material prior to sequencing. This method could help reduce the need to pool samples across locations and species, and maintain ecological information in future virome studies.

Methods

Mosquito collection and RNA extraction. We described study site characteristics and adult mosquito collection in detail in a previous publication. In summary, the study sites were located along a forest to urban landscape gradient in Nakhon Nayok province of central Thailand. Adult mosquitoes were collected during the rainy season of 2008 using a combination of trap types (BG Sentinel, Mosquito Magnet, CDC light trap, and CDC backpack aspirator). In total, over 83,000 adult mosquitoes were collected and transported to the laboratory on dry ice. Species identification based on available morphological keys revealed 109 species of female mosquitoes.

In this study, we included nine samples of three vector species: Ar. subalbatus, Cx. fuscocephala, and Ma. uniformis, each collected from rural, suburban, and urban habitats (Fig. 1). For each sample, we combined 25 female mosquitoes of the same species collected from the same study site during the same sampling period. We visually confirmed that these mosquitoes were not blood-engorged. Mosquitoes were then homogenized in 250 μl of Phosphate Buffered Saline using a Tissue lyser (QIAGEN, USA) and stainless steel beads before mixing with TRIzol LS (Invitrogen, USA) at the ratio of 1:7. Samples were kept at −80 °C until RNA extraction according to the manufacturer’s protocol.

Mosquito specific ribosomal RNA probes, and host ribosomal RNA depletion. In order to increase the relative proportion of microbial RNA, we used biotin-labeled mosquito-specific ribosomal RNA (rRNA) probes to capture and deplete mosquito rRNA prior to sequencing. The procedures for probe construc-
tion and rRNA depletion followed a published protocol. In short, the rRNA probes were created by reverse transcribing *Cx. pipiens* (of a laboratory colony) rRNA gene using a set of custom designed mosquito-specific small subunit (SSU) and large subunit (LSU) ribosomal RNA primers attached with T7 promoter sequences (MEGAscript T7 Transcription Kit, Invitrogen, USA). The primer sequences are listed in Supplementary Table 1. The beads were washed three times to improve depletion efficiency. The resulting rRNA-depleted RNA samples, along with their paired non-depleted RNA samples, were used for next steps. We designate rRNA-depleted sample “RD” and un-depleted control sample “UD” for the rest of this manuscript.

**Sequence processing and viral contig identification.** Raw sequences were filtered for quality and the Illumina adapters were trimmed using Trim Galore! v0.4.5 (https://github.com/FelixKrueger/TrimGalore) using default parameters. The remaining reads were assembled into contigs using Trinity v2.8.4 with default parameters. To identify viruses in the sample, the resulting contigs were compared to the NCBI protein database (downloaded September 25, 2018) using DIAMOND v0.9.22 with an E-value cutoff of 10^{-03}. The best-similarity match was identified as having the lowest E-value.

**Viral phylogenetic analysis and coverage estimation.** In order to increase the coverage of viral genomes, viral contigs belonging to the same virus across samples and across the two preparation types were assembled using Sequencher v5.1 (Gene Codes Corporation, USA). The resulting assembled genomes or partial genomes were checked manually. The open reading frames (ORF) were predicted using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and were confirmed to match viral proteins using NCBI blastp suite. To infer phylogenetic relationships between RNA viruses, selected viral amino acid sequences were aligned using MAFFT v7 employing the E-INS-i algorithm. The best-fit model of amino acid substitutions was determined using ProtTest v3.4.2. Phylogenetic analysis was performed using RAXML blackbox implemented in Cipres Science Gateway application. The resulting phylogenetic trees were visualized in iToL v4.4.1.

The sequence depth and coverage for each viral genome or partial genome was determined by mapping the raw, quality-checked reads to the nucleotide sequences of the viral genome or partial genome using Bowtie2 v2.2.6 and default parameters. Sambtools v1.8 was used to process the resulting SAM file from the Bowtie alignment to calculate the average read depth and coverage.

**Identification of endogenous virus elements (EVEs).** Because the genomes of the three mosquito species are currently unavailable, we cannot definitively exclude EVEs from our data. Instead, we manually checked and removed virus contigs closely related to known EVEs using NCBI blastx. For example, contigs similar to multiple sclerosis associated retrovirus were removed.

**Identification of non-viral microorganisms.** To classify non-viral reads, Kraken v2.0.7-beta was used. The NCBI’s Nucleotide (nt) database was used to build a Kraken-specific reference database. In order to confirm the genus of the organism, SortmeRNA was used to filter rRNA reads from the data. Then, the rRNA reads were used to search for matches using NCBI blastx against the nucleotide database with an E-value cutoff of 10^{-3}. The most similar match was identified as having the lowest E-value.

**Statistical analysis.** To compare virus occurrences across samples, we first normalized the number of reads (i.e. sampling effort) so that they all contained the same number of sequences (n = 418,000, which is the size of the smallest library; Supplementary Table 2). This is done by randomly selecting a subset of raw, quality-checked reads from each sample using Seqtk tool kit (https://github.com/lh3/seqtk). This data set was then assembled de novo using Trinity, searched for viral matches with Diamond, and sequence depths for contigs were determined using Bowtie2 v2.2.6 as described previously. A data matrix containing log-transformed read numbers that mapped to each virus were used as input for principal component analysis (PCA) to assess whether their
pattern of occurrence is determined by host species or habitat. PCA was performed using the “prcomp” function (R v3.5.3), which performs PCA on the given data matrix.

To compare parameter values such as average length of virus contigs, percentage, and number of reads between RD and UD samples, we used Mann–Whitney U test with the alpha level of 0.05. The non-parametric test was chosen because the data were not normally distributed (as revealed by Q–Q plots and the Shapiro–Wilk test). All statistical analyses, and data visualization were performed using R v3.5.3 and Rstudio v1.2.

Criteria used for identifying contaminating virus taxa. A given virus taxon was defined as an external contamination if it met all of the following: (1) it was found in the no-template controls (NTCs); (2) it was represented by ≤ 80 reads (we chose this cutoff number because a majority, or around 97%, of contigs found in NTCs was represented by 80 reads or less); (3) it was found in mosquito samples in a random pattern (i.e. its presence had no species-specific pattern or habitat-specific pattern); and (4) it is not similar to a previously characterized insect-specific virus. In addition, a given viral taxon was likely a cross contamination between samples if it met criteria (1)–(3) mentioned above. We indicated taxa that are likely contaminants or cross-contaminants in Fig. 2. The complete list of virus contigs found in NTCs is shown in Supplementary Table 3. The complete lists of non-viral taxa found in NTCs according to analysis using SortmeRNA and Kraken are shown in Supplementary Tables 4 and 5, respectively.

Results
Read numbers and length. The Illumina MiSeq sequencing generated over 13 million reads. Not including NTCs, the average number of raw reads per sample was 626,988 (SE = 65,936). After quality filtering, the average number of reads dropped to 616,915 (SE = 64,768). Our quality filtering step removed on average 1.53% of raw reads (Supplementary Figure 2). The quality filtered reads were then assembled to produce an average of 5032 contigs per sample (SE = 1216). The average number of assembled contigs per RD sample was significantly higher than in the UD samples (paired t-test, t = 4.2573, p value = 0.003; Supplementary Figure 2). The average read length after the filtering step across all samples was 184 bp (SE = 5). The average contig length was 308 bp (SE = 6). The numbers of reads and contigs for all samples are listed in Supplementary Table 2.

Mosquito viromes. At least 21 putative viruses were identified from all samples. These viruses belong to the order Bunyavirales (family Phenuiviridae, Jonviridae, and an unclassified clade), order Mononegavirales (family Rhabdoviridae and Ximmoviridae), family Flaviviridae, family Iflaviridae, an unclassified clade closely related to Luteoviridae and Sobemovirus (referred to as Luteo-Sobemo-related group8), unclassified clades related to family Narnaviridae, family Novaviridae, and family Picornaviridae (Fig. 2). In addition, we recovered a small number of virus reads (≤ 80) aligning to contigs that were classified in the following groups: Togaviridae, Totiviridae, Tombusviridae, Chuviridae, Orthomyxoviridae, Partitiviridae, and an unclassified clade closely related to nepoviruses. Notable amongst these was the insect-specific Negevirus-like virus, and the virus in the family
**Togaviridae**, which had 98% amino acid similarity to a mosquito-borne zoonotic virus: Getah virus of the genus **Alphavirus**. Detailed information about all viral contigs found in this study, and their most closely related viruses is listed in Supplementary Table 3.

**Virome structure across host species and habitat.** We used Principal Component Analysis to determine how mosquito viromes differ across host species relative to habitats. The results indicate that the virus communities in both the RD (Fig. 3a) and UD (Supplementary Figure 3) samples were grouped based on host species rather than the habitat of mosquito collection. This is in contrast to the bacterial community (Fig. 3b), where grouping patterns did not show association with host or habitat type.

**Depth and coverage: comparison between RD and UD samples.** We tested a laboratory method to deplete host ribosomal RNA from total RNA. Table 1 shows the comparison between samples with (RD) and without (UD) host rRNA depletion and the statistical tests used. The total number of virus contigs and virus groups was higher in RD than in the UD sample set. The average percentage of virus reads per RD sample was higher than in the UD sample, though this was not statistically significant (n = 18, p value = 0.094). The average percentage of viral reads per contig in the RD samples was significantly higher than UD samples (n = 292, p value = 0.023). Successful depletion was apparent only for the small subunit of mosquito ribosomal (SSU) gene.

**Table 1.** Comparison of characteristics between rRNA-depleted samples (RD) and undepleted samples (UD). Statistical analysis was performed using Mann–Whitney U test.

![Figure 3. Similarity of virus community (a) and bacteria community (b) from RD samples. A Principle Component Analysis was performed based on log-transformed read numbers of bacterial and virus taxa found in each mosquito species and habitat type.](image-url)
but not for the large subunit (LSU). The number of reads mapped to the SSU gene was significantly lower in RD samples than in UD samples (p value < 0.001), a difference that was not observed for the LSU.

**Evolutionary history.** Family Flaviviridae. Genus Flavivirus. Viruses of the family Flaviviridae possess positive-sense single-stranded RNA (ssRNA) genomes of approximately 9–13 kbp encoding a single polyprotein. We found at least two partial viral genomes from the insect-specific clade of the genus Flavivirus in our samples (Fig. 4). The first putative virus, Culex fuscocephala-associated flavivirus, found in all three Cx. fuscocephala samples, formed a clade with many flaviviruses isolated from Culex spp. We were able to recover >10 kbp of its genome. The most similar sequence (99% amino acid identity) belonged to a flavivirus isolated from multiple Culex species (Cx. tritaeniorhynchus, Cx. vishnui, or Cx. fuscocephala) collected in Myanmar. The second putative virus, Mansonia uniformis-associated flavivirus (Fig. 4), was represented by a short contig (207 bp) found in the suburban Ma. uniformis sample. It is related to Palm Creek virus isolated from Coquillettia xanthogaster in Australia (78% amino acid identity), and Nakiwogo virus isolated from Mansonia sp. in Uganda (45% amino acid identity).

Family Iflaviridae. Genus Iflavirus. The viruses of the genus Iflavirus possess linear positive-sense ssRNA genomes of approximately 9–11 kbp in length encoding a single polyprotein. All members infect arthropod hosts with the majority infecting insects. We found four non-overlapping partial genomic fragments of iflavivirus, provisionally called Armigeres subalbatus-associated iflavirus (Fig. 4), in the urban and suburban Ar. subalbatus samples. Based on our phylogenetic analysis, all four fragments are clustered with Armigeres iflavivirus found in an unknown Armigeres species collected in the Philippines. The percent amino acid identity to the reference Armigeres iflavivirus ranged from 48% to 79% (73–606 amino acids long).

Unclassified clade closely related to Luteoviridae and Sobemovirus. Both Sobemovirus and Luteoviridae are groups of plant viruses with linear and non-segmented positive ssRNA genomes of approximately 4 kbp and 6 kbp in length, respectively. A recent study found many new virus genomes in diverse invertebrate species classified to a novel clade that is phylogenetically divergent but related to Sobemovirus and Luteoviridae. At least three partial virus genomes identified in our samples belonged to this novel clade.

Using RNA-dependent RNA polymerase (RdRp) proteins to construct a phylogeny, the putative viruses identified in this study clustered with 3 different clades. The first putative virus, provisionally called Armigeres subalbatus-associated sobemo-like virus 1 (Fig. 4) found in all Ar. subalbatus samples fell within a clade containing Yongsan sobemo-like virus 1, Wenzhou sobemo-like virus 4, and Hubei mosquito virus 2. The second putative virus, provisionally called Culex fuscocephala-associated sobemo-like virus 2 (Fig. 4) was similar to Hubei sobemo-like virus 41 (95% and 97% amino acid identity). This virus was found in all Cx. fuscocephala samples. The last putative virus, provisionally called Armigeres subalbatus-associated sobemo-like virus 2 (Fig. 4), found in the urban Ar. subalbatus sample, formed a clade with Culex mosquito virus 6.

Narnaviridae and the closely-related Ourmia-like virus. Viruses in the family Narnaviridae possess a single molecule of non-encapsidated positive-sense ssRNA of 2.3–3.6 kbp. Two genera are currently recognized within this family: genus Mitovirus (fungi viruses) and Narnavirus (viruses of yeast). Members of the genus Ourmiavirus, closely related to the Narnaviridae, are plant viruses with genomes consisting of three positive-sense ssRNAs. At least 4 partial virus genomes could be classified within the Narnaviridae and Ourmiavirus in our study. The first virus, provisionally called Armigeres subalbatus-associated ourmia-like virus (Fig. 4), was found in large numbers (0.15–0.38% of total reads) in all three Ar. subalbatus samples. Its partial genome was similar to the sequence of Hubei mosquito virus 3 (99% amino acid sequence similarity) identified in a pool of multiple mosquito species collected in China. Our newly described ourmia-like virus clustered within a clade of ourmia-like invertebrate viruses.

The second virus, provisionally called Culex fuscocephala-associated narna-like virus (Fig. 4) was similar to the sequence of Zhejiang mosquito virus 3 (93% amino acid identity), which was identified in a pool of multiple mosquito species collected in China. This virus was found in all Cx. fuscocephala samples. The third virus, provisionally called Culex fuscocephala-associated mitovirus 1 (Fig. 4), was similar to Hubei narna-like virus 25 (68–69% amino acid identity). This virus was found in the rural Cx. fuscocephala sample. The fourth virus, provisionally called Culex fuscocephala-associated mitovirus 2 (Fig. 4) was similar to Erysiphe Necator mitovirus 1 (36% amino acid identity). This virus was also found in the rural Cx. fuscocephala sample.

Nodaviridae. The family Nodaviridae includes two genera, Alphanodavirus (infests insects) and Betanodavirus (infests fish). Genus Gammonodavirus infecting prawn and shrimp has also been proposed. Nodaviruses genomes consist of two molecules of positive sense ssRNA. RNA-1 (3.1 kbp) encodes protein A with functions such as polymerase, and RNA-2 (1.4 kbp) encodes protein alpha. Phylogenetic analysis showed that the partial RNA-1 found in our study (~2.5 kbp) formed a clade with Lunovirus (from a carnivore fecal virome study), Caninovirus (from a canid gut virome study), and Sanxia water strider virus 16 (from a pool of multiple water strider species collected in China). The most similar sequence was Sanxia water strider virus 16 (39% amino acid similarity). This virus, provisionally called Armigeres subalbatus-associated nodavirus (Fig. 4), was found in all three Ar. subalbatus pools. Another contig resembling capsid protein (~1 kb), likely representing the RNA-2 fragment of the same virus, was also found in all Ar. subalbatus samples.
Unclassified clade closely related to Picornaviridae. Sequences from a virus closely related to the family Picornaviridae was discovered in high numbers in the rural Cx. fuscocephala sample (1.89% of total reads). The
assembled partial genome of this virus, provisionally called Culex fuscocephala-associated picorna-like virus (Fig. 4), was most similar to the genome of Yongsan picorna-like virus 3 (70% amino acid identity) found in Aedes vexans from South Korea58. Picornaviridae is a large and diverse family containing viruses with positive sense ssRNA genomes ranging from 6.7 to 10.1 kb58. Recent metagenomic studies have found a great number of novel picorna-like viruses in invertebrates including mosquitoes6,14. It has been suggested only recently that a new family, Polycipiviridae, assigned to the order Picornavirales, should be adopted to include insect-associated picorna-like viruses59.

Order Bunyavirales. Order Bunyavirales includes viruses with segmented, negative-sense or ambisense single-stranded RNA (ssRNA) genomes distributed among 9 families (International Committee on Taxonomy of Virus, ICTV; Taxonomic Proposal 2016.030a-vM). The genomes consist of two to six segments encoding structural proteins, and one or more non-structural proteins60. Sequence analysis of the RdRPs indicated that the Bunyavirales contigs in our samples could be assembled into at least three distinct clades (Fig. 5). The first putative virus, provisionally called Culex fuscocephala-associated bunyavirus (Fig. 5), found in all Cx. fuscocephala samples, fell in an unclassified clade. This virus was similar to those found in Cx. pipiens collected in California (~93% amino acid identity)4,15. The second putative virus belongs to the genus Phasivirus in the family Phenuiviridae (Fig. 5). This putative virus, provisionally called Mansonia uniformis-associated phasivirus, was found in the suburb and

**Figure 5.** Phylogenetic relationships of the negative sense ssRNA viruses discovered in this study (labeled red) and other known viruses. The analyses were based on RNA-dependent RNA polymerase (RdRp) amino acid sequences. The numbers in parentheses are the lengths of the amino acid sequences.
urban *Ma. uniformis* samples. It was similar to Phasi Charoen-like phasivirus found in *Ae. aegypti* in Thailand and China (amino acid identity varies from 58 to 85% depends on the fragment)\(^1\). The third putative virus, provisionally called Culex fuscocephala-associated orthojonvirus (Fig. 5), was found in all three *Cx. fuscocephala* samples. The closest relative (32–53% amino acid identity) was the Jonchet virus (family *Jonviridae*) documented from mosquitoes in Côte d’Ivoire\(^6\).

**Order Mononegavirales: Rhabdoviridae and Xinmoviridae.** The family *Rhabdoviridae* includes viruses with genomes of 10.8–16.1 kb, usually with 5 genes encoding structural proteins\(^6\). The family is ecologically diverse with members infecting plants, fish, reptiles, birds, and mammals as well as arthropod hosts or vectors\(^6\). Our phylogenetic analysis of the L proteins (RdRp) suggested that there were at least two putative rhabdoviruses in our mosquito samples. The first virus, provisionally called Mansonia uniformis-associated rhabdovirus (Fig. 5), was found only in the suburban *Ma. uniformis*. This virus formed a clade with Menghai rhabdovirus (77–96% amino acid similarity), which was recently isolated from *Ae. albopictus* from China\(^6\). Another set of rhabdovirus L gene fragments was recovered in all three *Ar. subalbatus* pools (Armigeres subalbatus-associated rhabdovirus). These fragments showed similarity to known virus sequences (33–50% amino acid similarity) and the closest similarity was to Culex rhabdovirus. Because of their small sizes, the phylogenetic analysis of these L fragments did not result in a strongly supported clade and we cannot conclude whether these fragments belong to the same virus (Fig. 5).

We also recovered contigs similar to a virus in the newly proposed genus *Anphevirus* of the family *Xinmoviridae* (ICTV report 2017.016 M.A.v3), which consists of multiple insect viruses. The assembled genomic fragments found in our study (Fig. 5) were closely related to *Culex mononega*-like virus 2 (amino acid sequence similarity ranges from 33 to 50%). This virus, provisionally called Mansonia uniformis-associated anphevirus (Fig. 5), was found mostly in the rural *Ma. Uniformis* sample.

**Phylogenetic relationship of viruses across study sites.** In order to determine genetic relationships within those viruses that occurred across study sites, the partial genomes of each virus found in all three sites were subjected to phylogenetic analysis where genome coverage was sufficient. Of the three viruses that were found in high numbers in all sites, we document limited genetic divergence across sites of collection (Supplementary Figure 4).

**Non-viral reads.** The majority of non-viral reads, in both the RD and UD samples, were classified to phylum Arthropoda, averaging 79.80% and 81.08%, respectively (Supplementary Table 5). The average number of reads that could not be classified across all samples was 7.17% and 2.73% for RD and UD samples, respectively. The average numbers of non-virus reads classified to the Bacteria domain were 2.23% and 0.48% for RD and UD samples, respectively. The proportion of bacteria groups in all samples is shown in Fig. 6. The dominant bacterial group differed in each mosquito host species: in *Ar. subalbatus* the majority were in the class Gammaproteobacteria (average = 48.73% of all bacterial reads) while the majority of bacteria found in *Cx. fuscocephala* were in the class Alphaproteobacteria (48.19%). The composition of the bacteria community in *Ma. uniformis* samples was more evenly distributed, dividedly between Alphaproteobacteria (26.12%) and Gammaproteobacteria (19.87%). Other groups of bacteria found in mosquito samples included: phylum Actinobacteria, averaging 3.58% of all bacterial reads across all samples; phylum Firmicutes, 2.39%; class Betaproteobacteria, 1.68%; Bacteroidetes-Chlorobi group, 1.54%; and Delta-Epsilon Proteobacteria subdivision, 1.53%.

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**Figure 6.** The proportion of bacteria groups found in all mosquito samples and NTCs. RD = rRNA depleted, UD = rRNA not depleted. NTC1 and NTC2 refer to no-template controls for separate sequencing runs (Supplementary Table 2).
In terms of individual bacteria of note, Wolbachia (Alphaproteobacteria), a genus of common obligate intracellular bacteria found in a wide range of invertebrate taxa, was found in all samples as indicated by 16s rRNA contigs recovered. We also found rRNA reads of bacteria relating to common house flies and bee gut-associated Apis bacteri in all Cx. fuscocephala samples, but not other mosquito species (Supplementary Figure 5). Also restricted to the three Cx. fuscocephala samples were bacteria similar to Helicobacter sp. of the Epsilon-proteobacteria, and bacteria similar to the bee-associated Frischella sp. and Gilliamella sp. of the Gammaproteobacteria.

Multiple groups of fungi were detected (Supplementary Table 5), averaging 0.56% and 0.21% of all non-viral reads in RD and UD samples, respectively. Common divisions of fungal reads found in all samples were Ascomycota and Basidiomycota. The highest percentage of fungal reads was from the pool of Cx. fuscocephala collected from the rural habitat. The majority of fungi in this sample were classified into phylum Zoopagomycota (division Zygomycota), at 2.56% and 0.88% or all in RD sample and UD sample, respectively.

Other organisms within our samples included trypanosomatids, alveolates, plants, algae, platyhelminthes, nematodes, anelids, mollusks, and vertebrates (Supplementary Table 4). Initial analysis of ribosomal RNA reads suggested there existed multiple poorly known species of mosquito-associated eukaryotic microorganisms in our samples. For example, partial trypanosomatid rRNA reads, related to trypanosomatids of multiple insects, were found in all three mosquito species. In addition, partial 18s rRNA reads similar to Paratrypanosoma con-fusum, a trypanosomatid species recently found in Cx. pipiens guts, were detected in Cx. fuscocephala samples.

Multiple samples collected from rural and urban sites harbored 18s rRNA reads similar to nematode species in the Mermithidae, and Setariidae families. Of note is a partial 28s rRNA reads found (274 bp) found in the rural Ma. uniformis sample that is 100% similar to Setaria digitata, a mosquito-borne zoonotic nematode species infecting ungulates in Asia. Ma. uniformis sample collected in suburban site harbored reads likely belong to Brugia malayi (99–100% similarity, 215 and 431 bp).

Several rRNA reads were recovered in the urban Cx. fuscocephala sample related to trematode of the order Plagiorchiida such as Collyriclum faba, Paralechithodendrium longiforme, and Lecithodendrium instowi. C. faba is a bird parasite that has aquatic gastropods as the first intermediate host and mayflies as the second intermediate host. On the other hand, P. longiforme and L. instowi are bat parasites with unknown second intermediate hosts.

Lastly, 18s rRNA reads of Ascosregarina sp., a mosquito-specific apicomplexan parasite, were found in rural and urban Ar. subalbatus samples and were most similar to A. armigerei (99.4%, 1061 bp). The genus Ascogregarina parasitizes mosquitoes and sandflies, and is relatively host specific: A. taiwanensis infects Ae. albopictus, A. culicis infects Ae. aegypti, and A. armigerei infects Ar. subalbatus.

Discussion

In this study, we used a high throughput RNA sequencing metagenomic approach to characterize the mosquito-associate virome and microbiome. We identified at least 21 putative RNA viruses in three vector species: Ar. subalbatus, Cx. fuscocephala, and Ma. uniformis. We chose the three mosquito species because of their abundance and potential vector status in central Thailand. Ar. subalbatus transmits Japanese encephalitis virus, Wuchereria bancrofti, and dog heartworm Dirofilaria immitis. Their larvae have been found in nutrient enriched water including septic tanks and polluted stagnant water as well as less enriched bamboo stumps, artificial containers, and tree holes. Cx. fuscocephala, often associated with flooded rice field and agricultural lands, is among multiple Culex mosquito species that transmit Japanese encephalitis. Mansonia uniformis is a vector of Wuchereria malayi. They are associated with vegetated water habitat such as ponds and canals with floating vegetation, rice fields, and swamp forest.

According to the Principle Component Analysis, we found that the pattern of viruses in our mosquito samples was defined primarily by the host species rather than by geographical location. Viruses are obligate intracellular parasites that can only function when inside host cells, necessitating specific host cell recognition, entry, and manipulation of multiple host cell molecular mechanisms in order to complete their replication cycles. These properties limit viruses’ biological host ranges. It is not surprising that we observe this host species-specific rather than location-specific pattern. This is in contrast to the bacterial community, where grouping patterns did not show association with host or location. Even though the full factorial design in our study allowed us to investigate the relative importance of ecological factors in structuring mosquito virome, the small sample size limits our ability to generalize this finding.

On the other hand, the same virus species found in the same mosquito species but from different collection sites showed limited genetic divergence (Supplementary Figure 4), suggesting that the viruses are shared between mosquito populations, at least within the geographical scale of this study (the three study sites are within 20 km apart). Other studies comparing mosquito-specific RNA viruses isolated from wider geographical areas found that they showed genetic similarity across continents. The results from our study lead to important implications for the surveillance of emerging viral diseases: in order to capture as much viral diversity as possible, monitoring efforts should sample a wide diversity of mosquito species, rather than focus on a narrow set of mosquito species albeit over a larger geographic landscape.

We also tested a host rRNA depletion method which could reduce the cost of sequencing without the need to pool too many individuals into a single sample. Our analysis indicated that we successfully depleted rRNA of the mosquito’s SSU but not of the LSU. Even then, the host rRNA depletion as adopted in our study significantly increased the percentage of virus reads in the sample as well as the coverage for virus contigs. However, our caution is that depletion was time consuming such that the same gains in virus detection could have been achieved for less cost in time with an additional sequencing run, especially when the cost of sequencing may continue to come down in the future.
We found multiple putative viruses associated with certain mosquito species, suggesting that they are likely mosquito-specific. For example, Armigeres subalbatus-associated ourmia-like virus was classified to a novel insect virus clade closely related to plant viruses in the genus Ourmiavirus. It was found in high numbers in all Ar. subalbatus samples but not in the other two mosquito species. A recent NGS-based study found multiple insect viromes in well-defined clades distinct but related to both Ourmiavirus and Narnavirus. According to a recently published ICTV report, there could be a future establishment of a new virus family “Ourmiaviiridae”. The family would comprise a genus for ourmia-like mycoviruses, an existing genus containing plant viruses, and a genus for ourmia-like viruses isolated from invertebrates (which may contain the ourmia-like virus in our sample). Another putative virus, Culex fuscocephala-associated narna-like virus, was found in Cx. fuscocephala and not the other two mosquito species. This putative virus formed a monophyletic group with a virus found in Cx. pipiens and other arthropods. Although this group of viruses was closely related to mycoviruses, it likely contains a group of novel insect-specific viruses.

Another insect-specific virus, Ma. uniformis-associated anphevirus, was recovered mostly in the rural sample of Ma. uniformis, suggestive of habitat restriction. Interestingly, the newly proposed genus Anphevirus consists of multiple insect viruses including those isolated from Culex, Anopheles, and Aedes mosquitioes. A recent study showed that Aedes anphemivirus may reduce DENV replication in cell culture. Our phylogenetic analysis indicated that Ma. uniformis-associated anphevirus is separated from other mosquito anpheviruses (Fig. 4). Thus, we speculate that Ma. uniformis-associated anchemevirus could be a Mansonia-specific clade.

It is important to note that the virus names given in our manuscript are only provisional and not species names. The ICTV sets different species demarcation criteria that vary depending on virus groups; and the criteria often include information other than virus genetic sequences. Lacking relevant information for species assignment, we cannot classify the viruses found in our study into the same species of existing virus, nor can we establish a new species. Ideally, future studies should be done to assign each of these viruses to species.

Our non-targeted sequencing approach reveals multiple interesting mosquito-associated non-viral microorganisms such as a Bacteroidetes species (found in Cx. fuscocephala samples; Supplementary Figure 5) similar to the bee-associated Apibacter sp., and a not yet described digenean trematode species in which mosquitoes might serve as the second intermediate host (Supplementary Figure 6). The identification of these non-viral organisms is important in their own rights but it can also give further insight into potential hosts of viruses that might be found in the same mosquito pools. Even though the viruses were discovered in mosquitoes, they may infect other mosquito endosymbionts such as bacteria, fungi, and parasites, or ingested organic material such as plants and algae.

We have included no-template controls (NTCs) that were processed alongside mosquito samples from extraction to sequencing. Due to the high sensitivity of NGS and the well-documented risk of contamination in microbiome studies, control samples should always be included to assess contamination that can originate from the environment (e.g. reagents and kits, plastic consumables, dust, and humans), and cross-contamination between samples, occurring either during sample processing (e.g. aerosol contamination, barcode cross-contamination), or sequencing (e.g. barcode sequencing errors and ‘index hopping’)37. Despite its importance, not all NGS-based metagenomic studies include controls.

We detected reads belonging to multiple organisms in the NTCs. The proportion of reads in NTCs classified to each taxon is shown in Supplementary Table 5. We observed that abundant taxa were more likely to be recovered in the NTCs. Thus, we suspect limited cross-contamination between samples. Because NTCs have no starting material, even trace amounts of contaminated DNA could be picked up by NGS. On the contrary, mosquito samples contain large amounts of template DNA reads, and only a few contaminated reads would be sequenced. Other types of controls, such as mock community controls should be adopted in future studies along with NTCs. This type of control not only allows a more realistically detection of contamination, but also the quantification of sequencing error and other bias introduced during the library preparation processes.

Endogenous viral elements (EVEs) are defined as viral DNA sequences deriving from retroviruses, DNA viruses, or RNA viruses that present within the genomes of non-viral organisms, including mosquitioes. Unfortunately, we cannot definitively determine whether the contigs identified in this study originate from viruses or EVEs because the genomes of the three mosquito species are currently unavailable, and because EVEs possess non-specific characteristics that cannot be used for their identification. However, we expect that the RNA extraction protocol adopted in our study likely limited the inclusion of EVEs, and the potentially expressed small EVEs (e.g. EVE-derived piRNAs) in our purified RNA samples.

Mosquito-borne disease emergence is thought to be associated with socio-economic and ecological factors arising from urbanization and poverty. In this study, we characterized mosquito-associated viromes and microbiomes across a landscape gradient transformed by human activities and urbanization. In addition, the three vector species differ in their biology and ecology. We found that the mosquito-associated virome component was defined primarily by the host species rather than by the geographical locations or habitats, and multiple viruses spread between sample sites and mosquito populations over the scale of this study. Similar to Wolbachia, we know that elements of the vector virome and microbiome can impact vector fitness and competence: we recovered a virus closely related to Aedes anphemivirus previously reported to reduce vector competence to dengue. Thus, this study may help inform future use of insect-specific virus (ISVs) in the control of vectors and diseases. Because we found that mosquito species determine virome composition more than habitat, urbanization is likely to have the greatest influence on the distribution of microbes by facilitating invasive vector species. This finding is supported by our previous study indicating that under urbanization mosquito communities become less diverse and skewed towards invasive mosquito species. To test the influence of habitat alone on mosquito-associated virome, future studies should focus on one mosquito species at a time, and sample individually and more intensively across landscape types.
Data availability

The raw sequence reads generated in this study are available at the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA716099; BioSamples SAMN18394705-SAMN18394726. All virus contigs and some 16S and 18S rRNA contigs generated in this study have been deposited in GenBank under accession numbers: MW854367-MW854382, MW858073-MW858116, MW874579-MW874588, and MW879746-MW879751.

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Author contributions
P.T. participated in fieldwork study design, carried out fieldwork and mosquito identification, and performed data analysis. J.A.C. selected samples for sequencing and designed and performed all molecular laboratory experiments and N.G.S. P.K. participated in study design and coordinated and accommodated field experiments. B.A.W., D.D.K. and S.N.B. conceived the initial study and participated in study design and coordination. P.T. wrote the original manuscript. J.A.C., D.D.K., and S.N.B. provided substantial edits. All authors read, gave input, and approved of the final manuscript.

Competing interests
The authors declare no competing interests.

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