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Discovery of Mwinilunga alphavirus: a novel alphavirus in *Culex* mosquitoes in Zambia

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Abstract

Mosquito-borne alphaviruses are disseminated globally and cause febrile illness in humans and animals. Since the prevalence and diversity of alphaviruses has not been previously investigated in Zambia, reverse transcription PCR was employed as a broad-spectrum approach for the detection of alphaviruses in mosquitoes. From 552 mosquito pools, a novel alphavirus, tentatively named Mwinilunga alphavirus (MWAV), was discovered from a single *Culex quinquefasciatus* mosquito pool. The full genome of MWAV was subsequently determined, and pairwise comparisons demonstrated that MWAV represented a new alphavirus species. Phylogenetic analyses and a linear discriminant analysis based on the dinucleotide ratios in various virus sequences indicated that MWAV is related to a mosquito-specific alphavirus distinct from other known mosquito-borne alphaviruses due to its inability to replicate in vertebrate cell lines. Further analyses of these novel alphaviruses will help to facilitate a greater understanding of the molecular determinants of host range restriction and the evolutionary relationships of alphaviruses.

Keywords: alphavirus; mosquito; *Culex*; Zambia

Main Text

Alphaviruses, belonging to the genus *Alphavirus* in the family *Togaviridae*, can infect diverse vertebrate hosts, including mammals, birds, reptiles, amphibians and fish (Griffin, 2013). Most alphaviruses are mosquito-borne and are transmitted by mosquitoes from at least eight genera (Ortiz et al., 2005; Webb et al., 2008). Restricted interactions between viruses, their invertebrate vector species and vertebrate hosts usually occur in nature; however, some alphaviruses occasionally can cause widespread epizootics (Weaver and Reisen, 2010). Humans and/or animals infected by pathogenic alphaviruses exhibit febrile illnesses that may culminate either in encephalitis or arthritis, depending upon the viral etiology (Atkins, 2013).

In Africa, human diseases caused by the Old World alphaviruses, such as chikungunya virus (CHIKV), o'nyong-nyong virus and Sindbis virus (SINV), have been reported (Lwande et al., 2015). While the Republic of Zambia is located in southern Africa and vector mosquitoes of alphaviruses have been found widely in the country (Gaffigan et al.; Kent, 2006), human cases of chikungunya fever, o'nyong-nyong fever or Sindbis fever have not been documented in prior studies (Lwande et al., 2015; Rezza et al., 2017; Storm et al., 2014) (<https://www.cdc.gov/chikungunya/index.html>). Moreover, comprehensive detection of alphaviruses in mosquitoes has not been conducted in Zambia to date. Therefore, screening of vector mosquitoes in Zambia is crucial to better predict future outbreaks of alphaviral diseases.

The alphavirus genome consists of a single-stranded, positive-sense RNA, encoding nonstructural proteins (nsPs; nsP1-nsP4), which are important for transcription and replication of viral RNA (Lemm and Rice, 1993), and structural proteins (sPs; Capsid, E3, E2, 6k and E1), which are the main constituents of virions (Voss et al., 2010). Based on genetic and serological similarities, around 30 species of alphaviruses are classified into antigenic complexes (Powers et al., 2012; Powers et al., 2001). In addition, an

unclassified alphavirus, Eilat virus (EILV), has been discovered in *Anopheles coustani* (Nasar et al., 2012) and Tai Forest alphavirus (TALV) has been recently discovered in *Culex decens* (Hermanns et al., 2017).

EILV appears to infect only mosquitoes and differs from other known mosquito-borne alphaviruses which generally maintain their lifecycles between mosquitoes and vertebrate hosts, although EILV is phylogenetically grouped within the mosquito-borne clade (Nasar et al., 2014; Nasar et al., 2012). Studies of EILV have contributed to the identification of the molecular determinants responsible for host range restriction and provided new insights into the evolutionary history of alphaviruses. However, further studies on insect-only alphaviruses are required for a better understanding of the ecology and evolutionary relationships in the genus *Alphavirus* and the molecular determinants of host range restriction (Nasar et al., 2015a; Nasar et al., 2015b). In this study, we have focused on the detection of alphaviruses in mosquitoes collected in Zambia.

To analyze the prevalence of alphaviruses in mosquitoes in Zambia, a total of 9,699 mosquitoes were collected from various locations between 2014 and 2017 (Fig. S1, available in the online Supplementary Material). Mosquito collection was carried out in protected areas with the permission of the Zambia Wildlife Authority, now the Department of National Parks and Wildlife, Ministry of Tourism and Arts of the Republic of Zambia and the Excellence in Research Ethics and Science (ERES) Converge Ethics Committee IRB (No: 00005948). Species were at first identified morphologically using a stereomicroscope, and subsequently some species were genetically confirmed by PCR and sequencing of the cytochrome-oxidase subunit I gene (Folmer et al., 1994). Information on the collected mosquitoes is summarized in Table S1 (available in the online Supplementary Material). The largest numbers of mosquito species were within the genus *Culex*. After species identification, mosquito samples were divided into 552 pools containing 1 to 40 individual mosquitoes, belonging to the same species and

sampling location. Subsequently, the pools were homogenized with Minimum Essential Medium containing 2% fetal bovine serum (Sigma, St. Louis, MO) using the BioMasher (Nippi, Tokyo, Japan). Total RNAs were extracted from 100 µl of the homogenates using the Direct-zol kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol and the remaining homogenates were stored at –80 °C until use for virus detection.

We employed a one-step reverse transcription PCR (RT-PCR) assay for detection of a wide-range of alphaviruses in the extracted mosquito RNAs. Oligonucleotide primers for the RT-PCR assay were designed based on an alignment of the conserved region of nsP4 among defined alphavirus species. One-step RT-PCR was performed with the PrimeScript One Step RT-PCR Kit, Ver. 2 (Takara, Shiga, Japan) with 1 µl of extracted mosquito RNA and 1 µM (final concentration) of each of the primers, pan-Alpha-nsP4-6692F (5'-CAYACRYTRTTYGAYATGTCDGC-3') and pan-Alpha-nsP4-7152R (5'-GCRTCDA TKATYTTBACYTCCAT-3') in 15 µl reaction volumes, with the following thermocycling protocol: 50 °C for 30 minutes; 94 °C for 2 minutes; 43 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 30 seconds; and 72 °C for 5 minutes. The PCR products (approximately 460 bp) were visualized on a 1% (w/v) agarose gel stained with ethidium bromide. We confirmed that CHIKV and SINV nsP4 genes were also detected by our methodology (data not shown).

From 552 pools, one pool, which contained 19 *Culex quinquefasciatus* mosquitoes collected in Mwinilunga (North-Western Province of Zambia) in 2016, was found to be positive by RT-PCR assay. The amplified products were sequenced using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with the pan-Alpha-nsP4-6692F primer and pan-Alpha-nsP4-7152R primer, and the nucleotide sequences were analyzed using GENETYX, version 13 (GENETYX, Tokyo, Japan). Sequence comparison of the amplified fragment in the National Center for Biotechnology Information database employing BLASTN analysis showed 76% nucleotide identity to

TALV and 75% identity to EILV. The detected alphavirus was tentatively named Mwinilunga alphavirus (MWAV). Other known alphaviruses were not detected in the present study.

We sought to isolate MWAV from RT-PCR-positive mosquitoes using *Aedes albopictus* cells (C6/36), African green monkey kidney cells (Vero) and baby hamster kidney cells (BHK-21). Homogenized samples of mosquitoes were diluted five-fold with DMEM supplemented with 2% fetal bovine serum, penicillin, streptomycin, gentamycin and 2 mM L-glutamine (Sigma), filtered and used to inoculate onto cells. After inoculation of the mosquito homogenates, supernatants of the cells were cultured through three passages for three weeks. Viral production into the supernatant was checked by RT-PCR with extracted RNA from supernatants of cultured cells and the pan-Alpha-nsP4 primers after three blind passages; however, proliferation of MWAV was not detected in any of the three cell lines tested.

To determine the full-length MWAV genome sequence, RNA extracted from the mosquitoes was subjected to next-generation sequencing. A library was constructed by the Nextera XT DNA Library Prep kit (Illumina, San Diego, CA). Sequencing was performed with a MiSeq Reagent Kit v3 (600 cycles) and Illumina MiSeq System (Illumina) following the manufacturer's protocol. After trimming low-quality reads, the resulting reads were *de novo* assembled using a CLC Genomics Workbench 10 (CLC bio, Qiagen, Hilden, Germany) with default parameters. The sequences were confirmed by specific PCRs using MWAV-specific primer pairs. The 5'- and the 3'- terminal regions were amplified using the rapid amplification of cDNA ends (RACE) approach with the SMARTer RACE cDNA Amplification Kit (Takara) and MWAV-specific primers. Amplified products were directly sequenced in both directions by Sanger sequencing. The full-genome sequence of MWAV was determined and the amino acid sequences were predicted using GENETYX, version 13. The MWAV genome consisted of 11,547 nt,

coding two open reading frames (ORFs) (2,439 and 1,235 amino acids) in the positive sense (Fig. 1). The full-genome sequence of MWAV was deposited in the GenBank/EMBL/DDBJ database under accession number LC361437. The pairwise nucleotide identities of MWAV were 70% to EILV, 58% to TALV and 52% to SINV. BLAST analysis indicated that the deduced amino acid sequence of one ORF (2,439 amino acids) was related to that of nsPs from alphaviruses and the highest identity was 83% to EILV. The deduced amino acid sequence of the second ORF (1,235 amino acids) was related to that of sPs from alphaviruses and the highest identity was 76% to TALV. The pairwise amino acid identities of MWAV ranged between 64% (E2 protein) and 88% (nsP1) to EILV for the corresponding proteins, and between 68% (nsP3) and 87% (nsP4) to TALV for the corresponding proteins (Fig. 1). According to the species designation criteria of the International Committee on Taxonomy of Viruses (Powers et al., 2012), the genetic analysis of MWAV suggested that this represented a novel alphavirus species, closely related to EILV and TALV. Eilat-like viruses, including EILV, TALV and MWAV were detected from different mosquito species including *Anopheles coustani* (Nasar et al., 2012), *Culex decens* (Hermanns et al., 2017) and *Culex quinquefasciatus*, respectively. This supports the view that those viruses have mosquito-restricted host ranges (Nasar et al., 2014). In addition, the narrow mosquito vector range may potentially explain why MWAV could not be isolated using *Aedes*-derived and mammalian-derived cells.

Since conserved sequence elements (CSEs) have important roles in the lifecycle of alphaviruses (Fayzulin and Frolov, 2004; Hyde et al., 2015; Niesters and Strauss, 1990; Zhu et al., 2013), we investigated the sequence identities of CSEs with other defined alphaviruses. The CSEs of MWAV within the nsP1 51 nt, subgenomic promoter sequence and the 3'-UTR shared more than 90% nt sequence identities with EILV and TALV (Fig. S2, available in the online Supplementary Material). The identity of the CSE within the putative 5'-UTR was 80% to that of EILV, and mFold analysis suggested that the putative

5'-UTR of MWAV might form secondary stem-loop structures, similar to EILV (data not shown) (Zuker, 2003). In addition, the conserved amino acid motifs, such as the ribosomal binding site, conserved RNA-dependent RNA polymerase motifs of MWAV were similar to those of other alphaviruses (data not shown) and the putative protease cleavage sites resembled those of EILV (Fig. S3, available in the online Supplementary Material). These sequence similarities suggest that MWAV may possess similar biological properties to EILV, which also displays a narrow vector range in mosquitoes (Nasar et al., 2014).

The full-length nucleotide sequences encoding either the nsPs or sPs ORFs of MWAV and alphaviruses available from GenBank (Table S2, available in the online Supplementary Material), were aligned using the ClustalW protocol (Thompson et al., 1994). For the alignments, the C terminus of nsP3 and the N terminus of capsid sequences were excluded, since these regions have numerous insertions and deletions and extensive sequence divergence in previous studies (Forrester et al., 2012; Nasar et al., 2012). Phylogenetic trees were constructed by maximum-likelihood method using MEGA, version 6.0 (Tamura et al., 2013). The robustness of the nodes was tested by 1,000 bootstrap replications. In both phylogenies based on nucleotide sequences of nsPs and sPs, MWAV was classified into the same phylogroup with EILV and TALV (Fig. 2), suggesting that MWAV belongs to the same antigenic complex with these two viruses in agreement with the genome analysis (Fig. 1). In both the nsPs and sPs trees, Eilat-like viruses (EILV, TALV and MWAV) have the same ancestral virus within the Western equine encephalitis (WEE) complex, placed between Trocara virus (TROV) and Aura virus (AURAV), as has been reported previously (Hermanns et al., 2017; Nasar et al., 2012). Although Eilat-like viruses have been detected in the Old World, TROV and AURAV have also been isolated from mosquitoes in South American countries (Rümenapf et al., 1995; Travassos da Rosa et al., 2001). Similar to Eilat-like viruses, vertebrate hosts of both TROV and AURAV have not been identified (Forrester et al.,

2012).

To predict the host range of MWAV, a linear discriminant analysis was performed based on the dinucleotide ratios in various virus sequences, which has been reported previously to determine host specificity based on the frequencies of dinucleotide in flavivirus sequences (Colmant et al., 2017). Complete genome sequences from 53 mosquito-borne alphaviruses with a defined host (bird, horse, donkey, human, hamster and mosquito) and full length sequences of nsPs and sPs from MWAV, EILV, TALV, TROV and AURAV were used for the analysis (Table S3, available in the online Supplementary Material). The dinucleotide ratios were calculated using the formula $P_{XY} = f_{XY}/f_X f_Y$, in which f_X and f_Y denote the frequencies of mononucleotides X and Y, respectively, and f_{XY} denotes the frequency of dinucleotide XY (Karlin and Mrázek, 1997; Lobo et al., 2009). The linear discriminant analysis was performed in the R package (Colmant et al., 2017; Di Giallonardo et al., 2017) (<https://www.R-project.org/>). The analysis showed correlations of dinucleotide usage patterns between alphaviruses and their host ranges (Fig. 3). Mosquito-borne alphaviruses showed similar dinucleotide usage patterns regardless of their phylogenetic relationships and hosts. On the other hands, the dinucleotide usage pattern of Eilat-like viruses were also similar to each other, indicating that MWAV may be a mosquito-specific alphavirus. Interestingly, TROV and AURAV, who have no known vertebrate hosts showed a different pattern from those of mosquito-bone alphaviruses and Eilat-like viruses.

If Eilat-like viruses and phylogenetically related viruses have adapted to specific mosquito hosts and relied on vertical and/or venereal transmission, it is possible that these viruses have not been spread *via* vertebrate hosts to other mosquito species. Therefore, 1) Eilat-like viruses, and phylogenetically related viruses, may exist in different parts of the world and have lifecycles involving vertical and/or venereal transmission exclusively in invertebrate mosquito hosts, or 2) Eilat-like viruses might be sporadically transported

from other areas and based on a previous report, geographic introductions of alphaviruses may have repeatedly occurred (Forrester et al., 2012). It is possible that Eilat-like viruses and/or their ancestral viruses might have lost their ability to infect vertebrates after being spread to other mosquito species in the Old World. Analyses of the biological properties of MWAV and TALV after isolation will greatly help to clarify the factors that determine the host range and the evolutionary histories of the Eilat-like alphaviruses.

In summary, we have identified a novel alphavirus, tentatively named MWAV from *Culex quinquefasciatus* mosquitoes in Zambia. Genetic analyses revealed that MWAV is closely related to, but distinct from, Eilat virus, a mosquito-specific virus and key to the better understanding of the evolution of alphaviruses. Further epidemiological and functional studies investigating arthropod and animal host species will help to better characterize future alphaviral disease risk and also provide new insights into their ecology.

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Figure Legends

Fig. 1. Genome organization of Mwinilunga alphavirus

The nucleotide lengths of the UTRs and amino acid sizes of each protein are displayed under the genome. Amino acid sequence identities of each protein between Mwinilunga alphavirus and closely related alphaviruses are indicated.

Fig. 2. Phylogenetic analysis of Mwinilunga alphavirus with previously described alphaviruses

Phylogenetic trees were constructed using the maximum-likelihood method in MEGA, version 6.0 with 1,000 bootstrap replicates based on multiple alignment of nucleotide sequences of the (a) structural proteins and (b) nonstructural proteins. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site.

Fig. 3. Score plot of the linear discriminant analysis

The figure shows a scatterplot of the two discriminant scores that explain the largest amount of the components from the linear discriminant analysis (72.3% and 16.2% for LD1 and LD2, respectively). Markers represent values for individual sequences as follows: complete genome sequences from mosquito-borne alphaviruses (crosses), full length of nsPs and sPs sequences from Mwinilunga alphavirus (black squares), Eilat-like viruses (triangles) or alphaviruses with no known vertebrate hosts (circles).

Amino acid sequence identity to Mwinilunga alphavirus (%)									
Virus	nsP1	nsP2	nsP3	nsP4	Capsid	E3	E2	6k	E1
Eilat virus	88	83	69	87	75	73	64	83	77
Tai' Forest alphavirus	83	82	68	87	75	73	71	83	79
Sindbis virus	70	64	41	78	55	51	43	47	50

Fig. 1.

Fig. 2.

Fig. 3.

Table S1. Information of collected mosquitoes

Year	Place	Mosquito genus	Pool no.	Mosquito no.
2014	Lusaka	<i>Culex</i>	10	258
	Mongu	<i>Anopheles</i>	2	19
		<i>Coquillettidia</i>	6	19
		<i>Culex</i>	52	1399
		<i>Mansonia</i>	6	26
2015	Chipata	<i>Aedes</i>	1	1
		<i>Culex</i>	16	306
	Kazungula	<i>Aedeomya</i>	1	1
		<i>Aedes</i>	3	5
		<i>Anopheles</i>	9	38
		<i>Culex</i>	5	45
		<i>Mansonia</i>	1	2
	Livingstone	<i>Aedes</i>	3	12
		<i>Anopheles</i>	3	4
		<i>Culex</i>	27	774
		<i>Mansonia</i>	1	8
	Lusaka	<i>Aedes</i>	3	20
		<i>Anopheles</i>	1	1
		<i>Culex</i>	54	1574
		<i>Mansonia</i>	2	2
2016	Kitwe	<i>Culex</i>	10	275
		<i>Aedes</i>	3	4
	Mongu	<i>Anopheles</i>	25	366
		<i>Coquillettidia</i>	10	153
		<i>Culex</i>	23	413
		<i>Mansonia</i>	5	99
		<i>Uranotaenia</i>	1	2
	Mwinilunga	<i>Aedes</i>	4	4
		<i>Anopheles</i>	2	3
		<i>Culex</i>	6	40
		<i>Uranotaenia</i>	1	2
	Ndola	<i>Culex</i>	2	19
	Siavonga	<i>Aedes</i>	22	141
		<i>Anopheles</i>	3	3
		<i>Culex</i>	19	385
2017	Livingstone	<i>Aedes</i>	8	12
		<i>Anopheles</i>	16	73
		<i>Culex</i>	46	812
		<i>Mansonia</i>	2	2
	Mongu	<i>Aedeomya</i>	2	10
		<i>Aedes</i>	4	11
		<i>Anopheles</i>	66	1451
		<i>Coquillettidia</i>	9	62
		<i>Culex</i>	49	710
		<i>Mansonia</i>	7	132
		<i>Uranotaenia</i>	1	1
		Total	552	9699

Table S2. Reference sequences for phylogenetic analyses

Species	Acession Number
Aura virus	NC 003900
Barmah Forest virus	NC 001786
Bebaru virus	NC 016962
Cabassou virus	AF075259
Chikungunya virus	NC 004162
Eastern equine encephalitis virus (NA variant)	NC 003899
Eastern equine encephalomyelitis virus (NA variant)	U01034
Eilat virus	NC 018615
Everglades virus	AF075251
Fort Morgan virus	NC 013528
Getah virus	NC 006558
Highlands J virus	NC 012561
Madariaga virus	NC 023812
Mayaro virus	NC 003417
Middelburg virus	NC 024887
Mosso das Pedras virus	AF075257
Mucambo virus	AF075253
Ndumu virus	NC 016959
O'nyong-nyong virus	NC 001512
Pixuna virus	AF075256
Rio Negro virus	AF075258
Ross River virus	DQ226993
Sagiyama virus	AB032553
Salmon pancreas disease virus	NC 003930
Semliki forest virus	NC 003215
Sindbis virus	NC 001547
Southern elephant seal virus	NC 016960
Taï Forest alphavirus	NC 032681
Tonate virus	AF075254
Trocara virus	HM147991
Una virus	HM147992
Venezuelan equine encephalitis virus (VEE IAB)	KC344505
Venezuelan equine encephalitis virus (VEE IC)	KP282671
Venezuelan equine encephalitis virus (VEE ID)	NC 001449
Venezuelan equine encephalitis virus (VEE IE)	KC344501
Venezuelan equine encephalitis virus 71D-1252	AF075255
Western equine encephalitis virus	NC 003908
Whataroa virus	NC 016961

Table S3. Reference sequences for linear discriminant analysis

Accession number	Species	Isolation host	Accession number	Species	Isolation host
NC 018615	Eilat virus	Mosquito	NC 023812	Madariaga virus	Human
NC 032681	Taï Forest alphavirus	Mosquito	NC 003417	Mayaro virus	Human
NC 003900	Aura virus	Mosquito	KY302801	Ross River virus	Human
HM147991	Trocaria virus	Mosquito	JQ771794	Sindbis virus	Human
GU001935	Eastern equine encephalitis virus	Bird	JQ771797	Sindbis virus	Human
GU001913	Eastern equine encephalitis virus	Bird	JQ771798	Sindbis virus	Human
GU001914	Eastern equine encephalitis virus	Bird	KC344505	Venezuelan equine encephalitis virus	Human
FJ827631	Highlands J virus	Bird	KP282671	Venezuelan equine encephalitis virus	Human
GU167952	Highlands J virus	Bird	GQ287640	Western equine encephalomyelitis virus	Human
KT429024	Highlands J virus	Bird	KC344491	Venezuelan equine encephalitis virus	Hamster
KJ409555	Highlands J virus	Bird	KC344515	Venezuelan equine encephalitis virus	Hamster
KT429026	Highlands J virus	Bird	GU908223	Chikungunya virus	Mosquito
GU001917	Eastern equine encephalitis virus	Horse	AY726732	Chikungunya virus	Mosquito
GU001915	Eastern equine encephalitis virus	Horse	HM045805	Chikungunya virus	Mosquito
GU001920	Eastern equine encephalitis virus	Horse	HM045818	Chikungunya virus	Mosquito
U01557	Eastern equine encephalomyelitis virus	Horse	HM045820	Chikungunya virus	Mosquito
KT429021	Highlands J virus	Horse	HM045815	Chikungunya virus	Mosquito
KT429023	Highlands J virus	Horse	HM045819	Chikungunya virus	Mosquito
AY973944	Venezuelan equine encephalitis virus	Horse	HM045784	Chikungunya virus	Mosquito
AY986475	Venezuelan equine encephalitis virus	Horse	HM045785	Chikungunya virus	Mosquito
L01442	Venezuelan equine encephalitis virus	Donkey	GU361116	Sindbis virus	Mosquito
KJ554965	Western equine encephalitis virus	Horse	JQ771793	Sindbis virus	Mosquito
GQ287645	Western equine encephalomyelitis virus	Horse	JQ771799	Sindbis virus	Mosquito
GQ287643	Western equine encephalomyelitis virus	Horse	KC344525	Venezuelan equine encephalitis virus	Mosquito
KX702402	Chikungunya virus	Human	KC344512	Venezuelan equine encephalitis virus	Mosquito
KX496989	Chikungunya virus	Human	KC344500	Venezuelan equine encephalitis virus	Mosquito
KY703990	Chikungunya virus	Human	KJ554983	Western equine encephalitis virus	Mosquito
KY704955	Chikungunya virus	Human	KJ554989	Western equine encephalitis virus	Mosquito
KX000164	Eastern equine encephalitis virus	Human

Fig. S1.

(a)		Virus	5'-UTR
		Mwinilunga alphavirus	ACAUGGGGAUAGGCUAUUAACACACAAU-UAAACCCAGUACCAAUAGCCUCCACUUUCAUCGAAU
		Eilat virus	-----.....GC.C.UU.....U.AG...U.----GUA.CG.....U.....C
		Taï Forest alphavirus	-----.....CG.U.....C.U.....U.GU.GAU.....U.....
(b)		Virus	nsP1 51 nt CSE
		Mwinilunga alphavirus	ACAGGU CACUCGAAUGAC CACGCCAAUGC GAGGGCGUUCUCGCAUUGC GC
		Eilat virus	CAU.....C.....A..C.....C.....
		Taï Forest alphavirus	C.U.....C.....U..A..U.....
		Trocar virus	G.....G.C.....U..U.....C..A.....CUG..
		Aura virus	G.....U..U.....C..A..U..U.....CUG..
		Western equine encephalitis virus	G.....GAC.....U.....C..A.....U.....GUG..
		Sindbis virus	G.....A.....U..U.....C..A..A..U.....CUG..
		Eastern equine encephalitis virus	G.....GAC.....U..U.....U.....U.....CCUA..
		Venezuelan equine encephalitis virus	G.....GAU.....U..U.....C..A.....U.....CUG..
		Ross River virus	G.....A..C.....U..U.....C..A..U..U.....CUG..
		Semliki forest virus	G.....A..A.....U..A.....C..A..A..U.....CCUG..
		Una virus	G.....A.....U..G..C.....U.....CCU..
		Chikungunya virus	G.....A.....U..U.....U..A.....CUA..
		Barmah Forest virus	G..AC...A..A..C..U..U..AC.C.....U.....CCUU..
		Southern elephant seal virus	G.....A..U.....U.....C..A..U..U.....CUG..
		Salmon pancreas disease virus	CA.UAGGU.GU.U..C.....U...GCC..C..A..U...C..C.UG..
(c)		Virus	Subgenomic promoter
		Mwinilunga alphavirus	CCCUCUACAACUGACCUAAAUAGU
		Eilat virus	A.....
		Taï Forest alphavirus	A..G...GGUG.....
		Trocar virus	A.....G.A...U..C..
		Aura virus	A.....GGUG.U.....A
		Western equine encephalitis virus	GG.....G
		Sindbis virus	AU.....GGUG.U.....
		Eastern equine encephalitis virus	GG.....G
		Venezuelan equine encephalitis virus	.U.....GG..A...G..G.A
		Ross River virus	A.....GG.G.U.....A
		Semliki forest virus	A.....GG.G.U...G..U.G
		Chikungunya virus	.UU.G...GG.G.U.....G
		Barmah Forest virus	AU.....GGUG.U.....
		Southern elephant seal virus	.G.....GG..U.....A
		Salmon pancreas disease virus	GU..A...U...U.
(d)		Virus	3'-UTR
		Mwinilunga alphavirus	AUUUUGUUUUUAUAUUUC
		Eilat virus	.A.....C
		Taï Forest alphavirus	
		Western equine encephalitis virus	A.....
		Sindbis virus	C.....
		Eastern equine encephalitis virus	
		Venezuelan equine encephalitis virus	
		Ross River virus	U..G.U.....UAC
		Semliki forest virus	.A..G.....C
		Una virus	...GGU.....C
		Barmah Forest virus	...GU.....UAC
		Salmon pancreas disease virus	C.A...G.....A...UCAAUAC

Fig. S2.

Virus	nsP1/nsP2	nsP2/nsP3	nsP3/nsP4	Capsid/E3	E3/E2
Mwinilunga alphavirus	DVGG/ALVE	EIGA/PSY	GAGG/YIFS	TVEW/SAIV	RSKR/SAPG
Eilat virus	.I../....	GV../....	/....	/....	.AR../AVAP
Tai Forest alphavirus	/....	GV../....	/....	/....	.IR../.TQP
Trocar virus	.I.A/...D	G..C/....	.V../....	..K../..AT	.P../.TEL
Aura virus	.A.A/....	GS../....	.V../....	/.RAI	.HV../.T.T
Western equine encephalitis virus	EA.A/GS..	EA.R/..A.	E..A/....	SES../.LVT	.Q../.ITD
Sindbis virus	.I.A/....	GV../....	.V../....	.E../..AP	/.VID
Eastern equine encephalitis virus	EA.A/GS..	EA.R/..A.	E..A/....	SEP../.LAT	.TR../DLDT
Venezuelan equine encephalitis virus	EA.A/GS..	EA.C/....	D..A/....	CEQ../.LVT	.KR../.TEE
Ross River virus	RA.A/GV..	TA.C/....	R..A/....	.E../..AL	.HR../.VTE
Semliki forest virus	HA.A/GV..	TA.C/....	R..A/....	SE../..PL	.HR../.VS.
Una virus	RA.A/GV..	TA.C/..A.	R.../....	/..PL	.HR../.VT.
Chikungunya virus	RA.A/GII.	RA.C/....	R.../....	AE../.LAI	.QR../.IKD
Barmah Forest virus	RA.E/GV..	PA.S/..A.	R.../....	S.../..AA	.PK../.VAH
Southern elephant seal virus	RA.A/GV..	PA.T/..N.	R..A/....	/..LT	.GK../.VAS
Salmon pancreas disease virus	GA.A/TIID	MV.../..G.	.L.../....	AIP../TRAP	.RK../AVST

Fig. S3.

Figure Legends

Fig. S1. The map of study sites in Zambia

Geographic areas where mosquitoes were collected from 2014 to 2017 (black circles) are shown. The district where the alphavirus-positive mosquito pool was collected in North-Western Province is indicated by a black star.

Fig. S2. Comparison of deduced conserved sequence elements (CSEs) of alphaviruses

Alignment of the deduced nucleotide sequences of CSEs were analyzed with the Mwinilunga alphavirus and selected alphavirus sequences using MEGA, version 6.0. Alignment of CSE (a) in the 5'-UTR region, (b) in nsP1 51 nt ORF, (c) in subgenomic promoter and (d) in the 3'-UTR region. Nucleotides identical to Mwinilunga alphavirus sequence are shown with black dots.

Fig. S3. Comparison of the protease cleavage sites of alphaviruses

Alignment of the amino acid sequences of protease cleavage sites were analyzed with Mwinilunga alphavirus and selected alphavirus sequences using MEGA, version 6.0. Conserved residues are indicated with black dots.