Establishment of *Culex modestus* in Belgium and a glance into the virome of Belgian mosquito species

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Running Head: Establishment of *Culex modestus* in Belgium

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Abstract:

Culex modestus is a mosquito species with a relevant role in the transmission of West Nile virus and Usutu virus. Its presence has been reported across Europe, yet it is absent in Belgium. Field mosquitoes in the city of Leuven and surroundings were collected in the summer of 2019 and 2020. Species identification was performed by morphological features and partial sequences of the mitochondrial cytochrome oxidase subunit 1 (COI) gene. In order to examine the genetic structure of the *Cx. modestus* population found in Leuven, mitochondrial sequences of *Cx. modestus* mosquitoes collected in other 8 countries across Europe were retrieved from GenBank and confronted to the COI sequences from Belgian mosquitoes to construct a haplotype network.

Hereby, we confirmed the new establishment of *Cx. modestus* in the surroundings of Leuven, Belgium. Haplotype network analysis showed that the Belgian population is rather diverse, suggesting that it must have been establish in Belgium for some time. The Belgian population was most closely related to *Cx. modestus* populations from the UK and Germany. The NetoVIR protocol was used to study the virome of 8 pools of mosquitoes. At least 33 eukaryotic viral species were identified. Nine (near-)complete genomes belonging to 6 viral species were identified, named Culex totivirus Leu1, Leu2 and Leu3, Alphamesonivirus Leu4, Iflavirus Leu5 and Leu6, Negevirus Leu7 and Leu8, and Rhabdovirus Leu9, all of which were closely related to known viruses. In conclusion, with the introduction of *Cx. modestus* in Belgium, the evaluation of its potential role in the transmission of arboviruses that could cause disease in animals and humans is necessary.

Importance for non-specialist:

Culex modestus is a mosquito species that plays a role in nature as a 'bridge' vector, being able to transmit pathogens between birds, as well as from birds to mammals, including humans. In Belgium, this species was considered absent. Field mosquitoes were collected in urban, peri-urban and wetland areas in the summer of 2019 and 2020 in Leuven, and morphological and molecular methods were performed to confirm the presence of *Cx. modestus* in this region. The ability of mosquitoes to transmit pathogens can depend on a lot of factors, one of them being the natural virus composition in their bodies. For this purpose, we aimed to identify the whole virus group harbored by Belgian mosquitoes. This could provide more insight for mosquito and, therefore, disease control. Besides, the introduction of *Cx. modestus* may increase the risk of disease transmission. It is advisable to implement mosquito surveillance programs monitoring this species.

Introduction

The *Culex modestus* mosquito species was described for the first time by Eugenio Ficalbi (1889) in northern Italy (1) and is considered a rare species. In Europe, this species is distributed mainly in southern and central European countries. Field collections have reported the presence of *Cx. modestus* in France, Spain, Portugal, Germany, Romania, Serbia and Czech Republic; and more recently, also in more northern countries such as the United Kingdom (UK), Denmark and Sweden (2). In Belgium, this mosquito species was considered to be absent. Recent field studies in the UK have confirmed two characteristics of *Cx. modestus*: (i) its ornithophilic habit, i.e. feeding on resident and migratory bird species (3), and (ii) its mammalophilic and anthropophilic feeding behaviour, showing that *Cx. modestus* is also a major human-biting mosquito species similar to *Cx. pipiens* (4). Thus, *Cx. modestus* could play a role in nature as a 'bridge' vector, being able to transmit pathogens between birds in an enzootic cycle, as well as from birds to mammals, including humans, in an epizootic/epidemic cycle.

Previous studies on different *Cx. modestus* populations in Europe revealed that this species can act as a carrier of different pathogens, and likely is able to transmit these pathogens as well. In the south of France, *Cx. modestus* mosquitoes have been found to serve as amplifying vectors for seasonal West Nile virus (WNV), introduced by migratory birds (5). *Cx. modestus* mosquitoes collected in the Danube Delta region (border of Romania and Ukraine) were positive for *Plasmodium sp.* lineage Donana03 (avian malaria) (6). In addition, a prevalence of 5.1% of *Trypanosomatids* was detected in the gut of *Cx. modestus* collected in the Czech Republic between 1998 and 2002 (7). Furthermore, *Cx. modestus* is the vector and reservoir of Lednice virus (LEDV), a rare bunyavirus that causes viremia in wild birds. During the last sixty years, various European countries have reported the presence of LEDV in their *Cx. modestus* mosquito populations (8). Besides LEDV, also the Tahyna virus (TAHV) has been isolated from *Cx. modestus* in Czechoslovakia and in France (9).

Mosquito surveillance targeting *Cx. modestus* has been increasingly recommended for mosquito-borne disease control, because of its important role in the transmission of WNV and Usutu virus (USUV). The role in WNV transmission in Europe was demonstrated by the detection of WNV in this mosquito species during an outbreak in the Sardinia region (Italy) in 2011 (10). During this outbreak, the circulating virus strains belonged to lineage 1. It was the first report of an Italian WNV strain that caused clinical signs in the affected birds. The mosquito survey carried out in this area revealed that these virus strains were found in *Cx. modestus* mosquitoes. During the mosquito seasons 2015 and 2016, WNV lineage 2 has also been detected in *Cx. modestus* mosquitoes collected in the Lednice-Valtice Area (in southern Moravia) (11, 12). Regarding the vector competence of *Cx. modestus* for WNV, this mosquito species was found to be competent to transmit WNV experimentally. More than 90% of *Cx. modestus* mosquitoes developed a disseminated infection 14 days after an infectious WNV bloodmeal (13). Moreover, it is considered an extremely efficient vector, given that the disseminated infection and the transmission rates reached 89.2% and 54.5% respectively, after 14 incubation days (14).

The USUV has also been detected in field collected *Cx. modestus*, likely co-circulating with WNV (15). The USUV is another arbovirus with African origin that is principally transmitted by *Culex* mosquitoes. This virus belongs to the genus *Flavivirus*, as well as dengue, yellow fever, Zika, Japanese encephalitis and WNV (16). The virus is maintained in an enzootic cycle between ornithophilic mosquitoes and birds. In Europe, USUV was found by retrospective analysis of archived tissue samples from bird deaths in the Tuscany region of Italy in 1996 (17). In 2001, USUV-associated death of blackbirds was reported in Austria (18), Germany and the Netherlands (19, 20). In 2016, numerous wild birds, mainly Eurasian blackbirds (*Turdus merula*), were affected by a USUV outbreak in Belgium in the provinces of Limburg, Antwerp and Flemish Brabant (21). In 2017, the virus further spread to the west and by the summer of

2018, the whole country was affected (22). Despite the recent USUV outbreaks, it is not known which mosquito species are the vectors of USUV in Belgium. Therefore, we collected field mosquitoes using BG-sentinel traps in the city of Leuven and its surroundings in three different environment types (urban, peri-urban and wetland areas). Species identification was performed by morphological features (keys) (23) and partial sequences of the mitochondrial cytochrome oxidase subunit 1 (COI) gene (DNA barcoding region) (24).

To unravel the high diversity of mosquito-specific viruses (MSVs) harbored by Belgian mosquitoes, we performed a metagenomic sequencing approach using the Novel enrichment technique of VIRomes (NetoVIR) protocol (25). The virome of tropical mosquito species such as *Aedes aegypti* has been studied extensively. Whereas knowledge on the viral diversity in mosquitoes from more temperate regions is still scarce. A recent virome study identified novel RNA viruses in Swedish mosquitoes (26). However, the virome of mosquitoes from Western Europe, including Belgium, has not yet been studied. The study of viral diversity in mosquitoes is important since MSVs have the potential to modulate the vector competence of mosquitoes for different arboviruses (27). Therefore, we provide a first glance into the virome of mosquitoes collected in Belgium.

Material and Methods

Ethics statement

Permits for peri-urban and wetland mosquito field collections were obtained from the security responsible of KU Leuven. Permits for field collections in urban habitats were obtained from the landowners.

Mosquito collections

Adult mosquitoes were trapped with the Biogents Sentinel traps (BioGents GmbH, Germany), which were baited with BG-lure (BioGents GmbH, Germany) and containing around 2 kg of dry ice in the isolated box for CO_2 production. Two traps were rotated in three different habitat

types (urban (N 50°52'41, E 4°41'21), peri-urban (N 50°51', E 4°41'), and water reservoir wetlands (N 50°51', E 4°40'), in Leuven and surroundings (Supplementary Figure S1). Collections were performed from August to the beginning of October in 2019, when the weather was good, avoiding strong wind or heavy rain. Every 24 hours, the traps were emptied and repositioned between sunrise and sunset of the next day. Mosquitoes were individually stored at -80˚C until species identification. A second collection was performed in August of 2020 in the same geographic locations as described above to confirm the presence of certain species.

Species identification, sample preparation and DNA sequencing

All collected mosquitoes were identified by key points (23). Individual thoraces were removed using forceps for molecular identification and homogenized in 100 µL of phosphate-buffered saline (PBS) using tubes with 2.8 mm ceramic beads with a Precellys Evolution homogenizer. Nucleic acid extraction was performed by lysing the homogenate at 100°C for 10 minutes (28). Tissue debris was removed by centrifugation at 12 000 rpm for 3 minutes, and 50 µL of supernatant was collected into a new tube. A 710 bp region of the cytochrome oxidase subunit I (COI) mitochondrial gene was the target for amplification by polymerase chain reaction (PCR) using previously reported primers (29). Presence of the PCR product was checked on a 2% agarose gel by gel electrophoresis. DNA was purified with the Wizard® SV Gel and PCR Clean system (Promega). The DNA concentration of amplicon was measured by NanoDrop (ThermoFisher), after which samples were sent for Sanger sequencing to Macrogen Europe.

Mosquito sequence analysis and phylogeny

Sequences were edited and assembled with Bioedit version 7.2.5 (30) to obtain a single consensus sequence per individual mosquito. Through the BLAST tool, the generated COI sequences were compared to the NCBI database. Reference COI sequences for all mosquito species considered were selected according to Versteirt and colleagues (24), which employed reference sequences that were registered in the Barcode of Life Data (BOLD) Systems, and

downloaded from GenBank. For phylogenetic analysis, the COI sequences generated in the study and the reference sequences were aligned with MAFFT v7.471 (31) using the G-INS-I option. The resulting alignment was trimmed by trimAl v1.4.rev15 (32) on gappyout setting and phylogenetic informative regions of the alignment were selected with BMGE v1.12 (33) for phylogenetic inference. Maximum-likelihood (ML) trees were constructed using IQ-TREE v2.0.3 (34) with automatic selection of the best nucleotide substitution model and 1000 ultrafast bootstrap replicates. Finally, trees were visualized using FigTree v1.4.4.

Haplotype network

Haplotype inference and nucleotide diversity were calculated in ARLEQUIN, version 3.5.2.2 (35). The population genetic data was analyzed using the median-joining (MJ) network algorithm in PopART, version 1.7 (36, 37). The COI sequences for *Cx. modestus* included in the haplotype network were retrieved from the NCBI database. These sequences were selected based on the specimen's country of origin and the length of the COI fragment (29).

Pool design, sample preparation and sequencing for virome analysis

The samples of mosquito abdomens were grouped in pools for sequencing according to the morphological identification of mosquito species by key points and sample location (urban, peri-urban and wetlands). Abdomens were homogenized in 600 µL of PBS with 2.8 mm ceramic beads with the MINILYS tissue homogenizer, including a negative control (blank tube with PBS).

All pool samples followed the Novel enrichment technique of VIRomes (NetoVIR) sample preparation protocol optimized for viral metagenomics (38, 39). In brief, after homogenization, samples went through a centrifugation and filtration step to remove pro- and eukaryotic organisms and large organic debris. Next, a nuclease treatment (employing benzonase and micrococcal nuclease) was applied to remove free floating nucleic acids. Nucleic acids were extracted with the QIAamp Viral RNA mini kit (QIAGEN) to be further randomly amplified

using a modified Whole Transcriptome Amplification 2 (WTA2) kit procedure (Sigma-Aldrich). The products were purified and libraries were prepared using the NexteraXT Library Preparation kit (Illumina). Sequencing of the samples was carried out on a NextSeq 500 High Throughput platform (Illumina) for 300 cycles.

Bioinformatic analysis and identification of eukaryotic viruses

Quality and adapter trimming on raw paired-end reads was performed using Trimmomatic v0.39 (40). Next, contamination of samples was removed with Bowtie2 v2.3.4 (41) by mapping trimmed reads to a set of contigs present in the negative controls (reagent contamination). Remaining reads were *de novo* assembled into contigs using metaSPAdes v3.13.0 (42). To remove redundancy in the data, contigs were filtered on a length of 1000bp and subsequently clustered at 95% nucleotide identity over 80% of the length using Cluster-Genomes (https://bitbucket.org/MAVERICLab/docker-clustergenomes). All contigs were classified by DIAMOND (43) against NCBI's nr database (downloaded on 27 October 2020) on sensitive mode for taxonomic annotation. KronaTools (44) was used to parse the DIAMOND output file and find the least common ancestor for each contig (based on the best 25 DIAMOND hits). Contigs annotated as eukaryotic virus were retrieved using an in-house Python script. Pool magnitudes were obtained by mapping the trimmed and decontaminated reads to these eukaryotic viral contigs with BWA-MEM2 (45, 46). The resulting abundance table was further used for ecological analysis in R using the phyloseq (47), metagenomeSeq (48), vegan (49) and ComplexHeatmap (50) packages.

Recovery and phylogenetic analysis of (near-) complete meta-assembled genomes

To recover full eukaryotic viral genomes in the mosquito pools, viral species were selected based on the level of genome completion after metagenomic *de novo* assembly. If a viral genome was not yet fully complete after assembly, the reads from the mosquito pool were mapped to a selected reference sequence (based on the annotated species by DIAMOND and

Krona tools) with BWA-MEM2 (45, 46). The consensus sequence was subsequently retrieved with samtools and bcftools (51). For phylogenetic analysis, relevant reference complete genome sequences were chosen after BLASTn of the metagenomic assembled genomes (MAGs) and subsequently downloaded from GenBank. Alignment, trim, model selection, construction and visualization of phylogenetic trees was done as previously described for mosquito COI sequences.

Results

Mosquito species detected in Leuven, Belgium

A total of 107 mosquito specimens was collected in three distinct locations in Leuven in the summer of 2019. According to the DNA barcodes generated and morphological features, these mosquitoes belonged to eight mosquito species: *Cx. pipiens* (24.3%), *Cx. modestus* (48.6%), *Cx. torrentium* (0.9%), *Culiseta annulata* (0.9%), *Culiseta morsitans* (0.9%), *Ae. sticticus* (14.0%), *Ae. cinereus* (9.3%) and *Anopheles plumbeus* (0.9%) (Figure 1A). Interestingly, *Cx. modestus* was not reported before in Belgium, while in this mosquito collection it made up ~50% of the collected mosquitoes, from three different breeding sites. *Cx.* species were predominant in urban and peri-urban areas, whereas specimens found in the water reservoir wetlands belonged mostly to the genus *Aedes* (Figure 1B).

Establishment of *Cx. modestus* in Leuven, Belgium

A ML tree was built from the *Cx. modestus* COI barcodes obtained in Leuven and COI sequences of 20 other Culicid species described in (52). *Cx. modestus* barcodes from Leuven clustered with two reference *Cx. modestus* sequences that were included (KJ401305, MK971991). All sequences for *Cx. modestus* fell within one large well-supported monophyletic cluster, separated from other mosquito species, which suggests that they belong to the same species (Figure 2). To find out whether *Cx. modestus* is established in the region, field collections were performed in the summer of the consecutive year (2020) using the same

geographic locations as previously. Again, *Cx. modestus* mosquitoes were retrieved (Figure 2), confirming the establishment of this mosquito species in the area of Leuven.

Haplotype network of *Cx. modestus* mosquitoes

The dataset analyzed for haplotype inference was constructed employing 184 *Cx. modestus* partial COI sequences retrieved from NCBI corresponding to eight European countries, and including 40 partial high-quality COI sequences obtained from the molecular identification of field collected mosquitoes in Leuven (Supplementary Table S2, S3). 4 partial COI sequences from mosquitoes collected during the summer of 2020 were included as well.

Among the 228 COI sequences (639 bp), 97 haplotypes were found. The majority of haplotypes (88) were present only in the country of origin, while only 9 haplotypes were shared by two or more countries. Haplotype diversity ranged from 0.8182 in Spain to 1.000 in Denmark, Portugal, Serbia and Sweden (Table 1). This analysis revealed that haplotype diversity in Belgium was the second highest (0.9852) of all countries screened, followed by the U.K. (0.9252) and Germany (0.9013). Nucleotide diversity estimations ranged from 0.0058 in Spain to 0.0270 in Belgium. Belgium exhibited a nucleotide diversity of 0.0270, which can be considered moderate, but which is the highest in all included European countries.

Mitochondrial DNA genealogy of *Cx. modestus*

The median-joining network displayed the ancestry of *Cx. modestus* mosquitoes (Figure 3), where two lineages were visualized separated by 1 mutation step. Haplotypes from Spain and Portugal were found uniquely in lineage I, while haplotypes from Germany, the UK, Belgium and Sweden predominated in lineage II. Haplotypes from France, Serbia and Denmark were scattered across both lineages. The majority of haplotypes that were found in Belgium were located in between of three central haplotypes of lineage II, which contain samples from several countries: one is shared by Belgium, the UK and Serbia (Figure 2, “3”), another one is shared by Belgium, the UK and Sweden (Figure 2, “2”), and the biggest one is shared by Belgium,

Germany, the UK, France and Sweden (Figure 2, “1”). Haplotypes found in mosquitoes collected in Leuven during the summer of 2020 were observed in both lineage I (1 haplotype) and lineage II (3 haplotypes).

A peek into the virome of Belgian mosquitoes

We characterized the virome of 107 mosquitoes’ abdomens, divided into eight pools according to their morphological identification and representing the three different habitat types mentioned before. A total of 44,002,358 reads were obtained from all mosquito pools. Most reads (21,602,296; 49.1%) belonged to the urban group. Mosquitoes collected in peri-urban and wetland areas generated 13,891,285 (31.6%) and 8,508,777 (19.3%) reads, respectively.

In all pools, the proportion of reads mapping to the order Diptera ranged from 40.8 to 77.7%. Regarding the bacterial reads, the wetland samples had a higher mean proportion (3.83%), followed by the urban samples with 2.03%, while the peri-urban samples presented less than 1% of reads mapping to bacteria. The viral component was more variable, with an observable ascending trend when moving from the wetlands to peri-urban and urban areas. Wetland samples gathered a low proportion of viral reads (<2%), whereas viral reads in peri-urban areas accounted for 1.28 – 7.19%. Lastly, reads mapping to the viral component composed 7.45 – 44.69% in the urban samples.

After filtering the viral reads for eukaryotic viral species, the relative abundances in the mosquito pools are shown in Figure 4 per viral family. While the *Cx.* pools in the urban area were completely dominated by one viral family (*Mesoniviridae* and *Iflaviridae* for pool 1 and pool 2 respectively), the mosquito pools from the peri-urban and wetland habitats seemed to have a higher viral diversity. The peri-urban samples contained mostly viral reads from a Negev-related virus, namely Yongsan negev-like virus 1, and from the *Totiviridae* family, with Culex inatomii totivirus being the most abundant viral species. In the wetlands *Ae. cinereus* pool on the other hand, an unclassified Bunya-like virus was most abundant.

Comparing the eukaryotic virome across habitat type and mosquito genus

To compare the eukaryotic virome of our samples, we mapped all trimmed and decontaminated reads back to the selected viral contigs, extracted the abundance table and subsequently constructed a heatmap with the normalized counts for each viral species on a $\log_2$ scale (Figure 5). In total, 33 eukaryotic viral species could be detected across all samples (a viral species was considered present if it had at least one contig >1000 bp and if more than 500 reads map to it). According to the Bray-Curtis distance matrix, the eukaryotic viromes of the *Cx.* mosquito pools clearly clustered together per habitat type. However, except for the peri-urban *Cx.* pools, each remaining pool had a more unique viral composition and only a small number of viruses were significantly shared between samples. Nevertheless, the peri-urban mosquito pools had a majority of viruses in common, such as Culex inatomii totivirus and Yongsan negev-like virus 1 which were shared with high abundance, while Ista virus, Sonnbo virus and Fitzroy Crossing toti-like virus 2 were common in lower abundance.

Recovery of (near-)complete meta-assembled genomes

In total we managed to recover 9 (near-)complete genomes of 6 viral species in our metagenomic data. These viral species belong to the following families: *Totiviridae* (Culex inatomii totivirus in pool 4, 5 and 6), *Iflaviridae* (Yongsan iflavirus 1 and Culex iflavi-like virus 4 in pool 2), *Mesoniviridae* (Alphamesonivirus 1 in pool 1), *Rhabdoviridae* (Riverside virus 1 in pool 8) and unclassified Negev-related viruses (Yongsan negev-like virus 1 in pool 5 and 6), and their phylogenetic relatedness to closely related reference strains is shown in Figure 6.

dsRNA viruses

Totiviridae

This family of dsRNA viruses are known to infect fungi, plants and invertebrates. In this study, we found Culex totivirus Leu1, Leu2 and Leu3 (98.3% average BLASTx identity with Culex inatomii totivirus; LC514398.1) in all peri-urban mosquito pools. This novel totivirus was

recently described in *Cx. inatomii* mosquitoes in Japan (53), and our finding now confirms its association with mosquitoes as a host.

(+) ssRNA viruses

Mesoniviridae

When constructing a phylogenetic tree of the MAG annotated as Alphamesonivirus 1 (99.7 BLASTx % identity; MH520101.1), together with all reference sequences of the *Mesoniviridae* family, our complete Alphamesonivirus Leu4 genome formed a clade with Nam Dinh virus and Cavally virus. Both Alphamesoni 1 viruses are frequently linked to mosquitoes (54, 55). Interestingly, all known members of the *Mesoniviridae* family infect mosquito hosts.

Iflaviridae

Iflaviruses are a well-known group of picorna-like viruses that exclusively infect arthropods (56). We found two complete genomes of iflaviruses (Iflavirus Leu5 and Iflavirus Leu6, having a 98.3 and 97.1 BLASTx % identity with Culex iflavi-like virus 4 (MT096522.1) and Yongsan iflavirus 1 (NC_040587.1) respectively) in an urban mosquito pool consisting entirely of *Cx. pipiens* mosquitoes.

Negev-related

Negevirus is a proposed taxon for diverse and geographically widely distributed insect-specific viruses isolated from mosquitoes and phlebotomine sandflies (57). We recovered 2 full genomes annotated as Yongsan negev-like virus 1 (average of 95.1 BLASTx % identity; MH703054.1) from two peri-urban mosquito pools that mainly contained *Cx. modestus* mosquitoes, named Negevirus Leu7 and Leu8.

(-) ssRNA viruses

Rhabdoviridae

Rhabdoviruses are a diverse group of negative-sense ssRNA viruses known to infect both vertebrates and invertebrates as well as plants (58). Riversidevirus 1 was first described in

Ochlerotatus sp. mosquitoes in Central Europe (59) and, in this study, it was also detected (98.2% BLASTx identity; KU248086.1). Rhabdovirus Leu9 was identified in a pool containing mostly *Ochlerotatus* mosquitoes. This suggests a restricted host species range as, up to this date and to our knowledge, this virus has not been found in other mosquito species or other hosts yet.

Discussion

A mosquito survey in 2013 clarified that the mosquito fauna in Belgium is composed by twenty-three mosquito species belonging to five traditionally recognized genera, including twenty-one indigenous and two exotic species (*Ae. koreicus* and *Ae. japonicus*)(60). The five most abundant species were *Cx. pipiens* (61.62%), *Coquillettidia richiardii* (15.43%), *Ae. cinereus* (5.04%), *Anopheles claviger* (3.52%) and *Ae. vexans* (2.93%) (60). Amid the eight species that were collected in this study in Leuven, *Cx. pipiens*, *Cx. torrentium*, *Culiseta annulata*, *Culiseta morsitans*, *Ae. sticticus*, *Ae. cinereus* and *Anopheles plumbeus* have been reported as autochthonous species of Belgium according to the latest mosquito surveillance (52). The exotic invasive species such as *Ae. koreicus* and *Ae. japonicus* were not found in this study.

In contrast, *Cx. modestus* was not previously identified during mosquito surveys in Belgium (24, 52), although it accounted for almost half of the mosquitoes that were collected during our survey in 2019, from three different breeding sites. In addition, *Cx. modestus* mosquitoes were also caught during a collection carried out in the summer of 2020 in Leuven. This finding suggests a recent introduction and new establishment of this mosquito species in Belgium, potentially introduced from the UK or Germany. The appearance and spread of *Cx. modestus* in the UK has only been reported recently as well, although this species seems to be abundantly present in certain regions based on recent surveys (2017, 2019) (2). The hypothesis for not noticing its presence in UK before probably relies on the misidentification of *Cx. modestus* by

other mosquito species, such as *Cx. torrentium* (2). Due to the relatively small sample size gathered in this study, we cannot conclude that the sampling year impacted the results.

Along with the introduction of a new mosquito species in a region, its potential role in the transmission of arboviruses that could cause disease in animals and humans must be evaluated. The presence of *Cx. modestus* in Belgium could be problematic as it is one of most important vectors for *Dirofilaria spp.* such as *Dirofilaria immitis* (61). Furthermore, coexistence of *Cx. pipiens* and *Cx. modestus* may increase the risk of outbreaks of WNV and USUV. These two viruses are likely to co-circulate in the same habitat, where birds and *Cx. modestus* mosquitoes play their roles as hosts and vectors, respectively. In September 2020, enzootic transmission of WNV in the Netherlands, a neighbouring country of Belgium, was confirmed for the first time by detecting simultaneously the presence of the virus in a local common whitethroat, in field collected mosquito pools and in humans (62). The new introduction of *Cx. modestus* in Belgium may increase the risk of WNV transmission. It would be advisable to implement vector surveillance programs monitoring this mosquito species. In Europe, the higher biting activity displayed by *Cx. modestus* lasts from July until the beginning of the October. However, given the detection of Tahyna virus (an arbovirus) in hibernating *Cx. modestus* mosquitoes in France (9), winter collection can also be considered for the surveillance of mosquito-transmitted pathogens.

In order to examine the genetic structure of the *Cx. modestus* population found in Leuven, we gathered mitochondrial sequences of *Cx. modestus* mosquitoes collected in other countries across Europe and constructed a haplotype network using the MJ method based on 228 partial COI sequences. As recently reported (2), *Cx. modestus* populations across Europe are separated in two lineages. According to this network most Belgian haplotypes were connected to haplotypes from the UK and Germany, suggesting that the mosquito population in Leuven, Belgium could be derived from these two populations. There were three central haplotypes in

the lineage II that were shared by several countries. In lineage I, there is one central haplotype that was shared by individuals from Denmark, Spain, and Belgium. This data might indicate that *Cx. modestus* mosquitoes belonging to both lineages are present in Belgium, suggesting the occurrence of at least two independent introduction events.

Vector competence of the mosquito can be influenced by several factors. Bacterial symbionts such as *Wolbachia* have the ability to hinder infection of a variety of pathogens such as chikungunya virus, dengue virus, Zika virus, WNV and malaria-causing *Plasmodium* in different mosquito species (63). It is possible that viral symbionts discovered in mosquitoes may have a similar effect. For instance, the insect-specific virus Nhumirim virus was shown to inhibit the replication of WNV, St Louis encephalitis virus and Japanese encephalitis virus in C6/36 cells (64). As a first step into unveil the role of viral symbionts in the mosquito's vector competence, we investigated the virome of the collected mosquitoes. Of note, no USUV or WNV was detected in the collected *Cx.* mosquitoes. Furthermore, no Lednice virus was detected in the *Cx. modestus* samples, although this mosquito species was reported to be an important Lednice *Orthobunyavirus* vector (8). In total, 33 eukaryotic viral species could be detected across all our samples in this study, and we recovered 9 (near-)complete genomes of 6 viral species.

When comparing viral hits across the mosquito species and habitat types where they were collected, some similarities could be observed. Mosquito pools belonging to the same genus seemed to have more viruses in common, as shown by the clustering of the *Cx.* mosquito pools or the distinct virome profile presented by the pool composed of *Anopheles*/*Culiseta* (pool 3) compared to the other pools. Additionally, we observed a clustering of pools per habitat type. In this case, peri-urban mosquito pools harbored several viruses in common, and in great abundance, such as Culex totivirus Leu1, Leu2 and Leu3, and Negevirus Leu7 and Leu8, closely related to Culex inatomii totivirus and Yongsan negev-like virus 1, respectively. Also,

the 6 viral species of which the (near-) complete genome was recovered were previously reported as, or clustered together with, viruses associated to mosquitoes, which might hint at the preservation of a core mosquito virome. However, a larger sampling size is needed to suggest that the virome composition and its abundance differ according to genus, local acquisition and ecosystem, and habitat composition.

When comparing our results with a virome study on *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes collected from Guadeloupe, which is the largest island of the French West Indies in the Caribbean, there were two virus species (Hubei toti-like virus 10 and Hubei partiti-like virus 22) found to be shared with Belgian mosquitoes (65). The fact that the same virus species was found in mosquitoes collected in Belgium and in Guadeloupe could indicate a widespread global movement and/or long host–virus coevolution. Moreover, several viruses were shared with Northern European Swedish mosquitoes (Whidbey virus, Hubei partiti-like virus 22, Chaq virus-like 1, Ista virus, Wuhan Mosquito Virus 6, and Sonnbo virus) (26, 66). At the virus family/order level, the relative virome abundance of the Swedish *Cx. pipiens* was dominated by the Luteo-, Orthomyxo- and Nam Dinh virus. In contrast, the virome of Belgian *Cx. pipiens* was dominated by *Iflaviridae* (pool 2).

When mosquito samples are pooled, as we did in our study, the virome profile could be strongly skewed by one or a few high titer virus infection(s) from a single mosquito in the pool. In a study of Swedish mosquitoes, Pettersson et al. (2019) reported that 30% of all reads of one of the libraries composed of *Cx. torrentium* mosquitoes were annotated to Nam Dinh virus. From pool 1, we recovered the (near-) complete genome of Alphamesonivirus Leu4, which is a member of the *Mesoniviridae* family that contains the Nam Dinh virus. Considering what was reported in Swedish mosquitoes and that pool 1 was the only pool containing one individual of *Cx. torrentium*, we suggest that Alphamesonivirus Leu4 might have been harbored by this mosquito species, as it was not found in any other mosquito pool. For further research, the use

of the individual mosquito body is recommended to perform virome characterization. The feasibility of this approach on single mosquitoes has been evaluated and no significant differences in total reads number and viral reads proportion were found when compared to pooled mosquitoes samples (65).

In conclusion, we here report the introduction and establishment of *Cx. modestus* in the surroundings of the city of Leuven, Belgium. The virome of the collected mosquitoes was revealed by a metagenomics approach. As *Cx. modestus* is known to be a vector of WNV and USUV, surveillance for this mosquito species is recommended.

References

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Figures

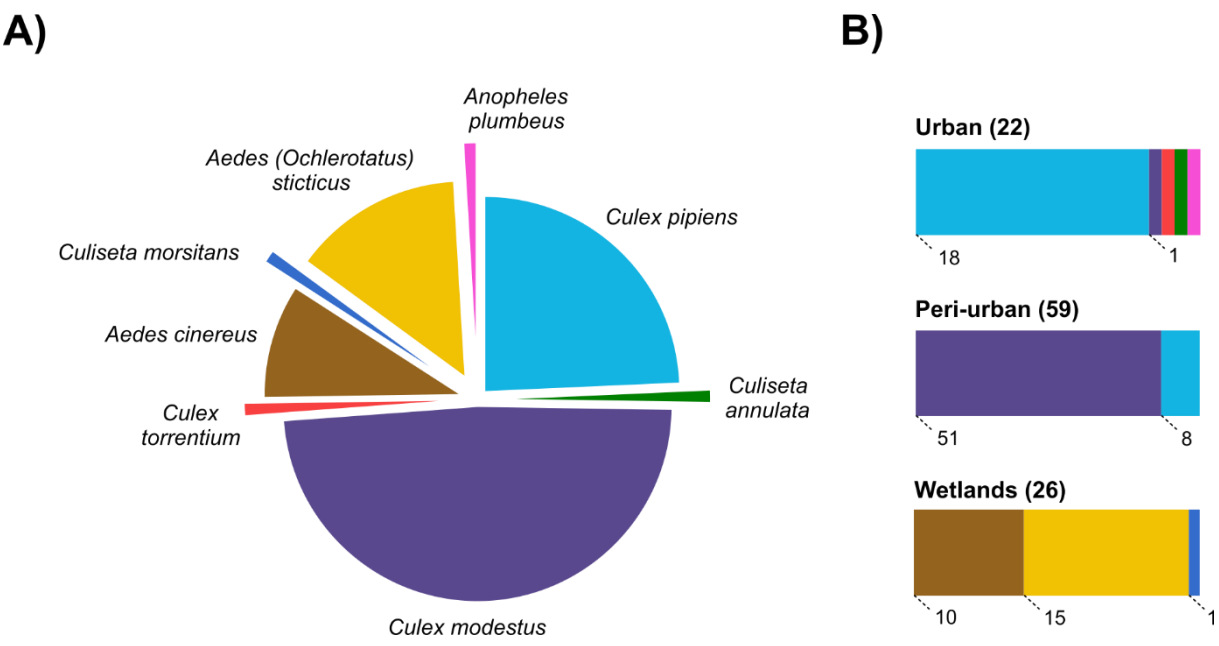

Figure 1. Mosquito species collected in Leuven, Belgium in 2019. A) Distribution of mosquito species captured during the summer of 2019 across all locations sampled in Leuven. B) Distribution of mosquito species across habitat types in Leuven. Mosquito species are marked in different colors. The number of specimens is indicated in the bar chart.

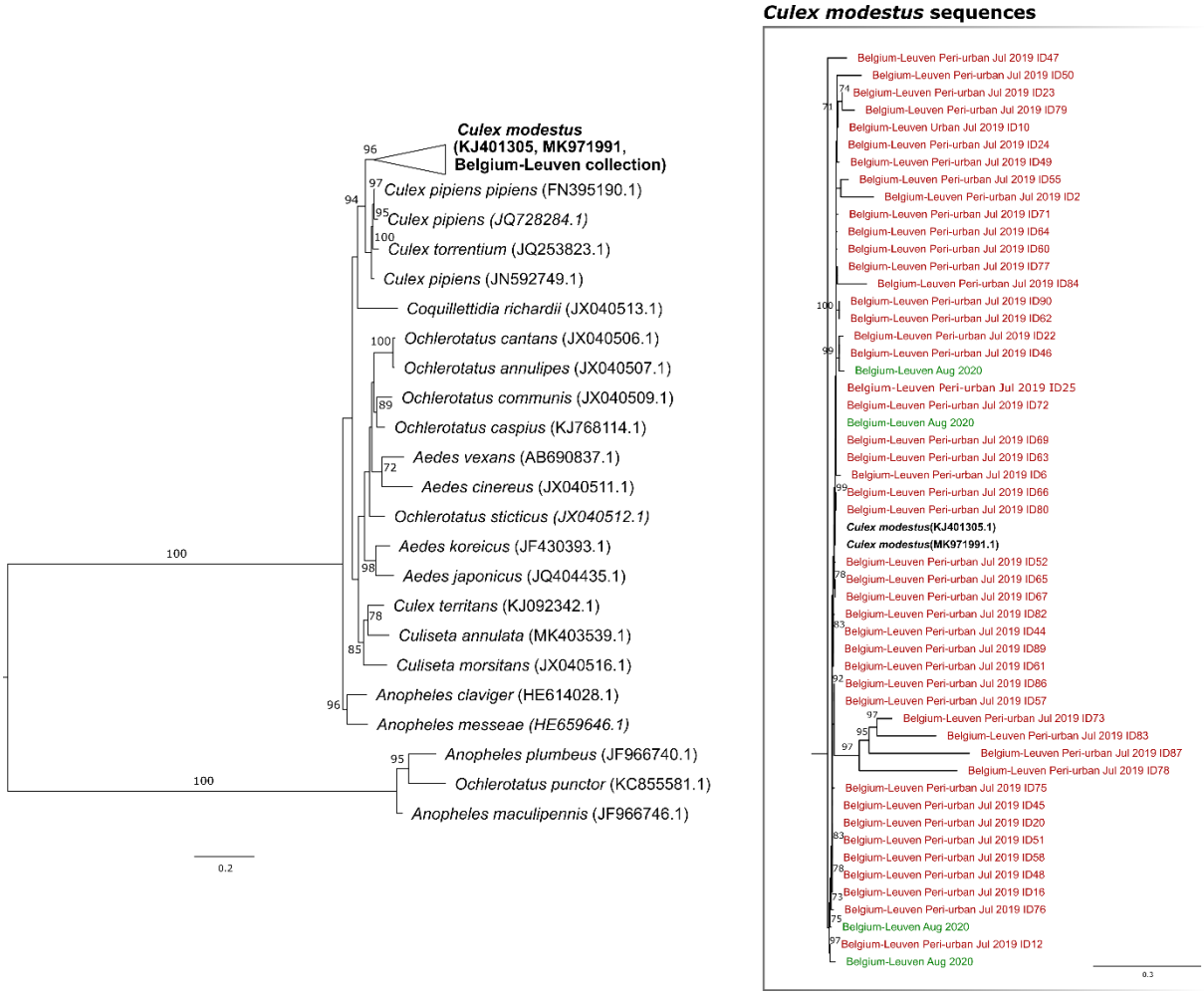

Figure 2. ML tree of the COI sequences of 21 Culicid species. Sequences derived from mosquitoes collected in Leuven are collapsed with the reference sequences for *Cx. modestus*. The collapsed branched is expanded in the panel on the right. Bootstrap values above 70% are shown above the branch points.

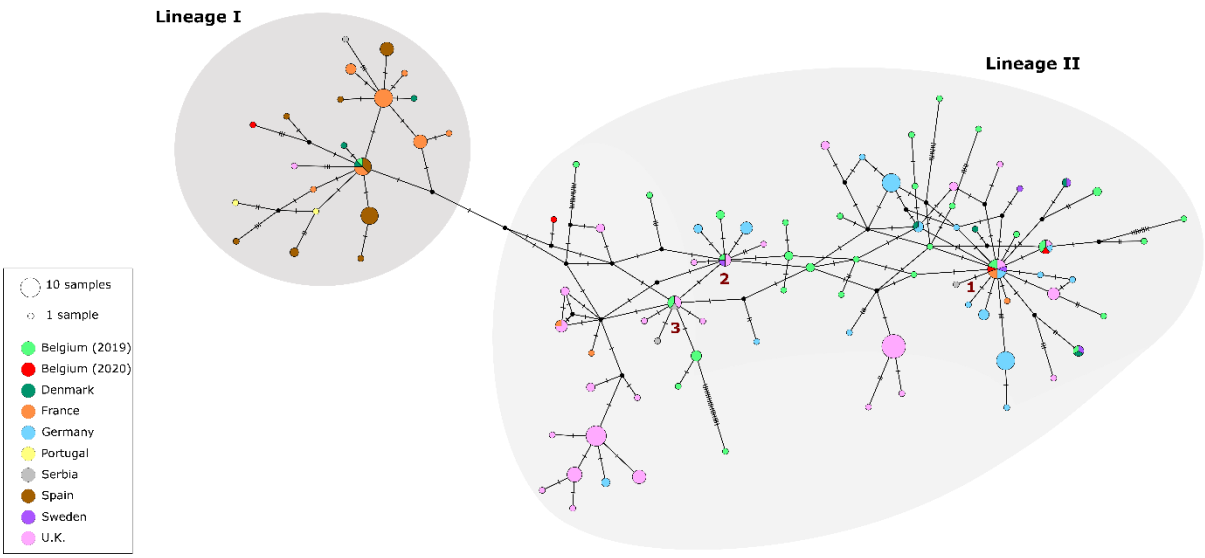

Figure 3. Median-joining network constructed with 228 COI sequences of *Cx. modestus* from 9 countries in Europe. Each circle represents a haplotype. The size of the circle corresponds to the number of specimens sharing that specific haplotype. Each country is represented by a color described in the legend. Mosquito collections in Belgium are separated per year to visualize the allocation of haplotypes in the network.

A)

Location	Pool ID	Mosquito species	*n*
Urban	1	*Culex pipiens*	6
		Culex modestus	1
		Culex torrentium	1
	2	*Culex pipiens*	12
	3	*Anopheles plumbeus*	1
		Culiseta annulata	1
Peri-urban	4	*Culex modestus*	5
		Culex pipiens	15
	5	*Culex modestus*	17
		Culex pipiens	3
	6	*Culex modestus*	19
Wetlands	7	*Aedes cinereus*	10
	8	*Aedes (Ochlerotatus) sticticus*	15
		Culiseta morsitans	1

B)

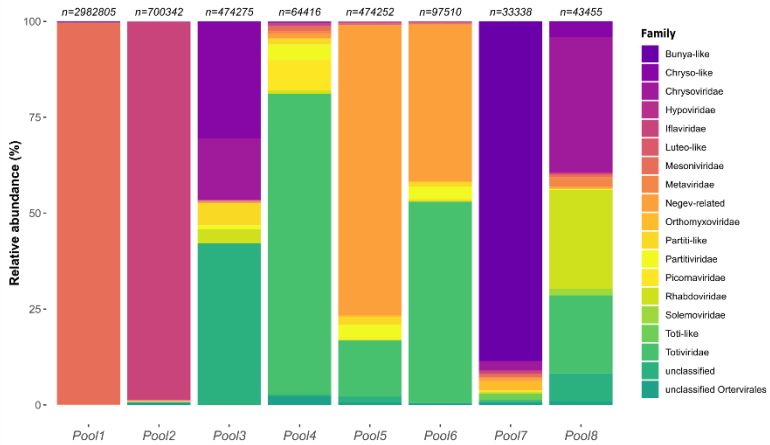

Figure 4. Summary information and viral composition of sequenced samples. A) Location, mosquito species and number of specimens present in each of the sequenced pools. B) Barplots representing the abundance of reads belonging to distinct viral families per pool. The number of eukaryotic viral reads per pool is given on top of each bar.

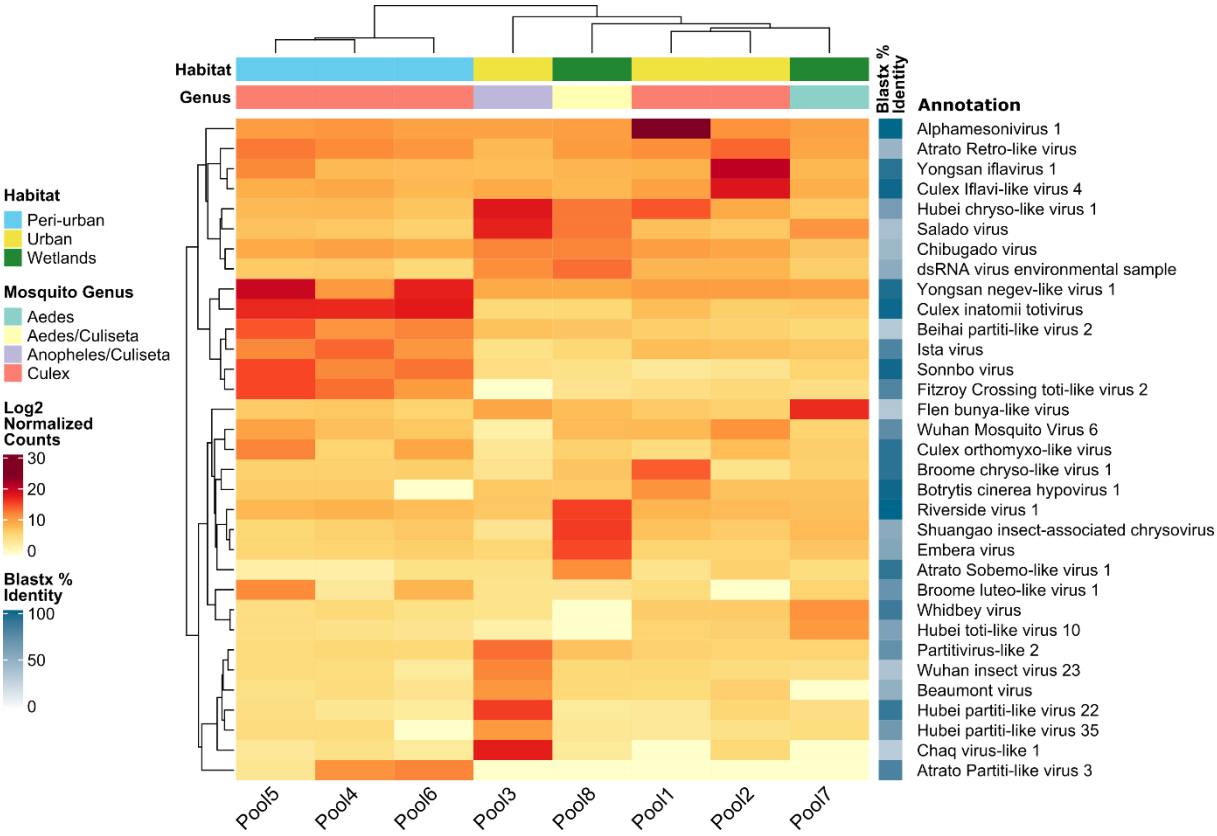

Figure 5. Heatmap of normalized read counts for eukaryotic viruses. The heatmap shows the normalized count on $\log_2$ scale of reads mapping to the assembled contigs of each eukaryotic virus. Next to the taxonomic annotation, obtained by DIAMOND and KronaTools, the average BLASTx identity for all contigs representing a viral species is depicted by the shaded blue boxes. Hierarchical clustering of the columns is based on the Bray-Curtis distance matrix calculated from the normalized read counts.

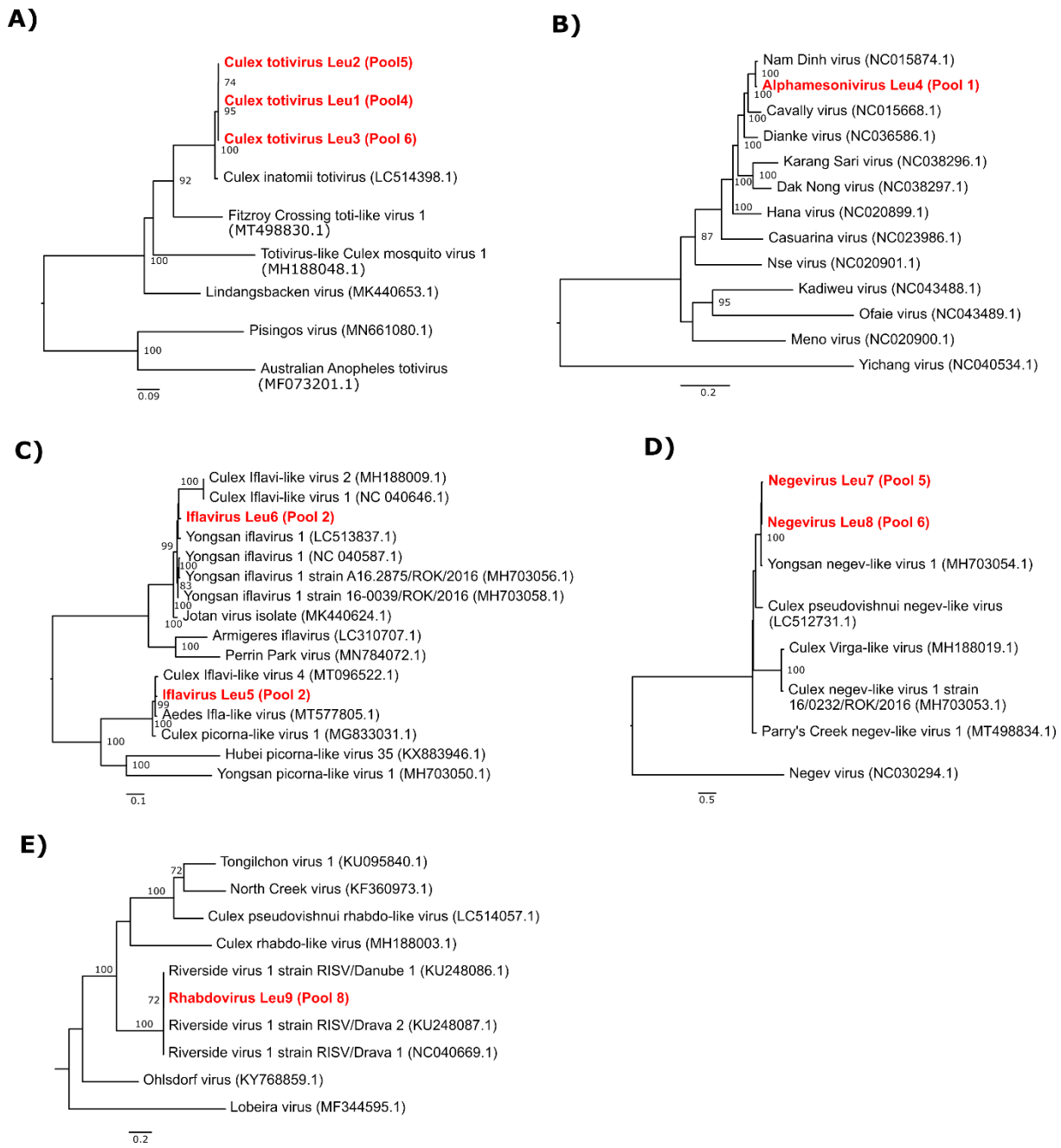

Figure 6. (Near-)complete meta-assembled genomes identified in mosquitoes collected during the summer of 2019. Bootstrap support values are shown next to the nodes. Complete MAGs are coloured in red. A) Midpoint-rooted ML tree of all complete genomes related to Culex inatomii totivirus, selected after BLASTn. B) Midpoint-rooted ML tree of all *Mesoniviridae* family members. C) Midpoint-rooted ML tree of all complete genomes related to our Yongsan iflavirus 1 and Culex Iflavi-like virus 4 genomes, selected after BLASTn. D) ML tree of all complete genomes related to Yongsan negev-like virus 1, selected after BLASTn.

Negevirus was used as the outgroup. E) Midpoint-rooted ML tree of all complete genomes related to the recovered Riversidevirus 1.

Tables

Table 1. Haplotype and nucleotide diversity of *Cx. modestus* from 9 countries in Europe.

Population	*n*	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Belgium	44	33	0.9852 ± 0.0082	0.0270 ± 0.0136
Denmark	7	7	1.0000 ± 0.0764	0.0174 ± 0.0103
France	28	11	0.8598 ± 0.0462	0.0069 ± 0.0039
Germany	42	17	0.9013 ± 0.0278	0.0113 ± 0.0060
Portugal	2	2	1.0000 ± 0.5000	0.0065 ± 0.0073
Serbia	4	4	1.0000 ± 0.1768	0.0182 ± 0.0125
Spain	22	8	0.8182 ± 0.0586	0.0058 ± 0.0034
Sweden	5	5	1.0000 ± 0.1265	0.0085 ± 0.0058
UK	74	28	0.9252 ± 0.0176	0.0107 ± 0.0057