Investigation of the life cycles of novel viruses in arthropods and molecular analyses of the intracellular proliferation mechanisms of Chikungunya virus

節足動物から検出された新規ウイルスの生活環の解析と蚊媒介性チクングニアウイルスの細胞内増殖の分子生物学的解析

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Contents

List of abbreviations

Arbovirus	Arthropod-borne virus	IgG	Immunoglobulin G
AURAV	Aura virus	IIF assay	Indirect immune fluorescence assay
B	Base		
bp	base pair	ISE6 cell	*Ixodes scapularis* embryo cell
BHK-21 cell	Baby hamster kidney cell		
BSA	Bovine serum albumin	KURV	Kuriyama virus
CHIKF	Chikungunya fever	LD	Liner discriminant
CHIKV	Chikungunya virus	MEM	Minimum Essential Medium
CPE	Cytopathic effect		
CSE	Conserved sequence element	MKWV	Mukawa virus
DAPI	4',6-diamidino-2-phenylindole	MWAV	Mwinilunga alphavirus
DENV	Dengue virus	NGS	Next generation sequencing
DMEM	Dulbecco's modified Eagle's medium		
		ORF	Open reading flame
dpi	days post-inoculation	PBS	Phosphate buffered saline
EEE	Eastern equine encephallitis	PCR	Polymerase chain reaction
EILV	Eilat virus	PFA	Paraformaldehyde
FBS	Fetal bovine serum	qPCR	quantitative PCR
FITC	Fluorescein isothiocyanate	RT-PCR	Reverse transcription polymerase chain reaction
H. flava	*Haemaphysalis flava*		
HTRV	Heartland virus	RACE	Rapid amplification of cDNA ends
HRP	Horseradish peroxidase		
I. persulcatus	*Ixodes persulcatus*	SFTSV	Severe fever with thrombocytopenia virus
IFA	Immunofluorescence assay		

SINV	Sindbis virus
TALV	Taï Forest alphavirus
TBPV	Tick-borne phlebovirus
$TCID_{50}$	50% tissue culture infectious dose
TPB	Tryptose phosphate broth
TROV	Trocara virus
UTR	Untranslated region
VEE	Venezuelan equine encephalitis
WEE	Western equine encephalitis
ZIKV	Zika virus

General Introduction

A variety of viruses have been detected in arthropods and these viruses maintain different life cycles. While arthropod-specific viruses have life cycles within arthropods, arthropod-borne viruses (arboviruses) can infect both arthropod vectors and vertebrate animals. Since arthropods are involved in multiple ecological systems interacting with other eukaryotes, including animals, plants, and fungi, some arboviruses might adapt to their surrounded environment and obtain infectivity and pathogenicity to vertebrates in evolutional processes.

Recently, arboviruses have geographically expanded their habitats and caused the public health concerns. The arboviruses are mainly consistent of three virus groups: the genus *Flavivirus* and *Alphaavirus*, and the order *Bunyavirales*. Arboviruses are spread through bites of virus-infected arthropods such as ticks and mosquitoes to vertebrate animals. Dengue virus (DENV), Japanese encephalitis virus (JEV), Zika virus (ZIKV) and Chikungunya virus (CHIKV) can be transmitted by mosquitoes. Severe fever with thrombocytopenia virus (SFTSV) and Heartland virus (HTRV) can be transmitted by ticks. These arboviruses are also transported from one place to another by infected travelers. Outbreak of transboundary arboviral diseases seriously affected public health worldwide including Europe and the Americas. For instance, it is estimated that 390 million people are infected by DENV annually and 96 million people have clinical symptoms. However, there are no available vaccines nor effective treatments for some arboviral diseases (i.e. DENV, ZIKV, CHIKV, SFTSV and HTRV infection), as the detailed virulence and intracellular proliferation mechanisms of these viruses are still unknown. To establish the effective measurements for arboviral diseases, epidemiological studies and basic experiments for the viruses are necessary.

In chapter 1, the genetic surveillance of phleboviruses was conducted in ticks in Hokkaido, to determine the existence of tick-borne phleboviruses (TBPVs), and discovered genetically unique phleboviruses, Mukawa virus (MKWV) and Kuriyama virus (KURV). In addition, serological screening of animals in Hokkaido district and experimental infection were performed to understand their ecological life cycles in nature and the pathogenicity in mammals.

In chapter 2, comprehensive detection of alphaviruses in mosquitoes was conducted in Zambia. Although human diseases caused by alphaviruses have been reported in Africa, the prevalence of alphaviruses has not been previously investigated in Zambia. A novel alphavirus, Mwinilunga alphavirus (MWAV), was characterized to know its life cycle in this study.

In this thesis, I discussed how to control arboviruses by performing epidemiological research.

Chapter 1

Infection of newly identified phleboviruses in ticks and wild animals in Hokkaido, Japan indicating tick-borne life cycles

Introduction

The recent discoveries of human-pathogenic tick-borne viruses have impacted public health by revealing the silent risks of emerging pathogens transmitted by ticks (Yu et al. 2011, McMullan et al. 2012, Kosoy et al. 2015). Ticks hardly spread disease outside their local habitats (Eisen and Eisen 2018, Eisen et al. 2017, Jongejan and Uilenberg 2004), and thus, early detection and characterization of novel viruses in local ticks is important to highlight the endemic threats of unrecognized pathogens. In the *Huaiyangshan banyangvirus* species, genus *Banyangvirus*, family *Phenuiviridae* [previously known as genus *Phlebovirus*, family *Bunyaviridae*] (King et al. 2018), two human-pathogenic TBPVs have been identified in the 2010s; SFTS virus emerged in east Asian countries (Yu et al. 2011, Kim et al. 2013, Takahashi et al. 2014) and HTRV in the United States (McMullan et al. 2012), followed by several discoveries of RNAs from novel TBPVs in ticks using pan-phlebovirus reverse transcription polymerase chain reaction (RT-PCR) (Matsuno et al. 2015, Papa et al. 2016, Papa et al. 2017, Prinz et al. 2017, Pereira et al. 2017) and/or next generation sequencing (NGS) (Tokarz et al. 2014, Li et al. 2015). Since epidemiological information of these novel viruses that have been identified only in ticks are limited, their potential risks of spillover into the human population have not been fully understood.

Screening of novel TBPVs in ticks is required especially in endemic countries of tick-borne diseases for differentiating novel TBPV infections from known diseases, as replication of a novel TBPV may cause nonspecific febrile illness. In Japan, SFTS and

tick-borne encephalitis (Lindquist and Vapalahti 2008, Takashima 1998, Yoshii, Kojima and Nishiura 2017) are reported as viral tick-borne diseases, and tick-borne diseases caused by bacteria or parasites such as Lyme disease (Takano et al. 2014, Sato et al. 2014, Saito et al. 2007), rickettsiosis (NIID 2017, Ando et al. 2010, Imaoka et al. 2011), and human granulocytic anaplasmosis (Ohashi et al. 2013), are also recognized. These diseases present non-specific febrile illness (Suttinont et al. 2006, Parola and Raoult 2001, Bakken and Dumler 2006). To identify the potential causes of febrile illness by novel TBPVs, clarification of TBPV's distribution and pathogenicity will be beneficial in case of their future emergence.

In the present study, the ecological characteristic of a previously reported TBPV, MKWV was examined (Matsuno et al. 2018). The previous study discussed the unique genetic and biological characteristics of MKWV, a tick-derived virus genetically similar to mosquito/sandfly-borne phleboviruses rather than other TBPVs. Even though the potential of MKWV to adapt to a human cell line has been shown, the life cycle of MKWV and its infectivity and pathogenicity in mammals is unknown. Here, serological screening and experimental infection was performed to understand the ecological life cycle as well as the pathogenicity of MKWV in nature.

The current epidemiological landscape of tick-borne diseases is complicated, as studies of known and novel pathogens in ticks as well as their correlations with each other in nature are limited. Thus, potential pathogens in ticks should be discovered using a comprehensive method in addition to studying each pathogen specifically. The present study also aimed to reveal a complete picture of TBPV distribution in a limited area in Japan (i.e. Hokkaido) by genetic screening of ticks using pan-phlebovirus RT-PCR (Matsuno et al. 2015).

Materials and Methods

Study site and sample collection

Only adult ticks (n = 1,481) were collected from various locations in Hokkaido, the northernmost island in Japan from 2013 to 2015 and used for genetic surveillance of TBPVs. Ticks (n = 67) collected in Mukawa area in the previous study were also included. Questing ticks (n = 1,217) were captured by the flagging method, and blood-sucking ticks were removed from wild Yezo-deer (*Cervus nippon yesoensis*) captured by hunters in 2013 (n = 54) and from wild raccoons (*Procyon lotor*) captured by local veterinary offices in 2015 (n = 210). Tick species were identified under a stereomicroscope based on morphologic features (Sasa and Aoki 1977).

Serum samples of Yezo-deer and raccoons were used for serological testing for MKWV. Serum samples of Yezo-deer (n = 50) hunted in Hidaka area from 2010 to 2011 (Fig. 1) were a part of the samples previously used for a serological study targeting hepatitis E virus (Tomiyama et al. 2009). Serum samples were also obtained from raccoons (n = 64) captured in Mukawa, Kuriyama, Atsuma, and Ebetsu areas from 2007 to 2010 as a part of raccoon population control programs implemented by the Hokkaido Government (Fig. 1). These samples were stored at −80°C until use.

RNA extraction from ticks

Each tick was washed once in 70% ethanol and soaked twice in distilled sterile water for 10 min. The samples were then homogenized with 100 µl of Dulbecco's modified Eagle's medium (DMEM) (Nissui) using a homogenizer (Tomy Seiko) twice at 3,000 rpm. Total RNA was extracted from 50 µl of the homogenate using blackPREP Tick DNA/RNA Kit (Analytic Jena) according to the manufacturer's protocol and the remaining homogenized samples were stored at −80°C until use for virus isolation.

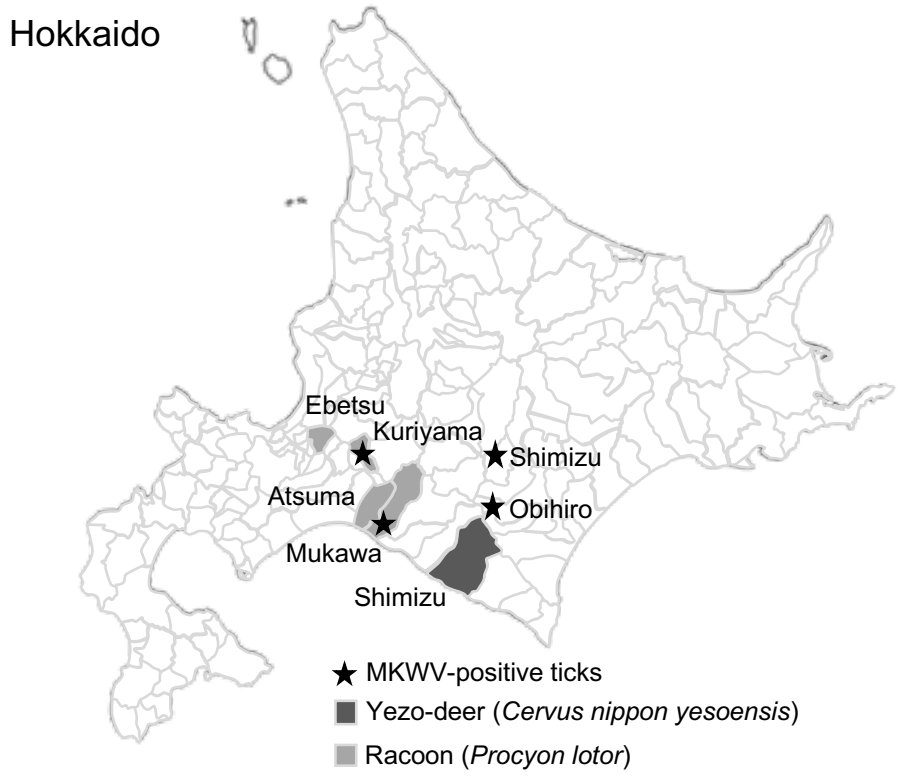


Fig. 1. Map of the present study sites.
Hokkaido is the second largest island in Japan and is geographically isolated from other prefectures. The areas where MKWV or KURV-positive ticks were captured from 2013 to 2015 (Mukawa, Kuriyama, Obihiro, and Shimizu) are indicated by black stars, areas where Yezo-deer (*Cervus nippon yesoensis*) were captured from 2010 to 2011 (Hidaka) are colored in dark gray, and areas where raccoons (*Procyon lotor*) were captured from 2007 to 2010 (Mukawa, Kuriyama, Atsuma, and Ebetsu) are colored in light gray.

Detection of TBPV RNAs

A one-step RT-PCR system reported previously (Matsuno et al. 2015) was employed to detect a wide range of TBPVs in the extracted tick RNAs. Briefly, around a 500-base pair (bp) fragment of the L segment RNA was amplified using a PrimeScript One step RT-PCR Kit Ver. 2 (Dye Plus) (TAKARA) from the tick RNA using the primer set; HRT-GOUL2759F (5′-CAGCATGGIGGIYTIAGRGAAATYTATGT-3′) and HRT-GOUL3276R (5′-GAWGTRWARTGCAGGATICCYTGCATCAT-3′). The amplified products were sequenced using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit on a 3500 Genetic Analyzer (ABI3500) (Thermo Fisher Scientific) using the HRT-GOUL2759F or HRT-GOUL3276R primer. The nucleotide sequences were determined using GENETYX version 13 (GENETYX).

Virus isolation

The remaining homogenates of RT-PCR-positive ticks were subjected to virus isolation. The *Ixodes scapularis* embryo-derived ISE6 cells were cultured in modified L-15B medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 5% tryptose phosphate broth (TPB) (Sigma), 0.1% bovine lipoprotein concentrate (MP Biomedicals), and 2% penicillin-streptomycin (Thermo Fisher Scientific) at 34°C as reported previously (Munderloh and Kurtti 1989). After ISE6 cells were cultured in 12-well plates for one day, the media were removed and 20 µl of tick homogenates were inoculated into cultured cells in 1 ml of the modified L-15B medium. One hour after inoculation, the media were changed, and the cells were cultured for seven days at 34°C under 5% CO_2. Following three blind passages (i.e. four weeks after the inoculation), viral production in the supernatant was confirmed using the RT-PCR described above.

Electron microscopy

Supernatants of virus-inoculated ISE6 cells were subjected to ultracentrifugation (27,000 rpm, 90 min) through 25% sucrose in phosphate buffered saline (PBS), following low-speed centrifugation to remove debris. The ultracentrifuged sample was adsorbed to collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH 5.8). Virus particles were observed under an H-7650 transmission electron microscope (Hitachi) at 80 kV.

Determination of nucleotide sequences of full-length viral genome

Viral RNA was extracted from the supernatants of ISE6 cells using QIAamp Viral RNA Mini Kit (QIAGEN) and the viral genome was amplified using PrimeScript One Step RT-PCR kit with specific primer pairs, which were designed based on the nucleotide sequence of MKWV (primer sequences available upon request). The 5′- and 3′- termini of each segment RNA were respectively amplified using the rapid amplification of cDNA ends (RACE) method (Li et al. 2005) with specific primers for MKWV. Amplified products were directly sequenced in both directions on the ABI3500. The full-genome sequence of each segment RNA was determined, and the amino acid sequences were predicted using GENETYX version 13.

Phylogenetic analysis

The determined full-length nucleotide sequences of the isolated viruses and those of other known phleboviruses available at GenBank, were aligned using MUSCLE implemented in MEGA version 6.0 (Tamura et al. 2013). Multiple sequence alignments were modified manually. Phylogenetic trees based on three segment RNAs were

constructed by the maximum-likelihood method using MEGA version 6.0. The robustness of the nodes was tested using 1,000 bootstrap replications.

Detection of viral antigens by immunofluorescence assay

Monolayers of ISE6 cells infected with TBPVs were fixed in ice cold acetone at three days post infection. After fixation, the cells were incubated with 1:1,000 diluted MKWV-infected mouse serum in the antibody dilution buffer: PBS containing 1.0% bovine serum albumin (BSA) fraction V (Roche Diagnostics), and 0.05% Tween 20 (Nacalai Tesque), for 1 hour at room temperature (approximately 24°C), followed by washing three times in PBS. The viral antigens were visualized by incubation with 1:1,000 diluted Fluorescein isothiocyanate (FITC)-conjugated Goat Immunoglobulin G (IgG) Fraction to Mouse IgG (MP Biomedicals) in the antibody dilution buffer for 1 hour at room temperature, followed by staining with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) (1:2,000 dilution) for 10 minutes. The cells were examined for staining under a fluorescence microscope (Olympus) with an appropriate barrier and excitation filters for visualizing FITC and DAPI (Chroma).

Detection of antibodies to MKWV by indirect immunofluorescence (IIF) assay

MKWV-infected and mock-infected Huh-7 cells (human liver carcinoma cells) were prepared for the IIF assay. Huh-7 cells were cultured in DMEM with 2% FBS, 2 mM L-glutamine (Sigma), and 2% penicillin-streptomycin at 37°C with 5% CO_2. Three days after MKWV infection, cells were fixed with 4% Paraformaldehyde (PFA) at room temperature for 10 minutes, followed by permeabilization with PBS containing 0.1% Triton X-100 (Nacalai Tesque) for 10 minutes. Serum samples of deer and raccoons were serially diluted 2-fold from 1:20 to 1:2,560 and added to both the fixed

MKWV-infected and mock-infected cells. The cells were incubated at 4°C overnight. After washing with PBS, the antigens on the slides were treated with Protein A/G-FITC (BioVision) at a dilution of 1:5,000 for 1 hour at room temperature. After another wash with PBS, the cells were examined under a fluorescence microscope with appropriate barrier and excitation filters for FITC. The titers of tested samples were recorded as the reciprocals of the highest dilutions showing positive staining.

Neutralization assay

IIF-positive serum samples were then used to detect neutralizing antibodies against MKWV. Serum samples were serially diluted 2-fold from 1:40 to 1:5,120 and mixed with an equal volume of MKWV at 500 50% tissue culture infectious dose ($TCID_{50}$). The mixtures were incubated at 37°C for one hour, and then added to cultured Huh-7 cells. Cells were incubated at 37°C with 5% CO_2 for four days, as inhibition of viral infection by MKWV-infected mouse serum used for positive serum control was clearly observed by the immunofluorescence assay. The titers were recorded as the reciprocals of the highest dilutions of serum samples that prevent infection. Titers of 1:40 or greater were considered positive results.

Experimental inoculation of MKWV into eight-week-old C57BL/6J mice

For the animal experiment, MKWV passaged four times in ISE6 cells (ISE6 cell-passaged) and MKWV passaged eight times in Huh-7 cells after the four passages in ISE6 cells (Huh-7 cell-passaged) were used. Huh-7 cell-passaged MKWV was inoculated intramuscularly (Group 1) or subcutaneously (Group 2), and ISE6 cell-passaged MKWV was inoculated subcutaneously (Group 3) at 10^5 $TCID_{50}$/mouse in a group of fifteen eight-week-old female C57BL/6J mice (Japan SLC). In total, 18

mice were inoculated with supernatants of Mock ISE6 cells or Huh-7 cells as negative controls. To analyze viral distribution in the blood and tissues of MKWV-inoculated mice, five mice from each group (three mice of each negative-control group) were sacrificed at 3, 7, and 14 days post-inoculation (dpi). The remaining mice were observed for their clinical signs, and their body weights were recorded for 14 days.

Detection of viral RNA and infectious titers in blood and tissues

Tissues (liver, spleen, and kidney) were collected individually from the mice and homogenized in DMEM supplemented with 2% FBS. Homogenates and whole blood of the three representative individuals in each group were serially diluted 10-fold with DMEM containing 2% FBS and inoculated into Huh-7 cells for titration. The remaining homogenates and whole blood were also subjected to RNA extraction with TRIzol LS (Thermo Fisher Scientific). After generation of cDNAs from the extracted RNAs with Superscript IV Reverse Transcriptase (Thermo Fisher Scientific), a quantitative PCR (qPCR) assay targeting MKWV was performed with the generated cDNA and a MKWV-specific primer set; Mukawa-L-6314F (5′-AGATCTTGTTGGGAAACACC-3′) and Mukawa-L6414R (5′-ACACAAAGTCCGCCCATTACCAATGAGATG-3′) using THUNDERBIRD SYBR qPCR Mix (TOYOBO). The reaction was performed on a Bio-Rad CFX96 system (Bio-Rad) and the MKWV titers were calculated as the $TCID_{50}$/g-equivalents of tissues or $TCID_{50}$/ml-equivalents of whole blood.

Results

Genetic surveillance of TBPVs in ticks collected in Hokkaido

To reveal the distribution of TBPVs in Hokkaido, ticks were collected from various locations and examined for TBPV genes by one-step RT-PCR. MKWV was identified in

a part of this screening (Matsuno et al. 2018). Based on morphological identification, ticks (n = 625) collected in 2013 comprised 9 species in 2 genera, ticks (n = 109) collected in 2014 comprised 5 species in 2 genera, and ticks (n = 747) collected in 2015 comprised 7 species in 2 genera (Table 1). TBPV genes were detected in *I. persulcatus* in 2013 (17/183); *I. persulcatus* in 2014 (3/41); and *Haemaphysalis flava* (1/14), *H. megaspinosa* (1/121), and *I. persulcatus* (10/219) in 2015. Only 1 TBPV was detected in an engorged *H. flava* tick collected from a raccoon in 2015 among engorged ticks. Although *I. ovatus* was dominant in Hokkaido throughout all years, no TBPV genes were detected. The homogenates of TBPV-positive ticks were further investigated based on the nucleotide sequences amplified by one-step RT-PCR. The homology values between nucleotide sequences were calculated with the corresponding partial sequences of MKWV L segment RNA. No SFTS virus-positive ticks were identified, and the nucleotide sequences derived from five *I. persulcatus* samples collected in Mukawa and Shimizu areas in 2013 [including MKW73 from which MKWV has been isolated (Matsuno et al. 2018)] were 100% matched to the corresponding partial sequences of MKWV MKW73 (Table 2). Interestingly, the nucleotide sequences in ticks collected from Kuriyama and Obihiro areas in 2015 demonstrated an 84% identity to the MKWV sequence and 100% identity with each other (Table 2). The putative MKWV-like virus has been named Kuriyama virus (KURV). MKWV and KURV RNAs were not detected in *I. persulcatus* collected from Honshu island, the largest island of Japan (Table 1).

Isolation and characterization of KURV

Homogenates of TBPV-positive samples were inoculated into ISE6 cells for virus isolation. Successful isolation of KURV was confirmed after 3 passages in ISE6 cells,

Table 1.
Screening of ticks for tick-borne phleboviruses in Hokkaido, Japan.

Place	Year	Tick species	Collection methods	Total	TBPV-positive	MKWV-positive[*1]
Hokkaido	2013	*Haemaphysalis concinna*	Flagging[*2]	7	0	0
		H. flava	Yezo-deer	8	0	0
		H. japonica	Flagging	27	0	0
			Yezo-deer	32	0	0
		H. longicornis	Flagging	4	0	0
		H. megaspinosa	Flagging	24	0	0
			Yezo-deer	3	0	0
		Ixodes ovatus	Flagging	323	0	0
			Yezo-deer	11	0	0
		I. pavlovskyi	Flagging	2	0	0
		I. persulcatus	Flagging	183	17	5
		I. tanuki	Flagging	1	0	0
			Total	625	17	5
	2014	*H. japonica*	Flagging	1	0	0
		H. megaspinosa	Flagging	1	0	0
		I. ovatus	Flagging	53	0	0
		I. pavlovskyi	Flagging	13	0	0
		I. persulcatus	Flagging	41	3	0
			Total	109	3	0
	2015	*H. flava*	Flagging	10	0	0
			Raccoons[*4]	4	1	0
		H. japonica	Flagging	33	0	0
		H. megaspinosa	Flagging	111	1	0
			Raccoons	10	0	0
		I. ovatus	Flagging	144	0	0
			Raccoons	136	0	0
		I. pavlovskyi	Flagging	34	0	0
			Raccoons	6	0	0
		I. persulcatus	Flagging	204	10	2
			Raccoons	15	0	0
		I. tanuki	Flagging	1	0	0
			Raccoons	39	0	0
			Total	747	12	2
Honshu island	2013	*I. persulcatus*	Flagging	12	1	0
	2014	*I. persulcatus*	Flagging	61	3	0

[*1] TBPV-positive samples with >80% nucleotide sequence identity to MKWV strain MKW73.
[*2] Questing ticks captured by the flagging method.
[*3] Blood-sucking ticks removed fromwild Yezo-deer (*Cervus nippon yesoensis*).
[*4] Blood-sucking ticks removed from wild raccoons (*Procyon lotor*).

Table 2.
Breakdown of MKWV-positive ticks.

Sample ID	Tick species	Year	Area	% Homology to MKWV[*1]
CZC69	*I. persulcatus*	2013	Mukawa	100
CZC73	*I. persulcatus*	2013	Mukawa	100
CZC74	*I. persulcatus*	2013	Mukawa	100
CZC209	*I. persulcatus*	2013	Shimizu	100
CZC215	*I. persulcatus*	2013	Shimizu	100
CZC80Q	*I. persulcatus*	2015	Kuriyama	84
CZC1957	*I. persulcatus*	2015	Obihiro	84

[*1]MKWV: The corresponding partial sequences of MKWV strain MKW73

without any cytopathic effects (CPE). No other putative TBPVs apart from KURV and MKWV were isolated. The KURV isolate KUR80Q originated from the tick CZC80Q collected in Kuriyama and was used in the following experiments. The virions of KURV KUR80Q were enveloped spherical particles (100-nm average in diameter) with surface spike proteins (Fig. 2A), indicating morphological similarity to MKWV and other bunyaviruses (Plyusnin et al. 2012).

Full-genome sequences of KURV RNA segments were determined by MKWV-specific primer sets followed by primer walking and the RACE method. KURV genome was consisting of 3 RNA segments: L segment, 6,444 base (b); M segment, 3,328 b; and S segment, 1,908 b. Each L or M segment RNA encoded 1 open reading flame (ORF) in the negative sense and the S segment RNA encoded 2 ORFs in the ambisense orientation. BLAST search indicated that the deduced amino acid sequences of KURV ORFs were related to those of proteins encoded in each segment from phleboviruses: RNA-dependent RNA polymerase (RdRp) on L segment, glycoproteins (Gn and Gc) on M segment, and nonstructural and nucleocapsid proteins (NSs and N, respectively) on S segment RNAs, respectively. The pairwise amino acid identities of KURV were 95% (L), 93% (glycoprotein precursor), 93% (N), and 74% (NSs) with MKWV. In the phylogenetic trees constructed based on all 3 segment RNAs, KURV was classified into the same phylogroup as MKWV (Fig. 2B). In all phylogenetic trees, the phylogroup of MKWV and KURV was branched together with the mosquito/sandfly-borne phlebovirus group and was closely related to mosquito/sandfly-borne phleboviruses rather than TBPVs. Despite their phylogenetically unique positions, MKWV and KURV were isolated from ticks and they replicated in ISE6 cells but not in *Aedes albopictus* mosquio-derived C6/36 cells like the tick-borne Uukuniemi virus (Matsuno et al. 2018) (data not shown for KURV in C6/36 cells). These results support the idea that the

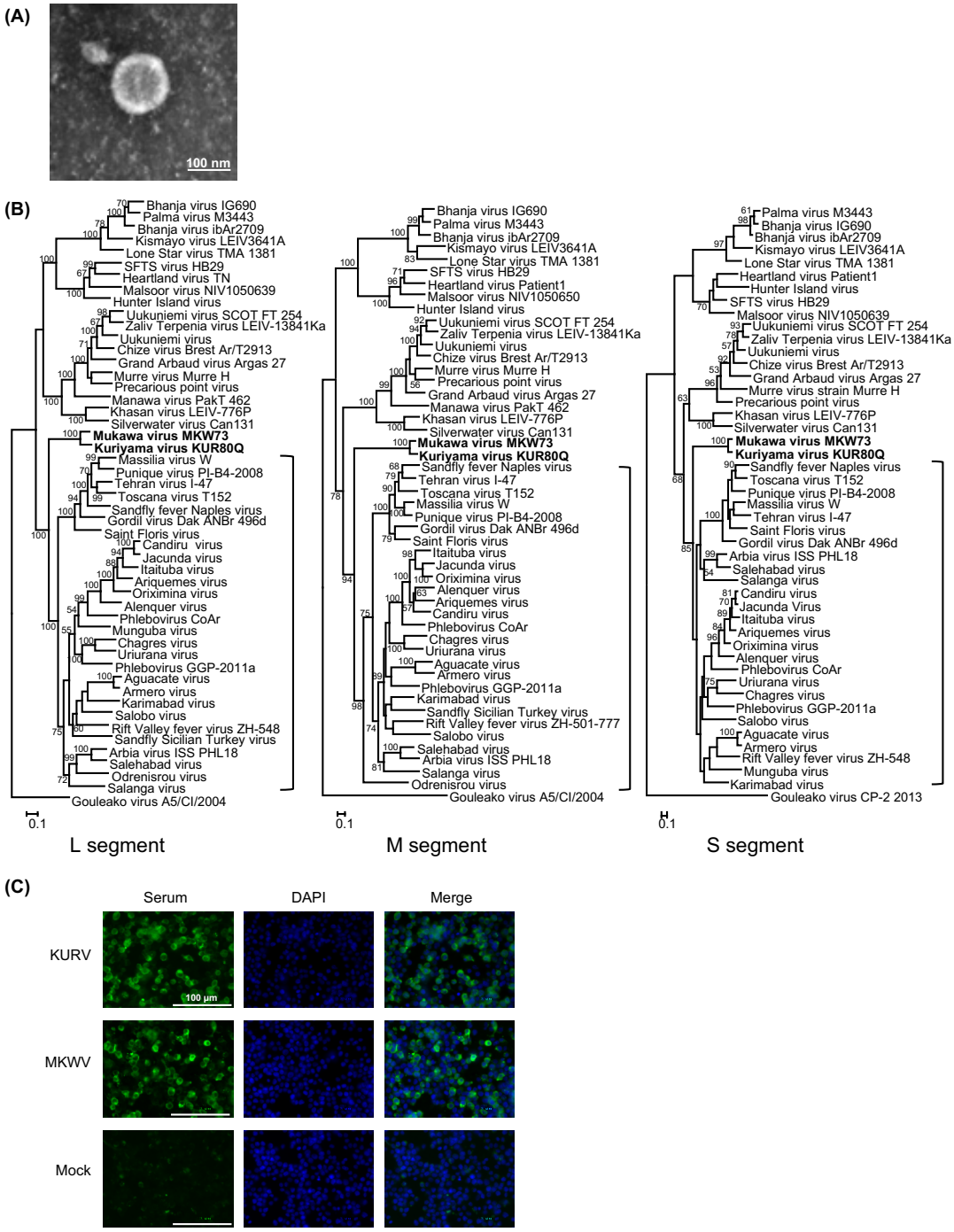


Fig. 2. Isolation and characterization of KURV.
(A) Electron micrograph of a KURV particle. (B) Phylogenetic trees constructed based on the full-length nucleotide sequences of 3 RNA segments of phleboviruses using the maximum-likelihood method in MEGA version 6.0. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site. A square bracket on the right side of each tree indicates mosquito/sandfly-borne phleboviruses. (C) The antigens of KURV and MKWV were detected in tick cell monolayer at 7 dpi using MKWV-infected mouse serum with indirect fluorescence-labelled antibodies. The antigens reacting with the serum were visualized in green; the blue color indicates nuclei stained with DAPI.

ancestor of MKWV and KURV evolved into mosquito/sandfly-borne phleboviruses from tick-borne viruses as discussed previously (Matsuno et al. 2018).

To compare the serological reactivity of KURV and MKWV, an immunofluorescence assay was conducted using MKWV-infected mouse serum. Immunofluorescence signals were mainly detected in the cytoplasm of both KURV- and MKWV-infected cells and the intensity of immunofluorescence signals was nearly identical to each other (Fig. 2C). The results indicated that the anti-MKWV serum cross-reacted with KURV antigens.

Serological survey

IIF assay was performed using serum samples from Yezo-deer and raccoons to investigate MKWV infections in wild animals in Hokkaido. Antibodies recognizing MKWV antigens were detected in 12% (6/50) of Yezo-deer and 27% (17/64) of raccoons, respectively (Table 3). Titers of positive sera ranged from 20 to 2,560 (Table 4). IIF-positive serum samples were then used for the neutralization assay to confirm MKWV infection. Serum samples of Yezo-deer (2/6) and raccoons (16/17) could neutralize MKWV; the seropositivity of anti-MKWV neutralizing antibodies was 4% (2/50) in Yezo-deer and 25% (16/64) in raccoons (Table 3). As described in the Materials and Methods section, titers of 1:40 or greater were considered positive results. Neutralizing titers ranged from 40 to 640 (Table 4). The neutralizing antibody-positive animals were found at least in 4 different areas in Hokkaido during the four-year period.

Evaluation of MKWV pathogenicity in eight-week-old C57BL/6J mice

Table 3.
Seropositivity against MKWV in wildlife captured in Hokkaido.

Animal species	Area	Year	IIF[*1]	NT[*2]
Yezo-deer (*Cervus nippon yesoensis*)	Hidaka	2010	5/27	1/5
		2011	1/23	1/1
		Total	6/50	2/6
Raccoons (*Procyon lotor*)	Mukawa	2007	1/7	1/1
		2010	0/6	-/-
	Kuriyama	2007	3/8	3/3
		2008	1/10	1/1
	Atsuma	2008	1/4	1/1
	Ebetsu	2007	5/10	5/5
		2008	3/10	2/3
		2010	3/9	3/3
		Total	17/64	16/17

[*1]IIF: Indirect immunofluorescence assay (Positive / Tested)
Titers of 1:20 or greater were considered as positive results.
[*2]NT: Neutralization assay (Positive / Tested)
Titers of 1:40 or greater were considered as positive results.

Table 4.
Detection of Anti-MKWV antibodies in wildlife in Hokkaido.

Sample ID	Animal species	Area	Year	IIF[*1] titers	NT[*2] titers
52863	Yezo-deer	Hidaka	2010	40	<40
52881	Yezo-deer	Hidaka	2010	40	160
52890	Yezo-deer	Hidaka	2010	20	<40
52900	Yezo-deer	Hidaka	2010	160	<40
53151	Yezo-deer	Hidaka	2010	80	<40
53195	Yezo-deer	Hidaka	2011	80	160
NP07-1	Raccoon	Ebetsu	2007	20	40
NP07-2	Raccoon	Ebetsu	2007	20	160
NP07-3	Raccoon	Ebetsu	2007	640	640
NP07-4	Raccoon	Ebetsu	2007	320	320
NP07-8	Raccoon	Ebetsu	2007	80	80
NP08-23	Raccoon	Ebetsu	2008	80	160
NP08-32	Raccoon	Ebetsu	2008	40	80
NP08-33	Raccoon	Ebetsu	2008	40	<40
NP10-7	Raccoon	Ebetsu	2010	20	40
NP10-10	Raccoon	Ebetsu	2010	80	160
NP10-11	Raccoon	Ebetsu	2010	2560	320
ATM08-30	Raccoon	Atsuma	2008	80	320
KUR07-4	Raccoon	Kuriyama	2007	40	40
KUR07-27	Raccoon	Kuriyama	2007	20	160
KUR07-28	Raccoon	Kuriyama	2007	40	160
KUR08-2	Raccoon	Kuriyama	2008	80	160
MKW07-4	Raccoon	Mukawa	2007	160	40

[*1]IIF: Indirect immunofluorescence assay
[*2]NT: Neutralization assay

To explore MKWV pathogenesis in adult mice, the MKWV isolate MKW73 was inoculated into groups of eight-week-old C57BL/6J mice, and their clinical signs were monitored until 14 dpi. None of the mice inoculated with MKWV demonstrated any clinical signs such as severe weight loss for 14 days. No infectious MKWVs were recovered from whole blood or tissues collected on 3, 7, and 14 dpi (data not shown), and MKWV RNA genomes were not detected in some tissues but not in whole blood (Fig. 3). On 7 and 14 dpi, MKWV RNA was detected in a limited number of organs that were inconsistent between animals (Fig. 3).

Discussion

Recently, an increasing number of viruses has been identified in ticks (Paules et al. 2018, Mansfield et al. 2017), and their potential to cause diseases in humans as well as animals is seriously discussed to disclose the impact on our public health. However, the ecological feature of these tick viruses has not fully addressed due to lack of comprehensive tick screening. The comprehensive screening of TBPVs in ticks in the present study was conducted on ticks collected in a limited area, i.e., Hokkaido, Japan, and discovery of novel viral RNAs suggested that a variety of TBPVs are still undiscovered in ticks worldwide. In this study, the genes of 2 putative TBPVs, MKWV and KURV, were detected from *I. persulcatus* ticks, and these viruses were isolated using the tick-derived ISE6 cell line. Though MKWV and KURV are genetically close to mosquito-borne viruses, replication of them in ISE6 cells indicated the major vectors of these viruses may be ticks. Infection of MKWV in mammals was also suggested by serological tests in wildlife and detection of viral RNA in animal experiments, while cross reactivity of antisera against MKWV to other endemic TBPVs may need to investigate carefully. Taken together, a tick-borne life cycle is indicated for MKWV.

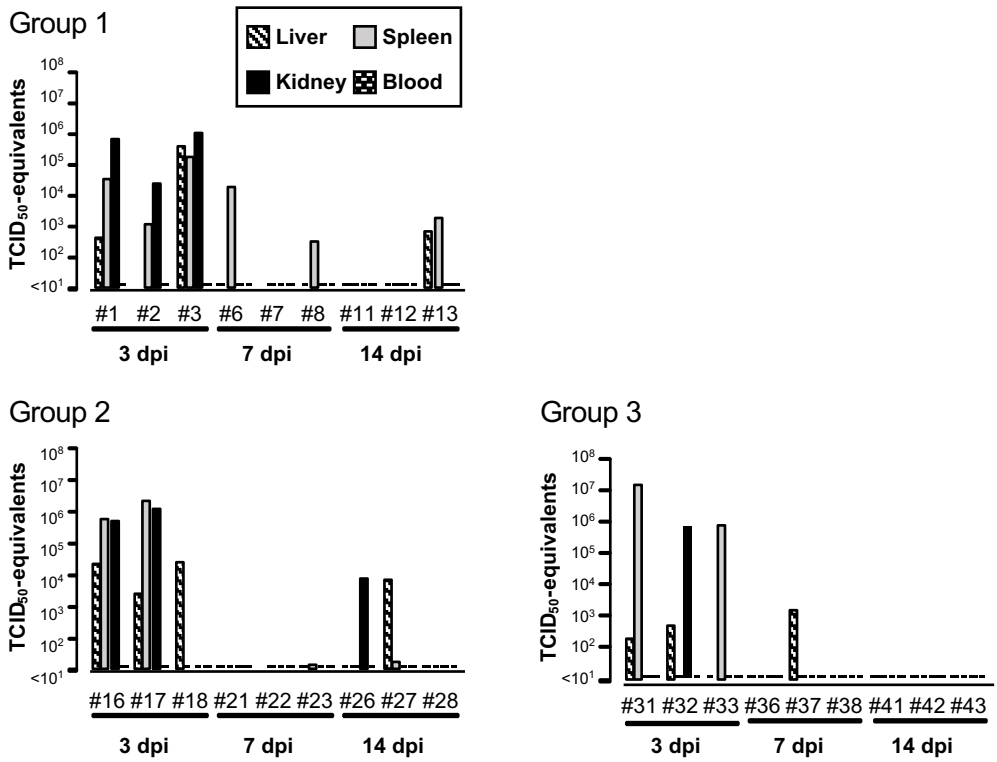


Fig. 3. Viral RNA loads in the MKWV-infected C57BL/6J mice.
Nine mice in each group were inoculated with 10^5 $TCID_{50}$ of Mukawa virus as follows: intramuscular inoculation with Huh-7 cell-passaged Mukawa virus (group 1), subcutaneous inoculation (group 2), or inoculation with ISE6 cell-passaged MKWV (group 3). At 3, 7, and 14 dpi, tissues (liver, spleen, and kidney) and whole blood collected from three individuals were subjected to reverse transcription followed by qPCR to quantify the viral RNA load. The load of MKWV RNA was indicated as the $TCID_{50}$/g-equivalents in tissues or $TCID_{50}$/ml-equivalents in whole blood. The IDs of mice are indicated as #number (1–63) below each graph.

Further experiments are demanded to confirm transmission of MKWV between ticks and mammals. However, the distribution of MKWV and KURV in ticks seemed sporadic and was not identical to the areas where antibody-positive animals were captured. Further investigation of *I. persulcatus* ticks using more sensitive detection methods for MKWV and KURV may be necessary to fill the gaps between genetic screening in ticks and serological studies in wild animals.

So far, both MKWV and its close relative KURV were detected only from *I. persulcatus* collected in Hokkaido, but not in Honshu island. While the number of *I. persulcatus* ticks collected in Honshu island was insufficient to prove the absence of MKWV and KURV using the pan-phlebovirus RT-PCR, the sporadic discoveries of MKWV and KURV in only a single tick species in limited areas suggested that their distribution could be restricted by host ticks as well as host animals. Testing on individual ticks allowed us to determine the detailed spatiotemporal distribution of MKWV and KURV rather than testing on pooled samples because a number of ticks collected in a single site is limited. So far, the detailed mechanism(s) underlying the emergence of similar viruses such as SFTS virus and HTRV, which are genetically related but discovered in different continents has not been clarified. Furthermore, selection pressure(s) to produce divergence in each TBPV species has also not been revealed yet. Since MKWV and KURV are closely related and were identified in a limited island, a spatiotemporal approach to reveal the evolutional pressures defining and maintaining the divergence of MKWV and KURV may be crucial to understand process of TBPV evolution.

To assess the potential risk of MKWV, understanding its virulence in humans and livestock animals is essential. Although serological evidences indicate that MKWV infection occurred in wild animals, no infectious virus was recovered upon experimental

infection in adult C57BL/6 mice, which show no visible clinical signs. However, as the virulence of a TBPV may vary depending on the host animal species, it is necessary to confirm the virulence of MKWV using other models. An immunocompromised mouse model such as interferon-α receptor knockout mice (Liu et al. 2014, Müller et al. 1994), may be a useful fatal model for assessing the potential of these viruses to replicate in an animal model. Infection of MKWV in mammals as well as arthropods suggested the potential risk of spillover to humans in the future. Thus, the seroprevalence of MKWV in humans and/or domestic animals is also interesting to assess the public health concerns.

In the present study, I revealed that divergent TBPVs have been maintained in ticks in Hokkaido, and demonstrated possible infection of one of the TBPVs in wildlife animals. Virus isolation is essential to conduct downstream experiments such as serological assays, experimental infections to laboratory animals, and biological characterization. Since most of recently reported novel TBPVs have not been isolated, establishment of isolation methods for these TBPVs is essential for comprehensively understanding their potential risks to public health.

Accession numbers

The reported nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession numbers LC133180.1, LC133178.1 and LC133179.1.

Summary

Recent discoveries of tick-borne pathogens have raised public health concerns on tick-borne infectious diseases and emphasize the need to assess potential risks of unrecognized tick-borne pathogens. First, to determine the existence of TBPVs, genetic surveillance of phleboviruses in ticks was conducted mainly in Hokkaido, the northernmost island in Japan from 2013 to 2015. Genes of 2 TBPVs, previously reported as MKWV and a newly identified relative of MKWV, KURV, were detected and the viruses were isolated from *Ixodes persulcatus* collected in Hokkaido, but not in *I. persulcatus* collected from other areas of Japan. These viruses were phylogenetically and antigenically similar to each other. Next, to investigate the infection of MKWV in mammals, serum samples from wildlife captured in Hokkaido from 2007 to 2011 were used for serological screening. Neutralizing antibodies against MKWV were detected in both Yezo-deer (*Cervus nippon yesoensis*) (2/50) and raccoons (*Procyon lotor*) (16/64). However, no infectious MKWV was recovered from laboratory mice in experimental infections, though viral RNAs were detected in their tissues. Thus, MKWV and KURV may maintain tick-mammalian life cycles in Hokkaido, suggesting their potential as causative agents of tick-borne diseases in mammals.

Chapter 2

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

Introduction

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 8 genera (Webb et al. 2008, Ortiz, Anishchenko and Weaver 2005). 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 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 (CHIKF), o'nyong-nyong fever or Sindbis fever have not been documented in prior studies (Lwande et al. 2015, Rezza, Chen and Weaver 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. 2001, Powers et al. 2012). In addition, an unclassified alphavirus, Eilat virus (EILV), has been discovered in *Anopheles coustani* (Nasar et al. 2012) and Taï 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 life cycles between mosquitoes and vertebrate hosts, although EILV is phylogenetically grouped within the mosquito-borne clade (Nasar et al. 2012, Nasar et al. 2014). 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. 2015b, Nasar et al. 2015a). In this study, I have focused on the detection of alphaviruses in mosquitoes collected in Zambia.

Materials and Methods

Study site and Mosquito collection

A total of 9,699 mosquitoes were collected from various locations (Fig. 4) between 2014 and 2017 (Table 5). Mosquito collection was carried out in protected areas with

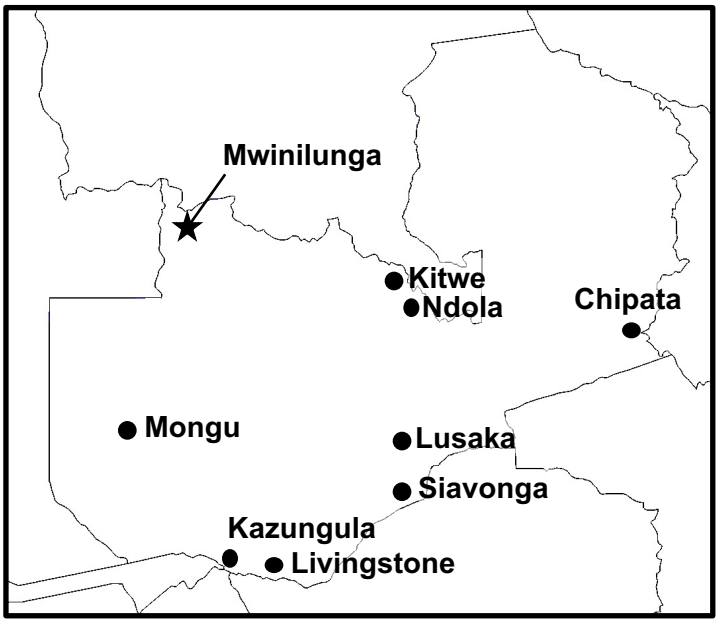


Fig. 4. The map of study sites in Zambia.
Geographic areas where mosquitoes were collected from 2014 to 2017 (black circles) are shown. The district where the alphavirus-positive mosquito pool was collected in North-Western Province is indicated by a black star.

Table 5. Information of collected mosquitoes

Year	Place	Mosquito genus	Pool no.	Mosquito no.
2014	Lusaka	*Culex*	10	258
	Mongu	*Anopheles*	2	19
		Coquillettidia	6	19
		Culex	52	1399
		Mansonia	6	26
2015	Chipata	*Aedes*	1	1
		Culex	16	306
	Kazungula	*Aedeomya*	1	1
		Aedes	3	5
		Anopheles	9	38
		Culex	5	45
		Mansonia	1	2
	Livingstone	*Aedes*	3	12
		Anopheles	3	4
		Culex	27	774
		Mansonia	1	8
	Lusaka	*Aedes*	3	20
		Anopheles	1	1
		Culex	54	1574
		Mansonia	2	2
2016	Kitwe	*Culex*	10	275
	Mongu	*Aedes*	3	4
		Anopheles	25	366
		Coquillettidia	10	153
		Culex	23	413
		Mansonia	5	99
		Uranotaenia	1	2
	Mwinilunga	*Aedes*	4	4
		Anopheles	2	3
		Culex	6	40
		Uranotaenia	1	2
	Ndola	*Culex*	2	19
	Siavonga	*Aedes*	22	141
		Anopheles	3	3
		Culex	19	385
2017	Livingstone	*Aedes*	8	12
		Anopheles	16	73
		Culex	46	812
		Mansonia	2	2
	Mongu	*Aedeomya*	2	10
		Aedes	4	11
		Anopheles	66	1451
		Coquillettidia	9	62
		Culex	49	710
		Mansonia	7	132
		Uranotaenia	1	1
		Total	**552**	**9,699**

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 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).

RNA extraction from mosquitoes

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 (MEM) containing 2% FBS (Sigma) using the BioMasher (Nippi). Total RNAs were extracted from 100 µl of the homogenates using the Direct-zol kit (Zymo Research) according to the manufacturer's protocol and the remaining homogenates were stored at −80°C until use for virus detection.

Detection of alphavirus genomes

I employed a one-step 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 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'-GCRTCDATKATYTTBACYTCCAT-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. CHIKV and SINV nsP4 genes were confirmed to be detected by this methodology (data not shown). The amplified products were sequenced using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) with the pan-Alpha-nsP4-6692F primer and pan-Alpha-nsP4-7152R primer, and the nucleotide sequences were analyzed using GENETYX, version 13.

Virus isolation

The remaining homogenates of RT-PCR-positive mosquitoes were subjected to virus isolation. Homogenized samples of mosquitoes were diluted five-fold with DMEM supplemented with 2% FBS, penicillin, streptomycin, gentamycin and 2 mM L-glutamine, filtered and used to inoculate onto *Aedes albopictus* cells (C6/36), African green monkey kidney cells (Vero) and baby hamster kidney cells (BHK-21). After inoculation of the mosquito homogenates, supernatants of the cells were cultured through 3 passages for 3 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 3 blind passages.

Determination of nucleotide sequences of full-length viral genome

To determine the full-length viral genome sequence, RNA extracted from the mosquitoes was subjected to NGS. A library was constructed by the Nextera XT DNA Library Prep kit (Illumina). 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) with default parameters. The sequences were confirmed by specific PCRs using specific primer pairs. The 5'- and the 3'- terminal regions were amplified using the RACE approach with the SMARTer RACE cDNA Amplification Kit (TAKARA) and specific primers. Amplified products were directly sequenced in both directions by Sanger sequencing. The full-viral genome sequence was determined and the amino acid sequences were predicted using GENETYX, version 13.

Phylogenetic analysis

The full-length nucleotide sequences encoding either the nsPs or sPs ORFs of detected virus and alphaviruses available at GenBank were aligned using the ClustalW protocol (Thompson, Higgins and Gibson 1994). Multiple sequence alignments were modified manually. 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.

A liner discriminant analysis

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, Trocara virus (TROV) and Aura virus (AURAV) were used for the analysis (Table 6). 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 (Lobo et al. 2009, Karlin and Mrázek 1997). The linear discriminant analysis was performed in the R package (Colmant et al. 2017, Di Giallonardo et al. 2017) (https://www.R-project.org/).

Results

Genetic surveillance of alphaviruses in mosquitoes collected in Zambia

To analyze the prevalence of alphaviruses in mosquitoes in Zambia, mosquitoes were collected from various locations and examined for alphavirus genes by one-step RT-PCR. Based on morphological identification, the largest numbers of mosquito species were within the genus *Culex*. From 552 pools, one pool, which contained 19 *Culex quinquefasciatus* mosquitoes collected in Mwinilunga (Fig. 4, North-Western Province of Zambia) in 2016, was found to be positive by RT-PCR assay. 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. Next, MWAV was attempted to isolate from RT-PCR-positive mosquitoes using C6/36, Vero and BHK-21 cells; however, proliferation of MWAV was not detected in any of the cell lines tested.

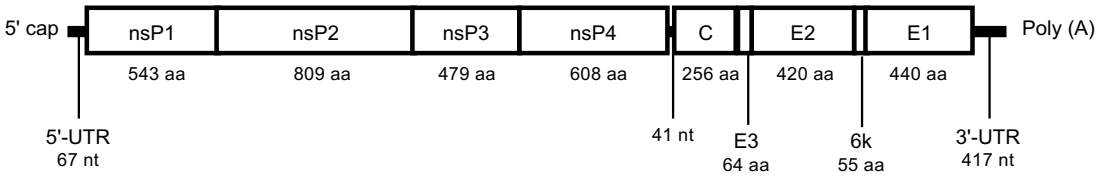
Table 6. Reference sequences for linear discriminant analysis

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	Trocara 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			

Characterization of MWAV

Full-genome sequences of MWAV were determined by NGS and MWAV-specific primer sets followed by the RACE method. The MWAV genome consisted of 11,547 bp, coding 2 ORFs (2,439 and 1,235 amino acids) in the positive sense (Fig. 5). 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 a 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. 5). 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.

Since conserved sequence elements (CSEs) have important roles in the lifecycle of alphaviruses (Hyde et al. 2015, Niesters and Strauss 1990, Fayzulin and Frolov 2004, Zhu, Zhao and Liang 2013), the sequence identities of CSEs were examined with other defined alphaviruses. The CSEs of MWAV within the nsP1 51 bp, subgenomic promoter sequence and the 3'-untranslated region (UTR) shared more than 90% nucleotide sequence identities with EILV and TALV (Fig. 6). 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



	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
Taï Forest alphavirus	83	82	68	87	75	73	71	83	79
Sindbis virus	70	64	41	78	55	51	43	47	50

Fig. 5. Genome organization of MWAV.
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 MWAV and closely related alphaviruses are indicated.

(A)

Virus	5'-UTR
Mwinilunga alphavirus	ACAUGGGGAUAGGCUAUAUAACACACAAU-UAAACCCAGUACCAAAUAGCCUCCCACUUUCAUCGAAU
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 b CSE
Mwinilunga alphavirus	ACAGGUCACUCCGAAUGACCACGCCAAUGCGAGGGCGUUCUCGCAUUGCGC
Eilat virus	CAU......C.....................A..C.....C........
Taï Forest alphavirus	C.U......C.....................U..A..U..............
Trocara 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............
Trocara 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	AUUUUGUUUUUAAUAUUUC
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. 6. Comparison of deduced CSEs of alphaviruses.
Alignment of the deduced nucleotide sequences of CSEs were analyzed with the MWAV and selected alphavirus sequences using MEGA, version 6.0. Alignment of CSE (A) in the 5'-UTR region, (B) in nsP1 51 nucleotide ORF, (C) in subgenomic promoter and (D) in the 3'-UTR region. Nucleotides identical to MWAV sequence are shown with black dots.

EILV (data not shown) (Zuker 2003). In addition, the conserved amino acid motifs, such as the ribosomal binding site, conserved RdRp motifs of MWAV were similar to those of other alphaviruses (data not shown) and the putative protease cleavage sites resembled those of EILV (Fig. 7). 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).

In both phylogenies based on nucleotide sequences of nsPs and sPs, MWAV was classified into the same phylogroup with EILV and TALV (Fig. 8), suggesting that MWAV belongs to the same antigenic complex with these 2 viruses in agreement with the genome analysis (Fig. 5). 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 TROV and AURAV, as has been reported previously (Nasar et al. 2012, Hermanns et al. 2017).

Prediction of the host range of MWAV

The host range of MWAV were predicted by linear discriminant analysis based the dinucleotide ratios in various virus sequences as previously reported. The analysis showed correlations of dinucleotide usage patterns between alphaviruses and their host ranges (Fig. 9). 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 was 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-borne alphaviruses and Eilat-like viruses.

Virus	nsP1/nsP2	nsP2/nsP3	nsP3/nsP4	Capsid/E3	E3/E2
Mwinilunga alphavirus	DVGG/ALVE	EIGA/APSY	GAGG/YIFS	TVEW/SAIV	RSKR/SAPG
Eilat virus	.I../....	GV../....	..../....	..../....	.AR./AVAP
Taï Forest alphavirus	..../....	GV../....	..../....	..../....	.IR./.TQP
Trocara 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. 7. Comparison of the protease cleavage sites of alphaviruses.
Alignment of the amino acid sequences of protease cleavage sites were analyzed with MWAV and selected alphavirus sequences using MEGA, version 6.0. Conserved residues are indicated with black dots.

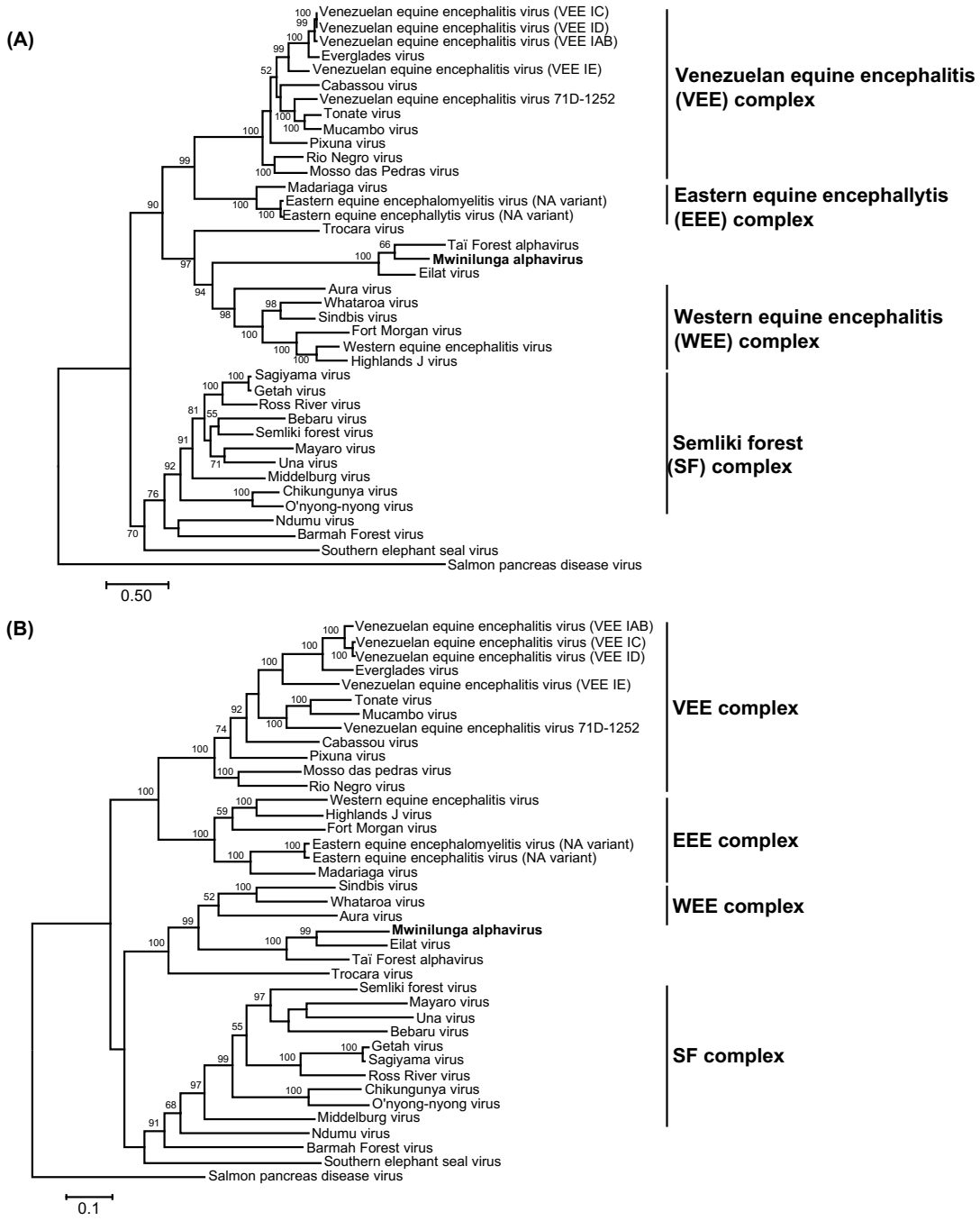


Fig. 8. Phylogenetic analysis of MWAV with previously described alphaviruses. Phylogenetic trees were constructed using the maximum-likelihood method in MEGA, version 6.0 with 1,000 bootstrap replicates based on multiple alignment of nucleotide sequences of the (A) sPs and (B) nsPs. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site.

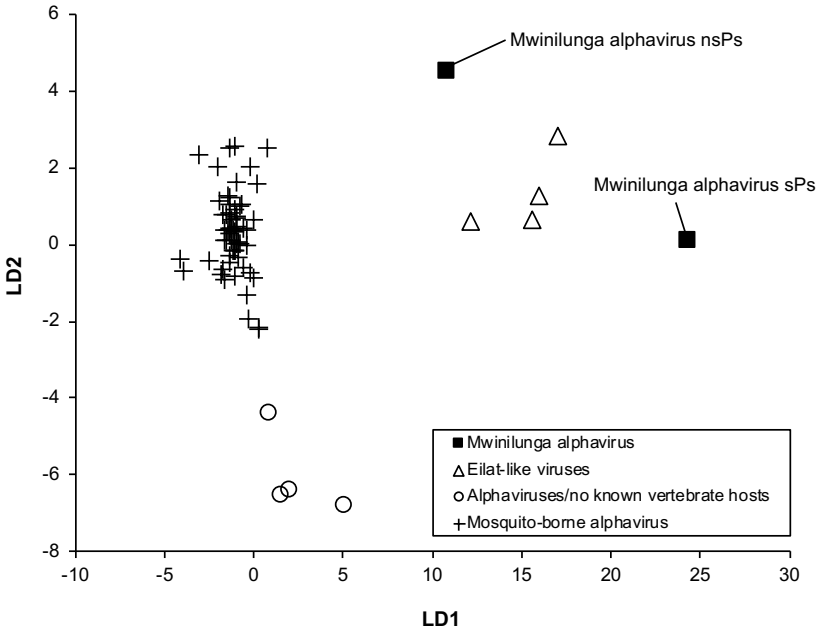


Fig. 9. Score plot of the linear discriminant analysis.
The figure shows a scatterplot of the two discriminant scores that explain the largest amount of the components from the linear discriminant analysis [72.3% and 16.2% for Liner discriminant 1 (LD1) and LD2, respectively]. Markers represent values for individual sequences as follows: complete genome sequences from mosquito-borne alphaviruses (crosses), full length of nsPs and sPs sequences from MWAV (black squares), Eilat-like viruses (triangles) or alphaviruses with no known vertebrate hosts (circles).

Discussion

Mosquito-borne alphaviral diseases have been reported largely in African countries, however the distribution of alphaviruses has not been examined in mosquitoes in Zambia to date. Here, my comprehensive and specific screening for alphaviruses has identified a novel alphavirus, tentatively named Mwinilunga alphavirus from *Culex quinquefasciatus* mosquitoes in Zambia. Complete genome sequence analyses revealed that MWAV is a novel alphavirus species highly similar to EILV, a mosquito-specific alphavirus, which is notably distinct from other known mosquito-borne alphaviruses due to its inability to replicate in vertebrate cell lines. Here, I report the existence of unique alphavirus, which will be a key to the better understanding of the evolution of alphaviruses.

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. And EILV were reported to display a narrow vector range in mosquitoes (Nasar et al. 2014). If MWAV can infect to only some mosquito species as same as EILV, this may potentially explain why MWAV could not be isolated using *Aedes*-derived and mammalian-derived cells. To acquire MWAV for the further analyses, different isolation methods using *Culex* mosquito and/or *Culex* mosquito-derived cells should be desired. Otherwise, to generate MWAV and chimeric viruses with other mosquito-borne alphavirus seems to be useful similar to the analyses of life cycle of insect-specific flaviviruses (Piyasena et al. 2017, Piyasena et al. 2019).

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, Strauss and Strauss 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). 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.

This study revealed that unrecognized alphaviruses still exist in mosquitoes. 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.

Summary

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, RT-PCR was employed as a broad-spectrum approach for the detection of alphaviruses in mosquitoes. From 552 mosquito pools, a novel alphavirus, tentatively named 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. 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.

General Conclusion

Viruses in arthropods possess a variety of life cycles. In this study, novel viruses were discovered from arthropods by RT-PCR screening and the molecular characteristics of novel viruses were examined. In chapter 1, two TBPVs, MKWV and KURV, were detected from *I. persulcatus* ticks. Importantly, infection of MKWV in wildlife was demonstrated by serological tests, while the sporadic discoveries of MKWV and KURV in only a single tick species in Hokkaido suggested that distribution of MKWV and KURV could be restricted in limited areas. In chapter 2, the existence of an alphavirus in *Culex quinquefasciatus* mosquitoes in Zambia was firstly reported by comprehensive and specific screening for alphaviruses. The detected virus is a novel alphavirus species, tentatively named Mwinilunga alphavirus, which seemed to have a mosquito-specific life cycle, distinct from other known mosquito-borne alphaviruses. Recent large-scale sequence analyses have revealed the existence of novel viral genomes; however, their ecological features have been left unknown in many cases, since most studies have accidentally discovered only partial viral genome fragments. By using tick-derived cell lines, two viruses (MKWV and KURV) were successfully isolated. Thus, downstream experiments could be conducted to know the infectivity and pathogenicity of these viruses in mammals. Establishment of efficient isolation methods is demanded for viruses in arthropods. On the other hands, MKWV was not isolated, since MWAV may possess a narrow host range. Therefore, generation of MWAV by reverse genetics method will be performed similar to that for insect-specific flaviviruses in the following study. Identification of the biological characters of novel viruses is essential for comprehensively understanding their potential risks to public health.

In consideration of the current impact of arbovirus infection on public health, a deeper understanding of the ecological features of arboviruses is demanded. There are

still uncharacterized viruses in arthropods. To draw the whole maps of network among viruses in arthropod, sensitive virus detection tool is necessary. In addition to that, following specific epidemiological and biological study is important to know their ecological feature. This study provides a base for both epidemiological and molecular analyses of arthropod viruses.

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Summary in Japanese

節足動物には様々なウイルスが存在し、それぞれ異なる生活環を形成している。節足動物に特異的に感染するウイルスが存在する一方で、節足動物媒介性ウイルス (アルボウイルス) は哺乳動物と節足動物の両者に感染する。近年、ジカ熱やデング熱といった蚊媒介性アルボウイルス感染症は、物質輸送のボーダーレス化に伴い、発展途上国を中心に世界規模で蔓延し、経済上の影響を与えてきた。加えてチクングニアウイルスなどのアルボウイルスは、アメリカやヨーロッパでも感染が認められ、日本でも毎年、輸入症例が報告されている。これら越境性のアルボウイルス感染症は公衆衛生上大きな問題となっているが、チクングニアウイルスやデング熱ウイルスなどのアルボウイルスは、未だ有効な対策法が確立されていない。今後、効果的なアルボウイルス感染症対策を構築するためにはウイルスの分布を把握する疫学調査が必要とされる。

そこで本研究では節足動物に存在するウイルスの分布状況を国内外で調査した。

第 1 章では、北海道のマダニを対象に網羅的なフレボウイルスの調査を実施した。RT-PCR によるフレボウイルス遺伝子調査の結果、ムカワウイルス及びクリヤマウイルスの 2 種のフレボウイルス遺伝子をシュルツェマダニから検出し、さらにマダニ由来細胞を用いてクリヤマウイルスを分離した。また、北海道の野生動物を対象とした血清疫学調査及びマウスを用いた感染実験の結果、ムカワウイルスは哺乳動物に感染し得ることが明らかとなった。

第 2 章では、これまでアルファウイルスの検出が報告されていないアフリカのザンビア国で、蚊を対象に網羅的にアルファウイルスを探索した。その結果、ネッタイシマカから新規アルファウイルスであるムウィニルンガアルファウイルスを検出した。決定したウイルス遺伝子全長配列を元に解析を実施した結果、

ムウィニルンガアルファウイルスは、他の蚊媒介性アルファウイルスと異なり、蚊特異的に感染するウイルスであることが明らかとなった。

近年のデングウイルス感染症やジカ熱感染症などの世界規模でのアルボウイルス感染症症例の増加を鑑みると、自然界におけるアルボウイルスの生活環、存続機構を考慮した対策が必須である。本研究では、アルボウイルス感染症対策に向けて節足動物に存在するウイルスの疫学調査の基盤を構築することができた。