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Author(s)	Kurnia, Yudistira Wahyu
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**Identification, Molecular Characterization, and Application
of a Novel Virus Isolated from Mosquito Larvae in Okushiri
Island, Japan**

(北海道奥尻島において蚊幼虫から分離された新規ウイルスの同定、性状解析および利用)

**Hokkaido University Graduate School of Agriculture
Division of Agrobiolology Doctor Course**

Yudistira Wahyu Kurnia

ユディスティラ ワヒュ クルニア

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ABSTRACT

Mosquitoes are one of the most important pest insects that have a serious impact on public health; they are the primary vector of serious infectious diseases such as malaria, dengue fever, chikungunya, Japanese encephalitis, and yellow fever. Vector control is an essential strategy for reducing the transmission of mosquito-borne diseases. However, vector control by chemical pesticides causes undesired effects. It also affects untargeted insects and stimulates appearance of pesticide-resistant insects. Viruses with following properties is ideal for biological control agents for mosquito-borne diseases: strong mosquitocidal activity, ability to naturally infect mosquitoes, and ability to propagate exclusively in mosquito cells *in vitro*. Therefore, exploring and characterization of viruses infecting mosquitoes in nature is demanded for developing environment-friendly biological pesticides to replace chemical pesticides.

In this study, a novel virus was isolated from field-collected *Aedes* larvae in Okushiri Island, Hokkaido, Japan, and was designated Okushiri virus (OKV). OKV replicated in the *Aedes albopictus* cell line C6/36 with severe cytopathic effects and released a large number of spherical viral particles 50-70 nm in diameter into the cell culture medium. The OKV had a positive-sense, single-stranded RNA genome that consisted of 9,704 nucleotides, excluding the poly(A) tail at the 3' terminus. The OKV RNA contained three major open reading frames (ORF1, ORF2, and ORF3). ORF1 encoded a putative protein of approximately 268 kDa that included a methyltransferase, FtsJ-like methyltransferase, helicase, and RNA-dependent RNA polymerase domains. ORF2 and ORF3 were inferred to encode hypothetical membrane-associated

proteins of approximately 45 kDa and 22 kDa, respectively. The genome organization and a phylogenetic analysis based on the predicted amino acid sequence suggested that OKV is a member of a new insect-specific virus genus *Negevirus*.

To enable genetic manipulation of the OKV genome, an infectious cDNA clone of OKV was constructed. OKV genomic RNA was synthesized *in vitro* from pFBOKV, a full-length OKV cDNA, in the presence or absence of cap analogue. Regardless of whether the 5' terminal is capped or not, transfection of *in vitro* synthesized RNA into mosquito C6/36 cells produced infectious progeny viruses. Subsequently, ORF3 in pFBOKV was replaced with GFP coding sequence to generate a construct designated as pO2GFP. C6/36 cells transfected with pO2GFP-derived RNA successfully expressed GFP, but failed to produce progeny viruses. Co-transfection of C6/36 cells with pO2GFP- and pFBOKV-derived RNA revealed that it is possible to produce infectious pO2GFP-derived progeny viruses by supplying OKV genetic elements and/or gene product deleted in pO2GFP even though the infectivity appeared to be low. To date, this is the first successful construction of a negevirus-based foreign gene expression system. The cDNA clones constructed and biological insights obtained in this study will be powerful tools allowing for reverse genetics of OKV and provide a basis to develop efficient negevirus-based expression vector systems.

Keywords: Negevirus, Okushiri virus, Identification, Characterization, Infectious clone

GENERAL INTRODUCTION

Arthropod-borne diseases has become significant problem for human health worldwide (Van Den Hurk *et al.*, 2012). The World Health Organization (WHO) estimated 500 000 people with severe dengue fever require hospitalization each year and about 2.5% of those affected die (Who, 2009). The cause of those diseases were arthropod-borne viruses (arboviruses); vertebrate-infectious viruses transmitted biologically (requiring replication in the vector) by mosquitoes, ticks, mites and other arthropod vectors (Bolling *et al.*, 2015). Among those vectors, mosquitoes became most urgent because they are primary vector for arboviruses causing world's prominent diseases described above

Chemical pesticides have been used as a means to control mosquitoes. Even though DDT and many synthetic insecticides have made great success in the control of mosquito-borne diseases such as malaria and typhus fever, the incidences of these infectious diseases are still extremely high in many parts of the world (Awoke and Kassa, 2006) Moreover, using chemical compounds to control insect pests such as mosquito larvae/adults may cause water pollution and/or air pollution and could lead to deteriorate the planet's health instead because of non-target controlling effects (Montesinos, 2003).

The development of a microbial pesticides using some of the non-culturable microorganisms including insect-specific viruses lead to a promising way to eliminate the possibility to affect untargeted insect species (Montesinos, 2003). Viruses are extremely small particles (about 20-500nm) composed of capsids and genetic materials either DNA or RNA and are simplest organisms capable of replication and production of virions. They lead

death of host's cells and host organism in particular host-virus combinations (Becnel *et al.*, 2007). The use of viruses to control insects was initially investigated by Balch and Bird (1944) and was proved its effectiveness in the 1940's through detection of disease in the pest called Sawfly (*Gilpinia hercyniae*) and of Steinhaus and Thompson (1949) in their report of principles of insect pathology (Ignoffo, 1973). They reported that water extract of diseased larvae caused same symptom that had been observed in diseased larvae and had killed the inoculated larvae. These observations provided an excellent starting point for further studies and developments of viral insecticides.

In past decades, the discovery of insect-specific viruses that replicates exclusively in insects such as baculoviruses (*Baculoviridae: Nucleopolyhedrovirus*), densovirus (*Parvoviridae: Densovirus*), iridoviruses (*Iridoviridae: Choloriridovirus*), and cytoplasmic polyhedrosis viruses (*Reoviridae: Cypovirus*) has been paid attention to their potential as biological control agents as well as novel vaccine platforms against arbovirus (Becnel *et al.*, 2007; Bolling *et al.*, 2015). However, molecular biology studies of these viruses are limited because cell lines suitable for investigating the molecular mechanisms of their multiplication and pathogenesis are not currently available. Therefore, detailed microbiological analysis of these viruses and industrial large-scale amplification have been inhibited (Montesinos, 2003). One exception is mosquito densovirus (MDV) which can propagate highly specifically and are virulent to mosquitoes *in vitro* and *in vivo*. These features make these viruses attractive candidates for biological control agents against mosquitoes (Carlson *et al.*, 2006). Since the host range of MDV cannot cover

all mosquito species and may not be a sufficient measure for insects resistant to MDV, isolation, characterization and application of novel mosquito viruses are still important steps for the development of environment-friendly mosquito management strategies.

Recently, novel insect-specific positive sense single-stranded RNA viruses have been isolated from naturally-infected mosquito and sand fly genera in geographically distant areas and were grouped to a new proposed taxon "Negevirus" (Vasilakis *et al.*, 2013). Negevirus rapidly and efficiently replicates in some mosquito cells in large amount (up to 10^{10} PFU/ml) (Vasilakis *et al.*, 2013), which implies the possibility that some of negeviruses can be used as a mosquitocide and/or a viral vector for gene expression in mosquito.

In this study, a small spherical virus, designated Okushiri virus (OKV), was isolated from mosquito larvae and identified as a novel virus belonging to the genus *Negevirus*. To enable genetic modification for further studies, an infectious cDNA clone for OKV and an OKV-based foreign gene expression system were constructed. The infectious cDNA clone system constructed in this study can be used as a basis for reverse genetics approach in basic and applied research of negevirus.

CHAPTER 1

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF OKUSHIRI VIRUS

1. INTRODUCTION

Mosquitoes are one of the most important pest insects that have a serious impact on public health; they are a primary vector of serious infectious diseases including malaria, dengue fever, chikungunya, Japanese encephalitis, and yellow fever. Vector control is an essential strategy for reducing the transmission of mosquito-borne diseases. Mosquito-specific viruses with mosquitocidal activity may be used as alternative insecticides for chemical pesticides. Although some mosquito viruses have been identified, molecular biology studies of these viruses are limited because cell lines suitable for investigating the molecular mechanisms of their multiplication strategy and pathogenesis are not currently available. Recently, insect-specific viruses belonging to the proposed insect virus group "Negevirus" have been identified (Vasilakis *et al.*, 2013). Negevirus replicated in some mosquito cells in large amount (up to 10^{10} PFU/ml) (Vasilakis *et al.*, 2013) implies possibility to develop biological control agents and viral vectors for gene expression in mosquito using some of these viruses. Nonetheless, experimental studies are still needed in order to elucidate the molecular mechanisms of their multiplication strategy and pathogenicity.

Here, a novel negevirus, designated Okushiri virus (OKV) has been isolated from pools of *Aedes* larvae collected in the field in Okushiri Island, Hokkaido, Japan. OKV was able to replicate in the *Aedes albopictus* cell line C6/36 with severe cytopathic effects and produced a large amount of spherical viral particles. Comparative analysis based on the amino acid sequence predicted from the nucleotide sequence indicated that OKV was a member of a new insect virus genus *Negevirus*.

1.2. MATERIALS AND METHODS

1.2.1 Mosquito larvae collection

Mosquito larvae were captured in small pools in the field during the mosquito season in 2010. The mosquito larvae collected were sub-grouped into three different genera (*Aedes*, *Culex*, and *Anopheles*) based on the characteristics of their external configuration and then stored at -80 °C in 1.5-ml centrifuge tubes with a maximum of 6 larvae per tube.

1.2.2. Cell cultures

The mosquito (*Aedes albopictus*) cell line C6/36 was used to isolate viruses and analyze their properties. These cells were cultured in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich, St Louis, MO, USA) containing 10 % heat-inactivated fetal bovine serum (FBS), 2 % Non-essential Amino Acids (NEAA, Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA), and were then maintained at 26 °C.

1.2.3. Virus isolation from mosquito larvae

Samples of larvae in 500 µl of ice cold MEM containing 2 % NEAA, 100 U penicillin / ml, and 100 µg streptomycin / ml were homogenized using the µT-01 bead crusher (Taitec) with stainless steel beads (2 mm, 70 g).

The homogenates were clarified by centrifugation at 1,000 x g at 4 °C for 5 minutes and the supernatants were passed through sterile 0.22 µm filters (Ultrafree MC, Millipore, Bedford, MA, USA). The filtrates were inoculated onto monolayers of C6/36 cells. The plates were incubated at 26 °C. After being inoculated, cell cultures were incubated for approximately 5 days, and then

observed using phase contrast microscopy. The supernatants of cells with cytopathic effects (CPE) were stored at -80 °C as virus samples. The supernatants from cells without CPE were also stored at -80 °C after a second blind passage.

1.2.4. Plaque purification

Plaque assay of OKV was performed on confluent C6/36 cell monolayers in 6-well plates. Duplicate wells were inoculated with 1ml aliquots of the serial 10-fold dilutions of the non-plaquepurified Okushiri virus in growth medium (MEM). Virus was added to the cells for 1 hr for viral adsorption. The inoculum was then removed by aspiration and the cell monolayers were overlaid with 2 ml of a medium consisting of a 1:1 mixture of 3% low-melting temperature agarose (SeaPlaque, Cambrex Biosciences) and Minimum Essential Medium Eagle (MEM, Sigma-Aldrich, St Louis, MO, USA) containing 2% of heat-inactivated fetal bovine serum (FBS), 2 % Non-essential Amino Acids (NEAA, Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA), and were then maintained at 26 °C for 48 hours to allow plaque development. The overlays were then removed and monolayers were fixed with 2 ml of 10% formaldehyde in PBS for 30 minutes. Cells were subsequently stained with 2% crystal violet in 30% methanol for 5 min at room temperature and excess staining dye was removed under slow running water. A plaque was picked for subsequent inoculation and plaque purification was repeated three times.

1.2.5. Purification of viral particles

Viral particles were purified from the culture medium of C6/36 cells inoculated with virus samples. The fluid was clarified from cell debris by low speed centrifugation, as above (1,000 x g at 4 °C for 5 minutes), and viral particles were precipitated by ultracentrifugation (250,000 x g at 4 °C for 5 hours). Pellets were suspended in TE buffer, layered onto a 10-40% sucrose liner gradient, and centrifuged at 99,600 x g for 2 hours. Viral particles that formed a white band in the gradient were resuspended in TE buffer, collected by centrifugation at 10,000 x g at 4 °C for 12 hours and resuspended in TE buffer.

1.2.6. Electron microscopic observations

Purified viral particles were used for electron microscopic observations. Viral particles were negatively stained and then observed using transmission electron microscopy TEM, Hitachi H-800 (Hitachi) as described elsewhere (Sugiharti *et al.*, 2010).

1.2.7. Characterization of the viral genome

RNA were extracted from the purified viral particles or supernatants of infected C6/36 cell cultures using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine if the purified virus is a RNA or DNA virus, the extracted RNA was treated with DNase I or RNase A at 37 °C for 20 minutes, denatured with formamide and formaldehyde, and electrophoresed on a 1.0 % GTG agarose gel (Lonza, Cologne, Germany).

The agarose gels were stained by GelStar™ Nucleic Acid Gel Stain (Lonza) and visualized under ultraviolet illumination.

1.2.8. Complementary DNA strand synthesis, sequencing, and analysis of genome organization

The first complementary DNA (cDNA) strand was synthesized from viral RNA extracted from purified viral particles with random primers or oligo dT primers using M-MLV reverse transcriptase (Takara Bio, Inc), followed by second-strand cDNA synthesis using DNA polymerase I after the RNase H treatment. Double-stranded cDNA was blunt ended using T4 DNA polymerase, ligated into the *Sma*I site in the pGEM 3Zf (+) plasmid (Promega), and sequenced. Some primers designed from the sequences obtained were used in PCR to synthesize additional viral genome sequences (Table 1). Sequences of the 3' and 5' terminal were obtained by Rapid Amplification of cDNA Ends (RACE) using the Gene Racer kit (Invitrogen) according to the manufacturer's instructions. In brief, the RNA extracted from OKV viral particles was divided into two portions and both were dephosphorylated with calf intestinal phosphatase (CIP). After dephosphorylation, one of the two was treated with tobacco acid pyrophosphatase (TAP) to remove possible cap structure. RNA oligo was then ligated to the 5'-terminus of both RNA, and n cDNA was synthesized from those RNAs by PCR using a primer set (Table 1) targeting the 5'-terminal sequence of about 1,200 nucleotides including the RNA oligo sequence, followed by agarose gel electrophoresis.

Viral genome sequences were analyzed using BLAST with the DDBJ, GenBank, and EMBL databases. Multiple sequence alignment among the

sequences obtained in this study and other RNA viruses found in the BLAST analysis was performed using the Clustal W program (Larkin *et al.*, 2007) included in MEGA5 (Tamura *et al.*, 2011) with the PAM matrix. A gap-opening penalty 10 and a gap-extension penalty 0.2 were used. A phylogenetic analysis was performed by MEGA5 with the Neighbor-Joining method (Saitou and Nei, 1987), and evolutionary distances were computed using the Poisson correction method. Bootstrapping was performed by resampling the data 1,000 times (Felsenstein, 1985). The accession numbers used in the phylogenetic analysis were shown in Table 2b. ORFs regions were predicted using ApE software (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>). The protein sequences were sought using a CD-Search included in PSI-BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul *et al.*, 1997). A homology search was also carried out by PSI-BLAST. Glycosylation sites were predicted using the NetNGlyc server 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) with a threshold value of 0.5. The programs TMHMM (<http://www.cbs.dtu.dk/services/TMHMM>) and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) (Hirokawa *et al.*, 1998) were adopted to analyze trans-membrane domains of the putative proteins.

1.2.9. Strand-specific RT-PCR

Total RNAs were extracted from purified OKV particles or OKV-infected C6/36 cells at 6 days post infection (dpi) using TRIzol reagent as described above. Their strand was tested by strand-specific RT-PCR (Craggs *et al.*, 2001). Total RNAs from uninfected cells were also extracted and used in strand-specific

PCR as a control. Cellular RNAs and RNA extracted from purified viral particles were treated with DNase I at 37 °C for 20 minutes and then reverse transcribed by PrimeScript RTase (Takara) using positive or negative strand-specific primers (Table 1) at 50 °C for 15 minutes. The remaining primers and nucleotides in the RT-PCR reaction tubes were digested by Exo/SAP-IT (USB Co., Cleveland, OH) at 37 °C for 15 minutes and then used for PCR. RNA 1 strand-specific PCR was carried out using a positive strand-specific primer set (OKV-F1, Tag) or negative strand-specific primer set (OKV-R1, Tag). PCR products were visualized by agarose gel electrophoresis.

Table 1 Primers used in this study

OKV-5 RACE-R	AACAGCGACAGCAAGCATCTTTAACTCGG
OKV-cDNA1-F	AACTTGAGTGTCTGACCATTTCCAGCG
OKV-cDNA2-R	AATCTCGAGATTGAACTTCAGACCG
OKV-3' RACE-F	TTGTTGATCGTAAACGACAGATGGACC
OKV-F1	AGAGACTCGAGAAGACCGAACAAGATCG
OKV-R2	TTGGGTGAAAAGATGGGATCTGACGG
Tag	<u>ATTGACTCAGCAATCGTAGTCAGCCG</u>
OKV-F1-Tag	<u>ATTGACTCAGCAATCGTAGTCAGCCG</u> AGAGACTCGAGAAGACCGAACA AGATCG
OKV-R1-Tag	<u>ATTGACTCAGCAATCGTAGTCAGCCG</u> TTGGGTGAAAAGATGGGATCTG ACGG

Sequences underlined: Tag sequences.

Table 2a GenBank accession numbers of viral genomic sequences used in the P22 trans-membrane region prediction

Virus	Accession Number
Piura virus	AFI24680.1
Negev virus	AFI24674.1
Negev-like virus #174	CRL87032.1
Brejeira virus	AIS40880.1
Ngewotan virus	AFY98074.1
Wallerfield virus	AIS40853.1
Dezidougou virus	AFI24671.1
Goutanap virus	YP_009094128.1
Citrus leprosis virus C	YP_654543.1
Citrus leprosis virus C type 2	AGM16556.1
Hibiscus green spot virus	YP_004928121.1
Blueberry necrotic ring blotch virus	YP_004901704.1

Table 2b. GenBank accession numbers of viral genomic sequences used for the phylogenetic tree

Virus	Accession Number
Piura virus	AFI24678.1
Negev virus	AFI24672.1
Negev-like virus #174	CCV01575.2
Brejeira virus	AIS40866.1
Ngewotan virus	AFY98072.1
Loreto virus	AFI24690.1
Tanay virus	YP_009028558.1
Wallerfield virus	YP_009001772.1
Dezidougou virus	AFI24669.1
Santana virus	AFI24675.1
Goutanap virus	YP_009094126.1
Citrus leprosis virus C	YP_654568.1
Citrus leprosis virus C type 2	AGE82887.1
Hibiscus green spot virus	YP_004928118.1
Ligustrum ringspot virus	ADM47770.1
Blueberry necrotic ring blotch virus	YP_004901700.1

1.3. RESULTS

1.3.1. Isolation of a viral agent from mosquito larvae

A sample pool of field-collected mosquito larvae was used for virus isolation in this study. Extensive CPE with cell globularization and destruction was observed in C6/36 cells inoculated with the homogenates of three (approximately 20%) field-collected mosquito larval pools 72 hours after inoculation (Fig. 1B). There was possibility to be a mixture of several different viruses at this point and plaque purification was repeated three times regarding a sample showing strong CPE. Plaques were clearly observed by holding the plate against the light without staining (Fig 1C). The plaque-purified OKV showed CPE indistinguishable from that of the non-plaquepurified sample on C6/36 cells (Fig. 1D).

Virus-like particles were observed in the culture medium of C6/36 cells with extensive CPE by an electron microscopic study, but not in that of control (uninoculated) cells (data not shown). These virus-like particles purified by sucrose gradient centrifugation were elliptical with a diameter of approximately 50 to 70 nm and some particles had small projection-like structures similar to those of Tanay virus (Nabeshima *et al.*, 2014) (Fig. 2). The purified particles were infectious to C6/36 cells and caused extensive CPE as above (data not shown). The virus isolated here was named Okushiri virus after Okushiri Island, where this virus was isolated.

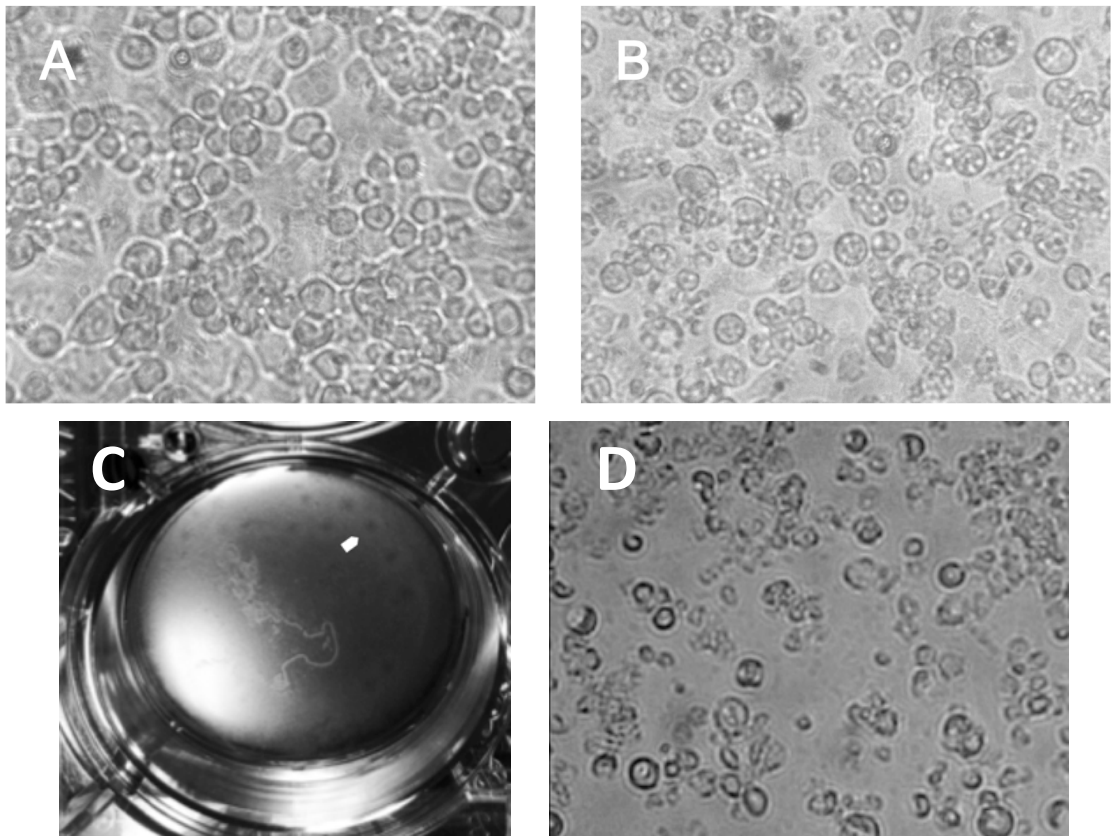


Figure 1. Phase contrast micrographs of control cells, uninfected C6/36 cells (A), and OKV-infected cells at 2 days post infection (B),. Plaque forming units were clearly visible on dilution of 10^{-7} (C). Plaque purified OKV-infected cells at 4 days post infection. White arrow indicate plaque forming unit.

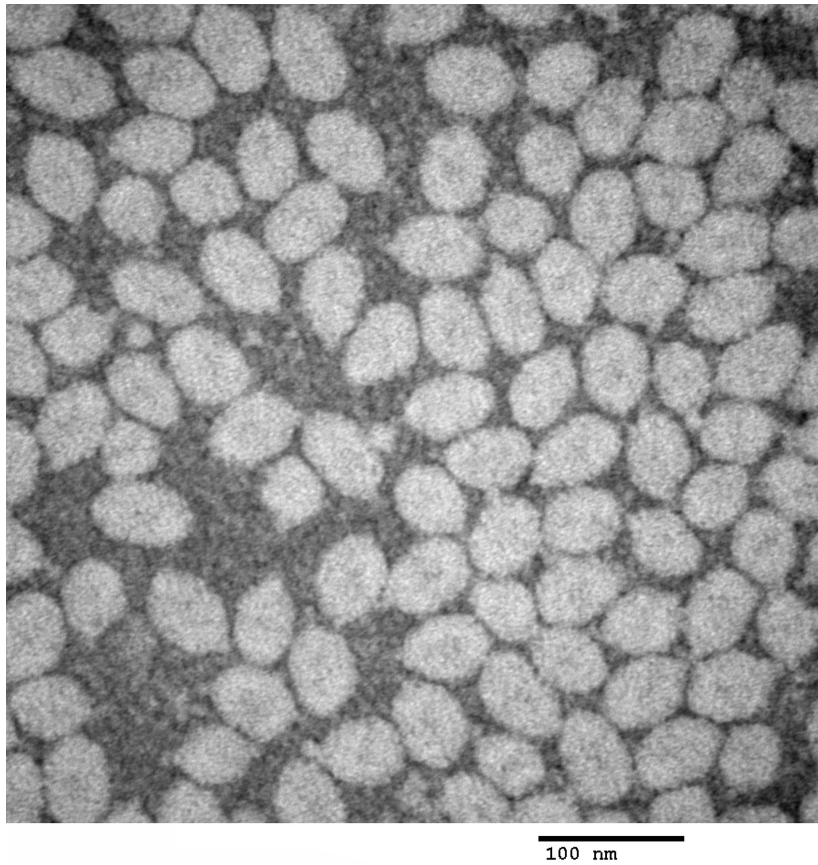


Figure 2. Electron micrographs of viral particles purified using sucrose gradient ultracentrifugation and visualized by negative staining (scale bar, 100 nm).

1.3.2. Characterization of viral nucleic acids

A major band, corresponding to a size of approximately 10 kb, and some smaller bands were detected in the supernatant of C6/36 cell cultures with CPE (inoculated) by agarose gel electrophoresis under denatured conditions (Fig. 3A). These bands were also detected in the analysis of nucleic acids extracted from purified viral particles (Fig. 3B). In order to further analyze the characteristics of these nucleic acids, they were subjected to a DNase I or RNase A treatment, followed by agarose gel electrophoresis. The bands disappeared when treated with RNase A, but not with DNase I, indicating that they were RNA (Fig. 3B). Purified viral particles were subsequently subjected to acridine orange staining and emitted a brilliant flame-red fluorescence (data not shown). These results suggested that the viral particles contained single-stranded RNA.

Strand-specific RT-PCR with primer sets designed from the sequence of 10 kb RNA (designated vRNA) was performed in order to determine whether OKV had positive or negative-stranded RNA. The positive strand- and negative strand-specific RT-PCRs using RNA extracted from OKV-infected C6/36 cells as the template amplified the sequences with expected sizes of approximately 2.4 kb each (Fig. 5). Suggested that OKV replicated in C6/36 cells. On the other hand, only PCR designed for detecting positive-stranded RNA amplified a sequence with the expected size when RNA from purified OKV particles was used as the template. Indicated that OKV had a positive sense single-stranded RNA.

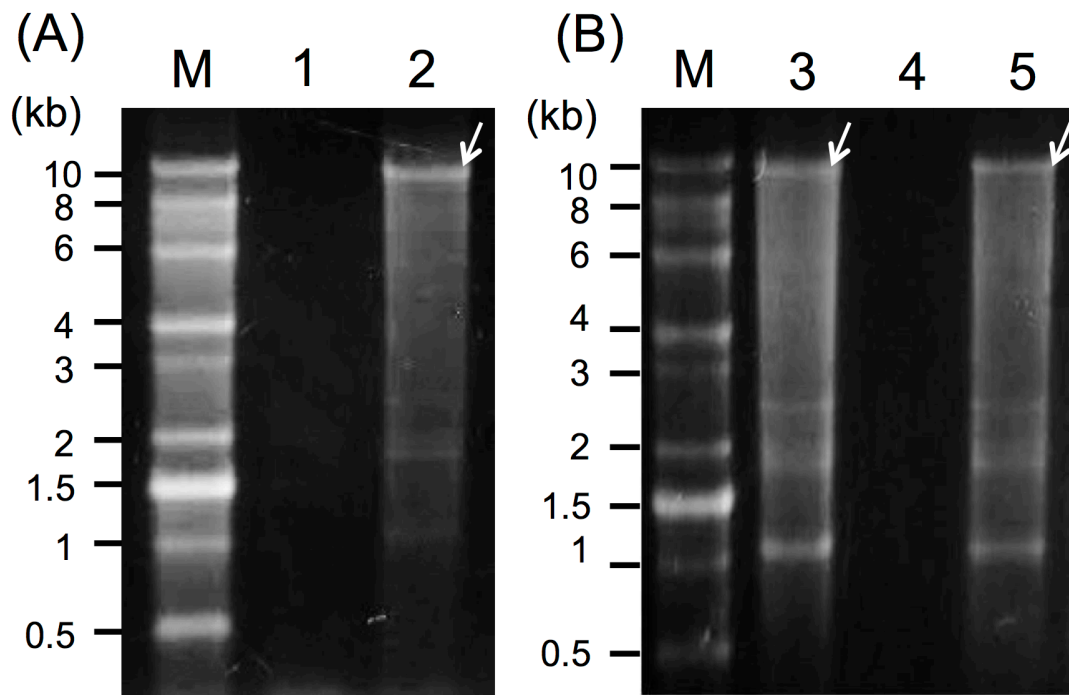


Figure 3. Agarose gel electrophoresis of OKV-specific RNA. Nucleic acids from the supernatant of OKV-inoculated C6/36 cells were separated by agarose gel electrophoresis (A). M: Size marker, 1: control (uninfected) C6/36 cells, 2: OKV-infected C6/36 cells. RNAs from purified viral particles treated with RNase A and DNase I (B). M: Size marker, 3: Control (no treatment), 4: RNase A-treated, 5: DNase I-treated. White arrows indicate OKV-specific RNA (vRNA).

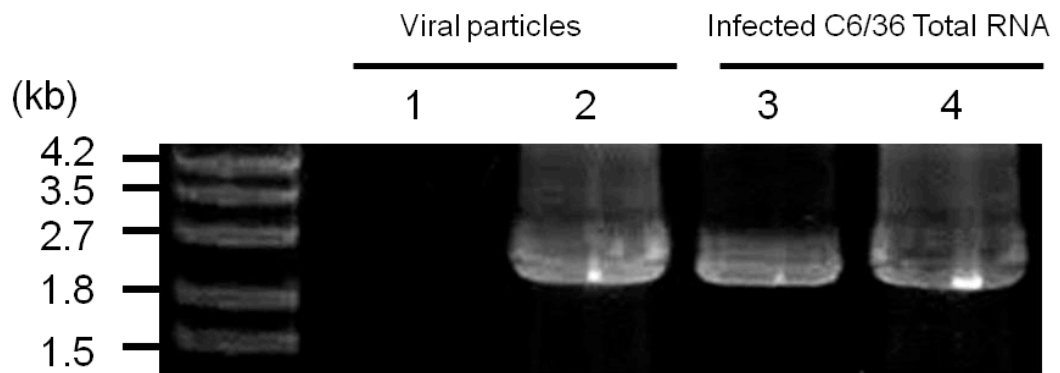


Figure 4. Strand-specific RT-PCR using RNA extracted from purified viral particles (Lanes 1 and 2) or OKV-infected C6/36 cells at one day post infection (Lanes 3 and 4). Lanes 1 and 3: negative-strand-specific RT-PCR, lanes 2 and 4: positive-strand-specific RT-PCR.

1.3.3. Organization of viral genome RNA (vRNA)

A sequencing analysis suggested that vRNA was composed of 9,704 kb nucleic acids (DDBJ accession number AB972669) and included three major ORFs (ORF1 at nt 262 to 7,347, ORF2 at nt 7,377 to 8,585, and ORF3 at nt 8,724 to 9,350) (Fig. 6A). A schematic diagram of the strategy for vRNA cloning is shown in Figure 6. cDNA fragments obtained by 3' RACE had a poly (A) tail at the 3' -end of vRNA in the range of 28 to 44 nucleotides.

The 5'-terminal sequence of vRNA was determined using 5' RACE. The cDNA was first synthesized from the OKV RNA without treatment with tobacco acid pyrophosphatase (TAP) for decapping. However, gel electrophoresis demonstrated that PCR product could not be amplified for this non-decapped RNA (Fig. 5). On the other hand, PCR using the decapped RNA as template generated three DNA fragments (Fig. 5). Three DNA fragment were extracted from agarose gel independently, cloned, and sequenced, resulted in the same sequence for the 5'-terminus and different sequence for the 3'-terminus (data not shown). These observations suggested that the cap at the 5'-terminus of vRNA interfered the ligation with the RNA oligo as a target sequence of a primer used in 5' Race (see MATERIALS AND METHODS). And two non-specific by-products in Figure 5 might be produced due to difference of melting temperature between RNA oligo primer provided by Generacer TM kit (about 74°C) and gene specific primer's (about 63°C).

A BLAST search using the amino acid translation of ORF1 indicated that four conserved domains: a methyltransferase domain (vMet, at nucleotide position 709 to 1,364), FtsJ-like methyltransferase (ribosomal RNA methyltransferase) domain (FtsJ, at nucleotide position 2,722 to 3,177), viral

RNA helicase domain (Hel, at nucleotide position 4,414 to 5,196), and RNA-dependent RNA polymerase domain (RdRp, at nucleotide position 6,046 to 7,212), were included. A BLAST search showed that RdRp amino acid sequences had high similarity to those from negevirus, Piura virus (PIUV), Brejeira virus (BRJV), Negev virus (NEGEV), NEGEV-like virus #174 (NEGEV174), Ngewotan virus (NWTV), Loreto virus (LORV), Santana virus (SANV), Dezidougou virus (DEZV), Wallerfield virus (WALV), Tanay virus (TANAV), and Goutanap virus (GANP) (Table. 3), and also those of positive sense single-stranded RNA plant viruses including Citrus leprosis virus C (CiLV-C), Citrus leprosis virus C type 2 (CiLV-C2), Ligustrum ringspot virus (LigRSV), and segmented double-stranded RNA plant viruses Hibiscus green spot virus (HGSV), and Blueberry necrotic ring blotch virus (BNRBV). CiLV-C has not yet been assigned to any family, but was classified into *Cilevirus*, while LigRSV has been proposed as a member of *Cilevirus* (Carlson *et al.*, 2006; Pascon *et al.*, 2006; Locali-Fabris *et al.*, 2012; Melzer *et al.*, 2013; Quito-Avila *et al.*, 2013). HGSV was very recently classified into *Higrevirus* (Adams *et al.*, 2014) and BNRBV is an unclassified virus proposed for genus *Blunervirus* (Pascon *et al.*, 2006).

ORF2 was inferred to encode a putative glycoprotein with an estimated size of approximately 45 kDa (designated P45) with two potential N-linked glycosylation sites and three trans-membrane domains, which is a common feature of hypothetical proteins encoded in ORF2 of negeviruses (Kallies *et al.*, 2014; Kuchibhatla *et al.*, 2014).

ORF3 was inferred to encode a hypothetical protein of approximately 22 kDa (designated P22) that showed high similarities ranged from 38 to 71 % in its

primary sequence with those of hypothetical proteins encoded in ORF 3 of NEGEV, NEGEV174, PIUV, BRJV, LORV, and NWTV. It also showed moderate sequence similarity (from 19 to 32 %) to P23/P24 of plant viruses HGSV (Melzer *et al.*, 2011), CiLV-C2 (Melzer *et al.*, 2013), CiLV-C (Locali-Fabris *et al.*, 2012), and BNRBV (Quito-Avila *et al.*, 2013). Furthermore, two to four trans-membrane domains were predicted for those proteins (Fig. 6B). This structural feature was also commonly observed in hypothetical proteins encoded in ORF3 of all other negevirus (Auguste *et al.*, 2014; Nabeshima *et al.*, 2014; Carapeta *et al.*, 2015; Kawakami *et al.*, 2015; Roy *et al.*, 2015).

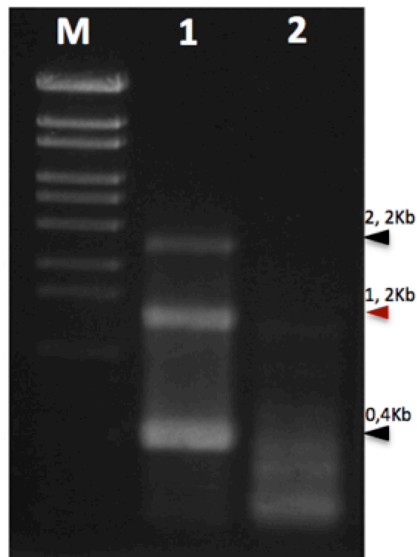


Figure 5. 0,8 % Agarose gel electrophoresis of PCR products from decapped and non-decapped purified OKV RNA (vRNA) using RNA-Oligo (GeneracerTM) and OKV gene specific primers targeting the 5'-terminal sequence (approx.. 1,200 nucleotides). Lane 1 and 2: PCR products from decapped vRNA and PCR products of cDNA from non-decapped vRNA, respectively. Arrowhead in red indicates amplified target sequence and arrowheads in black are non-specific by-products.

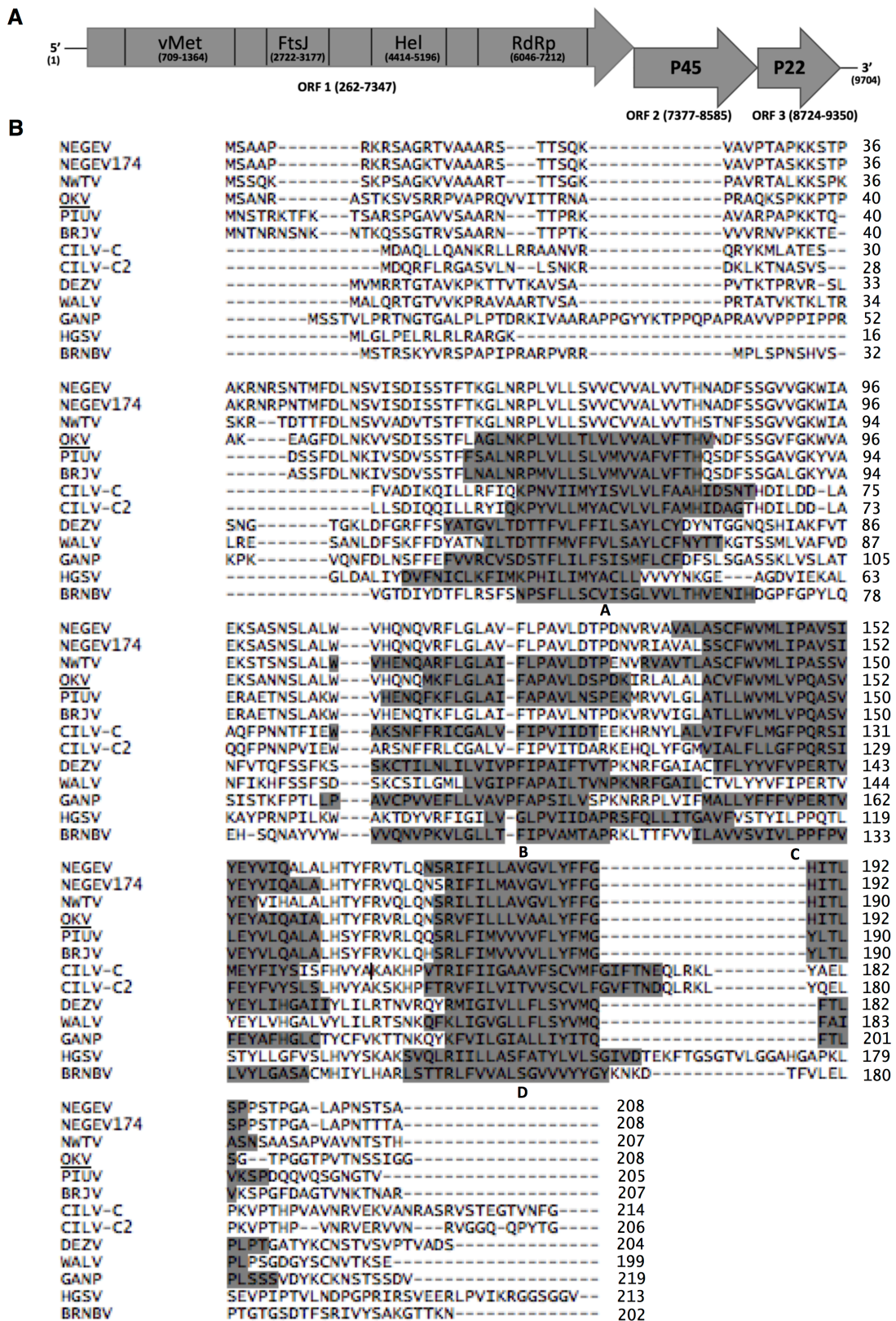


Figure 6. (A) Schematic drawing of OKV genome organization. Arrow shaped boxes indicate the positions of open reading frames (ORFs) with the indication of putative conserved domains. Numbers in parentheses indicate nucleotide positions in the ORFs and domains. (B) Predicted trans-membrane regions in proteins encoded in ORF3 of OKV and

negevirus (NEGEV, NEGEV174, PIUV, BRJV, and NWTV) and P23/P24 of negevirus-related plant viruses (CILV-C, CILV-C2, HGSV, and BNRBV). The amino acid sequence of OKV P22 was aligned with the deduced amino acid sequences obtained from NCBI GenBank using the Clustal W program (Larkin *et al.*, 2007) included in MEGA5 (Tamura *et al.*, 2011). Hypothetical trans-membrane regions (A-D) predicted by SOSUI (Hirokawa *et al.*, 1998) are shown in grey. GenBank accession numbers for the sequences used for alignment are given in Table 2a.

1.3.4. Phylogenetic analysis

For taxonomic characterization, phylogenetic analysis based on the conserved RdRp domain was performed. The results demonstrated that OKV formed a monophyletic clade together with LORV and the four sister species, NEGV, NWTV, BRJV, and PIUV, while the other members of the taxon *Negevirus* SANV, TANAV, GANP, DEZV, and WALV formed a different phylogenetic clade (Fig. 7). Phylogeny also revealed a sister group relationship between negeviruses and the plant-infecting viruses HGSV (*Higrevirus*), CiLV-C, CiLV-C2, LigRSV (*Cilevirus*), and BNRBV (*Blunervirus*) on a solitary branch. The phylogenetic relationship for these viruses did not contradict the results of the analysis based on whole ORF1 sequences (data not shown) and those previously reported (Vasilakis *et al.*, 2013; Auguste *et al.*, 2014; Kallies *et al.*, 2014; Nabeshima *et al.*, 2014; Carapeta *et al.*, 2015). The topology of these viruses was also well maintained in a phylogenetic tree including members of *Virgaviridae* as an outgroup, which was confirmed by an analysis based on a combination of the Bayesian approach with a maximum likelihood procedure in MEGA5 except that BNRBV showed a closer relationship with negeviruses in group II (data not shown).

Table 3. Pairwise comparison (%) of nucleotide identity (upper diagonal) and amino acid homology (lower diagonal) for RdRp region of negeviruses

	OKV	NEGEV	Negev-like #174	PIUV	NWTV	Brejeira	LORV	GANP	WALV	TANAV	DEZV	SANV
OKV		70	73	74	70	74	72	*	*	*	*	*
NEGEV	79		98	74	75	73	71	*	*	*	*	*
Negev-like#174	79	100		74	75	72	70	*	*	*	*	*
PIUV	83	79	79		73	77	93	*	*	*	*	*
NWTV	80	88	88	84		72	75	*	*	*	*	*
Brejeira	75	77	74	90	74		69	*	*	*	*	*
LORV	63	61	64	61	64	60		*	*	*	*	*
GANP	31	34	35	42	35	31	31		79	88	80	74
WALV	32	36	32	48	33	32	31	70		66	81	77
TANAV	31	35	32	43	33	31	31	66	63		83	83
DEZV	32	35	33	48	33	31	31	76	75	70		78
SANV	31	35	32	45	33	32	30	59	60	59	63	

*) no significance similarity found

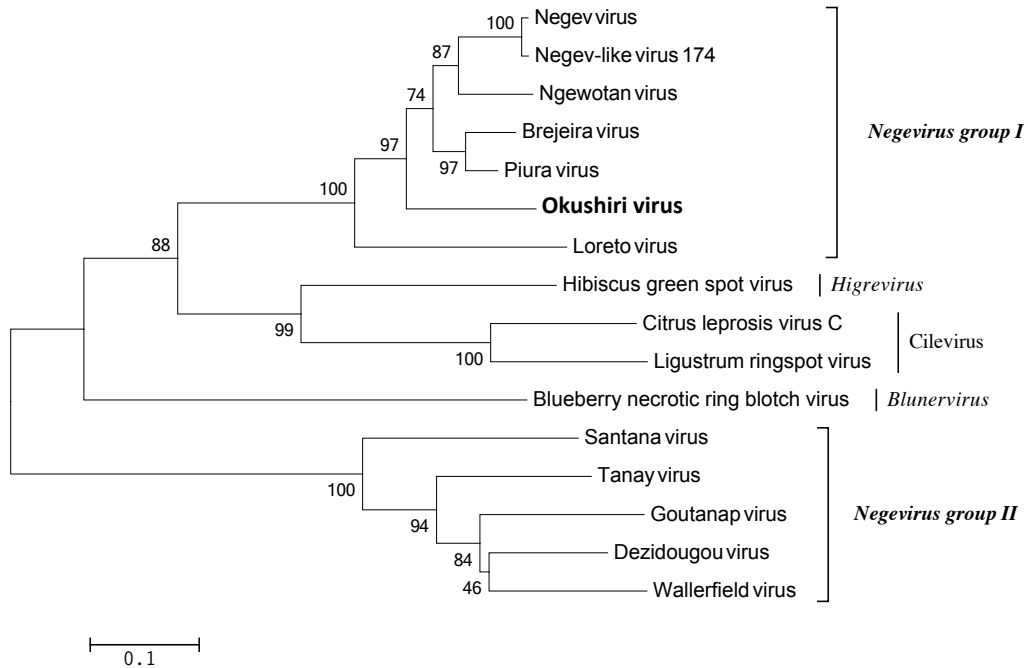


Figure 7. Phylogenetic analysis based on the conserved amino acid sequence of the RdRp region. The analysis was inferred using the Neighbor-Joining method based on the alignment of the RdRp region (amino acid positions 1900-2342) of OKV ORF1 (DDBJ accession number AB972669) with the deduced amino acid sequence obtained from NCBI GenBank. The bootstrap values were obtained from 1,000 resampling and are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale indicates the number of nucleotide substitutions per site. The sequence of OKV from this study is in boldface. GenBank accession numbers for the sequences used to construct the phylogenetic tree are given in Table 2b.

1.4. DISCUSSION

A virus showing extensive CPE in *Aedes albopictus* C6/36 cells was isolated from field-collected *Aedes* larvae in Okushiri Island, Hokkaido, Japan and was designated Okushiri virus (OKV). Plaque assay of Dengue virus and *Aedes* flavivirus using C6/36 cells were reported to be unsuccessful in some researches due to the significant partial lysis of the C6/36 cells during staining (Zamree *et al.*, 2005; Hoshino *et al.*, 2009). However, plaque purification of OKV using C3/36 cells was performed successfully by omitting the staining process (Fig. 1C). The plaque-purified virus showed CPE indistinguishable from that of non-plaquepurified one (Fig 1D), suggesting a viral agent causing strong CPE in C3/36 cells was OKV.

OKV virion was spherical in shape with approximately 50 to 70 nm in diameter with small short projection. This viral particle contains positive-sense single-stranded RNA of 9,704 nucleotides composed of three separate ORFs (Fig. 6A). Some lower molecular RNAs were detected by electrophoresis, even in the experiment using RNA extracted from purified OKV particles (Fig. 3B). These lower RNAs have also been detected in NEGV. These results implied the possibilities that these smaller RNAs were segmented genome of OKV (segmented RNA virus) or host RNAs packaged in these viral virions (Bernard *et al.*, 1994; Rumenapf *et al.*, 1995; Vasilakis *et al.*, 2013). Experiments described in chapter 2 revealed that OKV genome is non-segmented. .

Strand-specific PCR confirmed that OKV replicated in C6/36 cells with severe CPE (Fig. 1 and 4). The genome organization and amino acid sequences encoded in the ORFs showed high similarities to those of viruses in

a new taxa of insect-specific, positive-single stranded RNA viruses, negevirus (Fig. 7). In 5' RACE, decapping treatment was necessary to amplify the 5'-terminus of OKV RNA suggested the existence of the 5'-terminal cap structure in the viral RNA (Fig. 5).

OKV genome contained three major ORFs. ORF1 contained sequences conserved in the domains of Met, Hel, and RdRp of viruses in the groups including plant viruses (*Virgaviridae*, *Bromoviridae*, and *Closteroviridae*), mosquito-borne viruses (*Togaviridae*; *Alphaviruses*), mammalian-infecting viruses (*Hepeviridae*; *Hepevirus* and *Togaviridae*; *Rubivirus*), and newly isolated insect viruses belonging to negevirus (Kallies *et al.*, 2014; Nabeshima *et al.*, 2014). Phylogenetic analysis clearly subgrouped negevirus into two major clades (Fig. 7), which was consistent with the negevirus phylogenetic topologies presented in previous studies (Vasilakis *et al.*, 2013; Auguste *et al.*, 2014; Kallies *et al.*, 2014; Nabeshima *et al.*, 2014; Carapeta *et al.*, 2015). OKV belonged to one of the two major negevirus clades (group I) together with NEGV, NWTV, BRJV, PIUV, and LORV (Fig. 7).

Domains of predicted OKV proteins were also conserved in other negevirus. Potential glycosylation sites and/or trans-membrane domains were predicted in P45 (ORF 2) and P22 (ORF 3) of OKV and other negevirus. These results implied that OKV ORF 2 and ORF 3 encoded putative membrane-associated proteins. However, further elucidation is required to determine their roles in detail. Furthermore, although purified OKV particles appeared to have small projection-like structure (Fig. 2) as is observed in Tanay virus (Nabeshima *et al.*, 2014) and in the members of Coronavirus, a

positive-sense single stranded RNA virus group have virion comprised of trimers of spike glycoprotein of which plays role in viral entry (Gallagher and Buchmeier, 2001; Attoui *et al.*, 2011), other experiments are required. Taken together, these results suggested that OKV was a member of negevirus.

The phylogenetic analysis using RdRp showed that OKV had high evolutionary relationships not only to negeviruses but also to some plant viruses (Fig. 7). These observations was consistent with the relationships between negeviruses and *Higrevirus*, *Cilevirus*, and *Blunervirus* that was previously argued for other negeviruses (Vasilakis *et al.*, 2013; Auguste *et al.*, 2014; Kallies *et al.*, 2014; Nabeshima *et al.*, 2014; Carapeta *et al.*, 2015). The relationships between these viruses may also be speculated based on the previous findings in which P22 of negeviruses and the putative structural proteins P23/P24 of the plant viruses (Pascon *et al.*, 2006; Auguste *et al.*, 2014) shared topological and sequence similarities (Fig. 6B). Recent study demonstrated that plant virus-like mosquito viruses isolated from mosquitoes (*Culex* spp.), referred to as CuTLV had the ability to cause CPE in the mosquito cell line C6/36, and shares sequence similarities to plant viruses in the family *Tymoviridae* (Wang *et al.*, 2012). These findings indicated the possible transmission of some plant viruses by mosquitoes through the behavior of feeding on plant nectar and juices (Müller and Schlein, 2005). These findings is supported by previous reports demonstrating that viruses of the genus *Cilevirus* (Locali-Fabris *et al.*, 2012), *Higrevirus* (Adams *et al.*, 2014), *Blunervirus* (Quito-Avila *et al.*, 2013), and family *Tymoviridae* might be transmitted by mites, such as *Brevipalpus* sp. (Roy *et al.*, 2015) and insects such as leafhoppers (Martelli *et al.*, 2002). However, a virus with ability to replicate in and/or to be transmitted

between plants and mosquitoes has not yet been identified to date. OKV isolated from *Aedes* larvae may be transmitted by feeding behavior at the larval stage (horizontal infection) and/or through their parents (vertical infection). However, infection of the plant tissue (*Nicotina benthamiana*) with OKV has not succeeded yet (Nakahara, unpublished data). Moreover, Vasilakis et al. reported studies of oral infection of *Ae. aegypti* and *Ae. albopictus* with NEGV showed that the virus would only be disseminated at inoculation of 10^8 to 10^{10} PFU/ml of virus. However, it seems unlikely that floral nectars or fruit juices would contain such high viruses titers (Vasilakis et al., 2013). If these experimental studies are indicative for negevirus, than the vertical transmission is more likely. Further studies on replication mechanisms and life cycles of negevirus are required for understanding evolutionally relationships between negevirus and plant virus.

1.5 CONCLUSION

In conclusion, a novel negevirus has been isolated in Japan, referred to as OKV, and carried out genomic characterization. OKV formed one of the two phylogenetic clades of negevirus with the viruses isolated from Israel (NEGV), Portugal (NEGV174), North America (NEGV), South America (BRJV, PIUV, LORV), and Indonesia (NWTV). The worldwide distribution of negevirus, from the tropics to subarctic regions, with possible phylogenetic relationships to plant viruses has prompted us to speculate about unknown intimate relationships between mosquitoes and plants. Further studies are needed in order to elucidate the nature of negevirus for a deeper understanding of the potential risk of mosquitoes not only to public health, but also to agriculture.

CHAPTER 2

GENERATION OF AN INFECTIOUS CDNA CLONE OF OKUSHIRI VIRUS AND ITS DERIVATIVE CAPABLE OF EXPRESSING AN EXOGENOUS GENE

2.1 INTRODUCTION

Infectious cDNA clones of RNA viruses have played critical roles in studying functions of viral genetic elements by enabling genetic manipulation of their RNA genomes (Yamshchikov *et al.*, 2001; Isawa *et al.*, 2012; Ishikawa *et al.*, 2015). Their derivatives such as viral expression vectors have also been used for diverse applications including live imaging of viral infection with a reporter gene and expression of a foreign gene. Construction of infectious and genetically tractable clones opens a wide range of studies and applications of RNA viruses.

Negevirus were first reported by Vasilakis *et al.* about strains isolated mainly from mosquitoes in subtropical countries Israel, Peru, Brazil, US, Cote de Ivoire and tropical country Indonesia (Vasilakis *et al.*, 2013). Isolation of novel negevirus were then continuously reported in following different geographical areas: West Africa (Kallies *et al.*, 2014), Mexico (Kallies *et al.*, 2014), Trinidad (Auguste *et al.*, 2014), Philippines (Nabeshima *et al.*, 2014), Portugal (Carapeta *et al.*, 2015), Amazon (Nunes *et al.*, 2015) and subarctic Hokkaido Japan (Kawakami *et al.*, 2015), suggesting that negevirus has a geographically and climatically wide range of distribution as well as genetic diversity (Vasilakis *et al.*, 2013; Kawakami *et al.*, 2015). In this kind of situation, further studies are needed in order to elucidate biological properties and the nature of negevirus, for a deeper understanding of the potential risk of their host (mosquitoes) not only to public health, but also to agriculture. Reverse genetics using an infectious cDNA clone could be a powerful strategy to elucidate functions of negevirus genetic elements. Furthermore, infectious clones provide the foundation for developing useful tools based on negevirus. However,

construction of an infectious clone for negeviruses was reported only for NEGV strain M30957 to date (Gorchakov *et al.*, 2014).

In this study, a full-length infectious cDNA clone of OKV and a derivative to express green fluorescent protein (GFP) were generated. The OKV genome was cloned under the control of T7 promoter in pFastBac. *In vitro*-transcribed viral RNA from the full-length OKV cDNA clone produced progeny viruses when transfected into C6/36 cells. The infectious cDNA clone was further modified to express GFP by replacing ORF3 with the GFP coding sequence. RNA transcribed *in vitro* from the modified infectious clone successfully expressed GFP in the transfected cells. However, infectious progeny viruses were not recovered from the transfected cells, which suggested that ORF3 was necessary to produce infectious progeny viruses. Consistent with the hypothesis, co-transfection of *in vitro* synthesized RNA from the recombinant viral and the wild-type viral cDNA clones providing genetic information of ORF3 produced progeny viruses carrying GFP ORF and showed GFP fluorescence in the infected cells.

2.2 MATERIAL AND METHODS

2.2.1. Cell cultures

Aedes albopictus cell line C6/36 cells were cultured in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich, St Louis, MO, USA) containing 10 % heat-inactivated FBS, 2 % Non-essential Amino Acids (NEAA, Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA), at 28 °C under 5% CO₂ exposure.

2.2.2. Virus

The virus used was Okushiri virus (DDBJ accession number AB972669) as described in chapter 1 (1.2.3 and 1.2.4).

2.2.3. Plasmid constructions

For the construction of an infectious cDNA clone of OKV, RNA was extracted from the purified viral particles using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The extracted viral RNA was treated with DNase I (Takara Bio Inc., Shiga, Japan) at 37 °C for 20 minutes. One µl out of 15µl of DNase I-treated RNA was then used as a template for cDNA synthesis with PrimeScript RT reagent Kit (Takara Bio Inc.). Five overlapping regions of the viral genome (Fig. 8A and B) were separately amplified from viral cDNA by PCR using TaKaRa Ex Taq DNA polymerase (Takara Bio Inc.) with following thermal cycles: 95°C for 2 minutes; 30 cycles of 95°C for 15 sec, a temperature within 52-60°C adjusted for the T_m value of each primer pair for 15 sec, and 72°C for a period within 1.5-2.5 min adjusted for expected PCR product length; and 72°C for 5 min. The scheme to amplify

the five fragments and primers is summarized in Fig. 8 and Table 4, respectively. All five PCR products were sub-cloned separately into pGEM-T Easy (Promega, Madison, USA). Sequences of the fragments were verified by Sanger sequencing. Following sequence analysis, the five sub-clones were digested at the artificial (*NotI* and *HindIII*) or selected OKV endogenous restriction sites (*SpeI*, *PstI*, *SphI*, and *Bsp1407I*). The full-length OKV genome was assembled by sequentially ligating the fragments into the plasmid FastBac (Invitrogen) (Fig. 8C). The plasmid carrying the full-length OKV cDNA clone was designated pFBOKV. All the cDNA cloning was performed using DH5 α (Invitrogen).

A pFBOKV derivative carrying GFP coding sequence was generated as follows: First, *NheI* or *StuI* restriction sites were artificially generated at the junction between ORF2 and ORF3 by PCR using primer pairs OKV-29F and O2NheIR, and O3StuIF and OKV-3'HindIII, respectively (Table 4). The *NheI*-*StuI* digested GFP fragment from *Pontellina. sp*-derived GFP protein gene (Lonza) was then introduced to the *NheI*-*StuI* digested pFBOKV. The resultant plasmid was designated as pO2GFP.

2.2.4. *In vitro* RNA transcription

pFBOKV or pO2GFP was digested using *HindIII* and then purified via ethanol precipitation. *In vitro* transcription was performed using HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs) with or without m7G(5')ppp(5')G cap analogue (New England Biolabs) according to manufacturer's instructions. One μ g of the purified linear plasmid was used as a template for *in vitro* transcription. The template DNA in the reaction was

digested using DNaseI (Takara Bio Inc.).

2.2.5. Transfection and infection

C6/36 cells were seeded in a 24-well microplate with 450µl of FBS-free medium one day before transfection or infection. For transfection, 50µl of Lipofectamine 2000 (Life Technologies) was used per well with RNA. The medium was removed after incubation for 1 hour. Cells were washed three times with 550µl of 1x PBS followed by incubation with supplementing medium containing 2% FBS at 28°C. Cells were maintained with 5% CO₂.

2.2.6. Quantitative real-time PCR

Viral RNA was extracted from culture medium at different time points. One hundred ng of extracted RNA was used as the template for reverse transcription in 10µl volume with the PrimeScript RT reagent Kit (Takara Bio Inc.) according to the manufacturer's instructions. One µl of the synthesized cDNA was used for RT-qPCR using SYBR II Premix Ex Taq (Takara Bio Inc.) and LightCycler Nano Instrument (Roche) according to the manufacturer's instructions. Primer pairs, OKV-qpcrF and OKV-qpcrR, OKV-35F and OKV-36R, or GFP-qpcrF and GFP-qpcrR were designed to target 3' untranslated region, ORF3 region of OKV genome, or GFP region, respectively (Table 4). Quantitation cycle values for both cDNA and a set of serially diluted plasmids were obtained and copy numbers of cDNA were estimated based on calibration curves calculated using quantitation cycle values for the plasmids.

2.2.7. Quantification and visualization of GFP expression

GFP expression from C6/36 cells transfected or co-transfected with pFBOKV- and/or pO2GFP-derived RNA was quantified by measuring fluorescence intensity of GFP using infinite M200 PRO (Tecan, Männedorf, Switzerland). Microscopic observations on transfected or infected cells were performed using a Leica MZ FLIII fluorescence stereomicroscope.

Table 4. Primer used in this study

T7-5'OKV	TAATACGACTCACTATAGATAAAACAAATACACAAGTGAA ACTCAATTGGACCC
NotI-T7	GCGGCCGCTAATACGACTCACTATAGATAAAACAAATACA
OKV-4R	CCGAGTTAAAGATGCTTGCTGTCGCTGTT
OKV-3F	TTTACGATACGCCAGTCCATAAGCGG
OKV-12R	GCTATGCGTGAGTGTCTCGCTCTCA
OKV-7F	ACTCCAGAAGCCAGTATAACAAGAGGC
OKV-20R	CGAGACATGATCGTTCCTGACTT
OKV-19F	TTTCATGCGAGACTACAACATGCGCGAGC
OKV-30R	GGACGTTATTGACCGTAATTGGTT
OKV-26F	ATGACAAAACCTTGGTCGACATGACATGG
OKV-3'HindIII	CGCTACGTAACGGCATGACAGTAAGCTT
OKV-29F	TTTATAGCAAGCTGCGAATGCG
O2NheI	CGGGCTAGCAGTGAGAATGATTATGAAAAATT
O3StuI	AGGCCTGCCGAACCTCACTTCTTGAG
OKV-qpcrF	TTCATGAGATGTAGGCCGCGCTT
OKV-qpcrR	AATAATATAATGACCCCGGAGAAGCCGGGG
OKV-35F	AAGTGGTTATTACCACTCGG
OKV-36R	CCTGACCCTCGTCCTGGTCGTCGCT
GFP-qpcrF	ACCAACACCCGCATCGAGAAGTAC
GFP-qpcrR	ACCACCTTGAAGTCGCCGATCA

2.3. RESULTS

2.3.1. Construction of a cDNA clone of full-length OKV genome

A full-length OKV genome clone has been constructed as summarized in Fig. 8. Following reverse transcription of OKV genomic RNA, five cDNA fragments (1.6, 1.8, 2.7, 1.5, and 2.1 kb) covering the entire OKV genome were amplified by PCR (Fig. 8B) and were sub-cloned. Nucleotide sequences of the cloned fragments were verified by Sanger sequencing. To assemble the full-length cDNA, all the five sub-clones were digested at the introduced (*NotI* and *HindIII*) or endogenous (*SpeI*, *PstI*, *SphI*, *Bsp1407I*) restriction sites, and were serially inserted into pFastBac (Fig. 8C). The resulting plasmid carrying cDNA for a full-length OKV genome was designated as pFBOKV. The nucleotide sequence of OKV genome in pFBOKV was confirmed by Sanger sequencing.

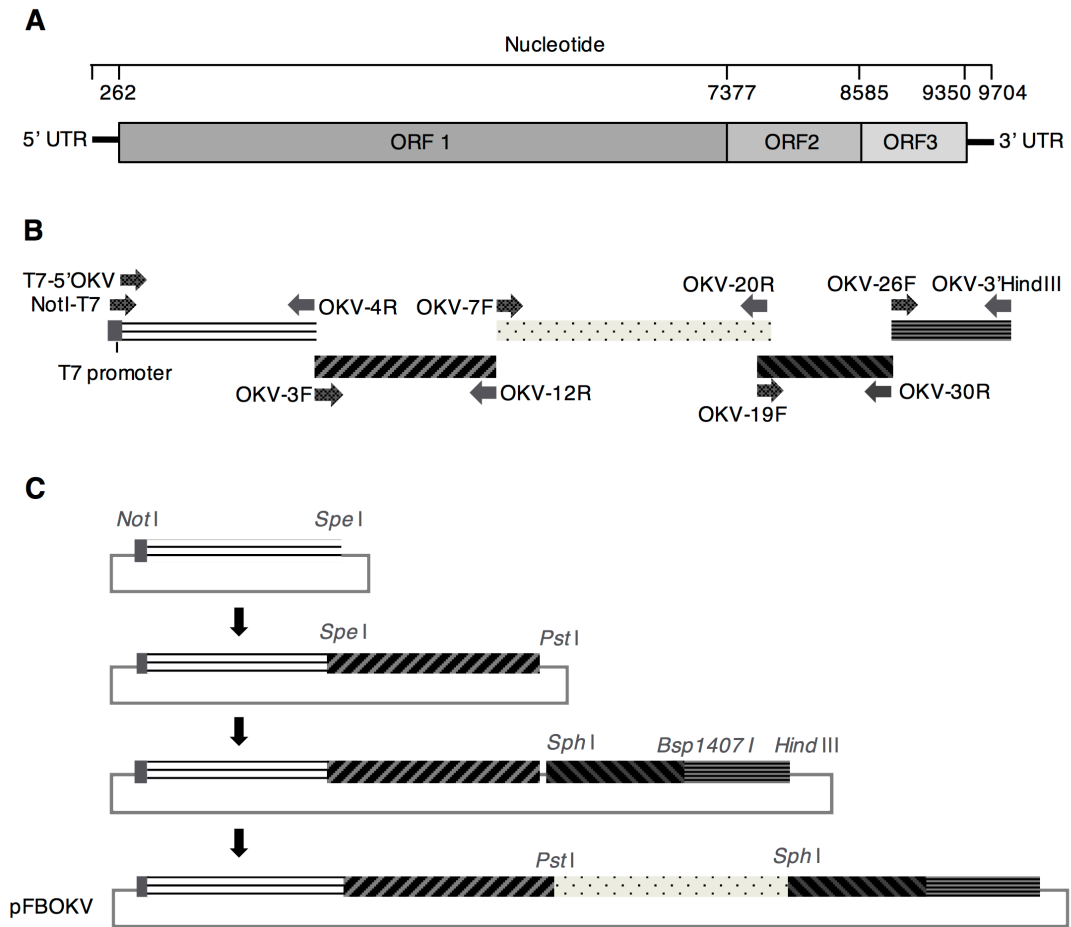


Figure 8. A schematic representation of construction of a full-length OKV cDNA clone. A, Organization of OKV genome. B, The scheme for amplification of sub clones. Primer pairs used for RT-PCR and resulting cDNA fragments are shown. C, The scheme for assembling the full-length OKV genome from the sub clones. Restriction enzyme sites used for the plasmid construction are indicated.

2.3.2. OKV cDNA clone-derived RNA is infectious regardless of the presence or absence of 5'-terminal cap structure

Positive-sense single-stranded RNA for OKV full-length genome was generated from linearized pFBOKV using *in vitro* transcription with T7 RNA polymerase. In a previous experiment, it was shown that the NEGV infectious cDNA clone did not require cap analogue to produce infectious RNA *in vitro* (Gorchakov *et al.*, 2014). However, it was not clear if the observation for NEGV could be applied to OKV. Therefore, we carried out *in vitro* transcription from the cDNA clone with or without cap analogue to generate capped or uncapped RNA products. These two types of RNAs were transfected into C6/36 cells and infectivity of the RNAs was evaluated by measuring the amount of viral RNA in culture media (Fig. 9A). Reverse transcription-quantitative PCR (RT-qPCR) showed that profiles of RNA genome copy number in culture medium were comparable between cells transfected with capped RNA or parental viral RNA (Fig. 9A). On the other hand, amount of viral RNA in culture medium from cells transfected with uncapped RNA a day post transfection (d.p.t.) was lower compared with those of cells transfected with other RNA while, from 2 d.p.t., the amount of progeny viruses derived from all the tested inoculum became comparable (Fig. 9A). This observation suggested uncapped RNA was less efficient to start infection cycle but was able to produce progeny viruses. To examine if progeny viruses from cells transfected with uncapped RNA were equivalently infectious to those derived from capped and parental viral RNAs, RNA purified from progeny viruses were used as inoculum. Three hundred ng of RNA purified from progeny viruses were used for transfection of C6/36 cells and kinetics of progeny virus production were measured using RT-qPCR.

Infection with progeny viruses derived from capped, uncapped, or parental viral RNA showed indistinguishable progeny virus production (Fig. 9B). Pathogenicity of the progeny viruses was also assessed based on cytopathic effect. The progeny viruses derived from the cDNA clone showed cytopathic effects indistinguishable from the one caused by the parental OKV (Fig. 9C to F). Taken together, these observations suggested that RNA from the full-length OKV cDNA clone was able to produce infectious OKV that recapitulated the parental OKV and that production of capped RNA is not essential to produce infectious progeny viruses.

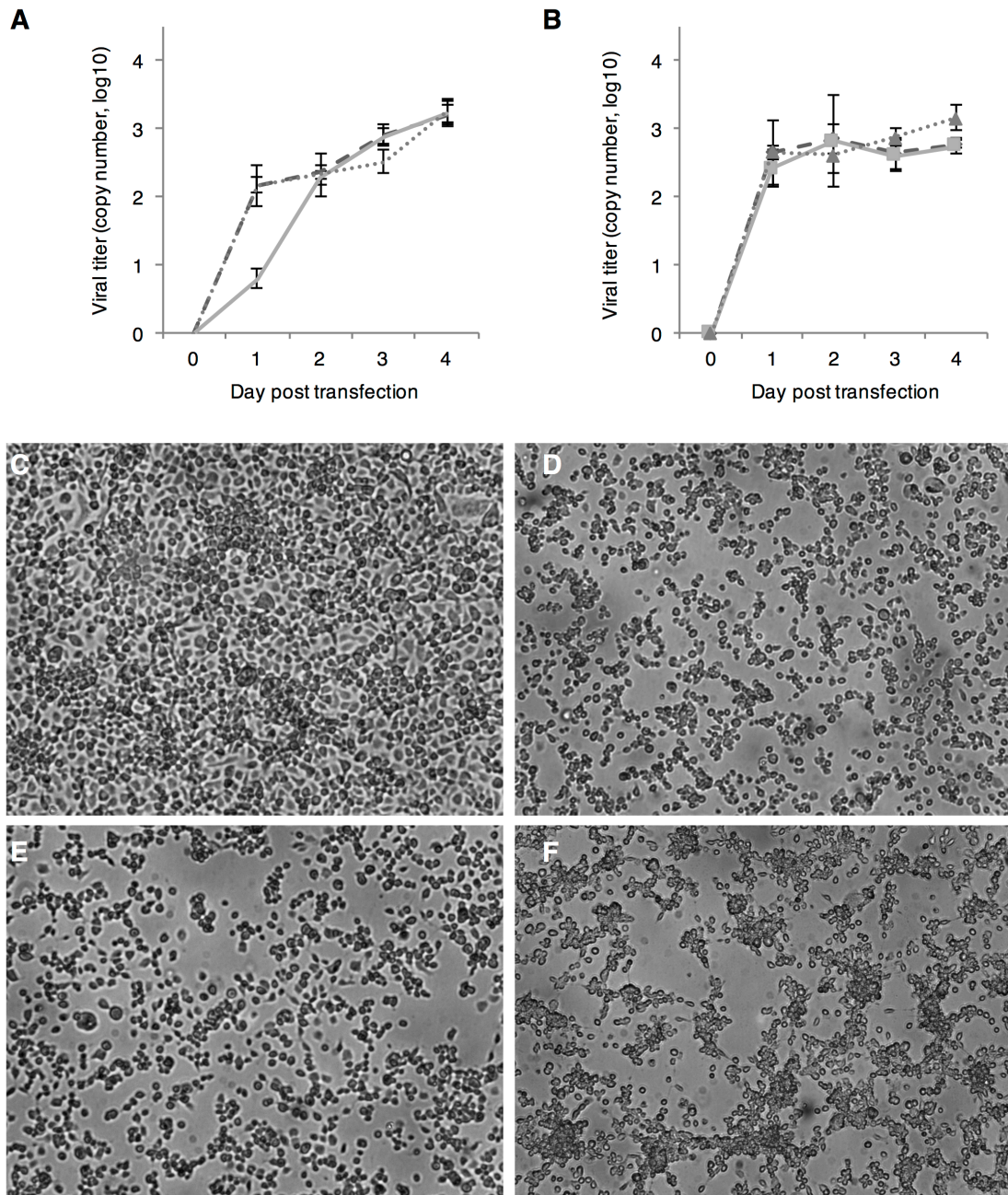


Figure 9. Production of infectious progeny viruses by transfecting RNA derived from OKV cDNA clone into C6/36 cells. A, Profiles of viral RNA amount in culture medium after transfection of *in vitro* transcribed pFBOKV RNA. RNA for transfection was prepared by *in vitro* transcription using T7 RNA polymerase with or without cap analogue or by purification of parental OKV RNA from viral particles. Amount of viral RNA in culture medium was quantified using RT-qPCR B, Profiles of viral RNA amount in culture medium after transfection with RNA of pFBOKV-derived or wild-type progeny viruses. Three hundred ng of RNA was used for transfection. The transfected RNA was prepared by purification of viral RNA from progeny viruses obtained after blind passages three times. Each data point represents the mean copy number and standard deviation obtained from triplicates. For both A and B, black dashed, grey solid, and grey dotted lines indicate capped, uncapped pFBOKV-derived, and parental

viral RNA, respectively. C to F, Cytopathic effects produced by pFBOKV-derived progeny viruses. Micrographs were taken 3 days post infection. C, mock-inoculated C6/36 cells; D, parental OKV-infected cells; E, C6/36 cells infected with progeny viruses produced from capped pFBOKV-derived RNA; F, C6/36 cells infected with progeny viruses produced from uncapped pFBOKV-derived RNA.

2.3.3. Replacement of ORF3 with GFP in the cDNA clone enabled GFP expression from OKV genomic RNA but compromised production of progeny viruses

The expression systems of exogenous genes in insect cells based on self-replicating RNAs have been reported for several positive-sense single-stranded RNA viruses including flavivirus (Corver *et al.*, 2003; Ishikawa *et al.*, 2015; Liljestrom *et al.*, 1991; Lo, *et al.*, 2003; Molenkamp *et al.*, 2003; Scholle *et al.*, 2004; Yamshchikov *et al.*, 2001) and coronavirus (Almazán *et al.*, 2000; Casais *et al.*, 2001). In such experiments, the replacement of viral gene dispensable for viral multiplication with an exogenous gene could be one choice (Liljestrom and Garoff, 1991; Varshavsky and Khromykh, 1999; Yamshchikov *et al.*, 2001; Corver *et al.*, 2003; Lo *et al.*, 2003; Molenkamp *et al.*, 2003; Scholle *et al.*, 2004; Ishikawa *et al.*, 2015). In this study, ORF3 encoding putative structural protein P22 in pFBOKV was replaced with GFP ORF for development of a negevirus-based expression vector. The resultant plasmid was designated pO2GFP (Fig 9A). Capped RNA was prepared by *in vitro* transcription from linearized pO2GFP. Two µg of the RNA was transfected into C6/36 cells and GFP expression was evaluated by quantification of GFP fluorescence using Infinite M200 Pro (Tecan) (Fig. 10B) and microscopic inspection (Fig. 11A to C). GFP fluorescence in cells transfected with pO2GFP-derived RNA slightly increased at 1 d.p.t. and substantially from 2 d.p.t. during the observation (Fig. 10B, 10A and 10B). However, despite of the increase of GFP fluorescence from transfected cells, progeny virus production was not detected by RT-qPCR on viral RNA in culture medium (Fig. 10C and D). These inconsistent observations indicated that increase of GFP fluorescence in cells transfected with pO2GFP-

derived RNA was expression and accumulation of GFP in transfected cells rather than increase of number of cells infected with pO2GFP-derived progeny viruses carrying GFP.

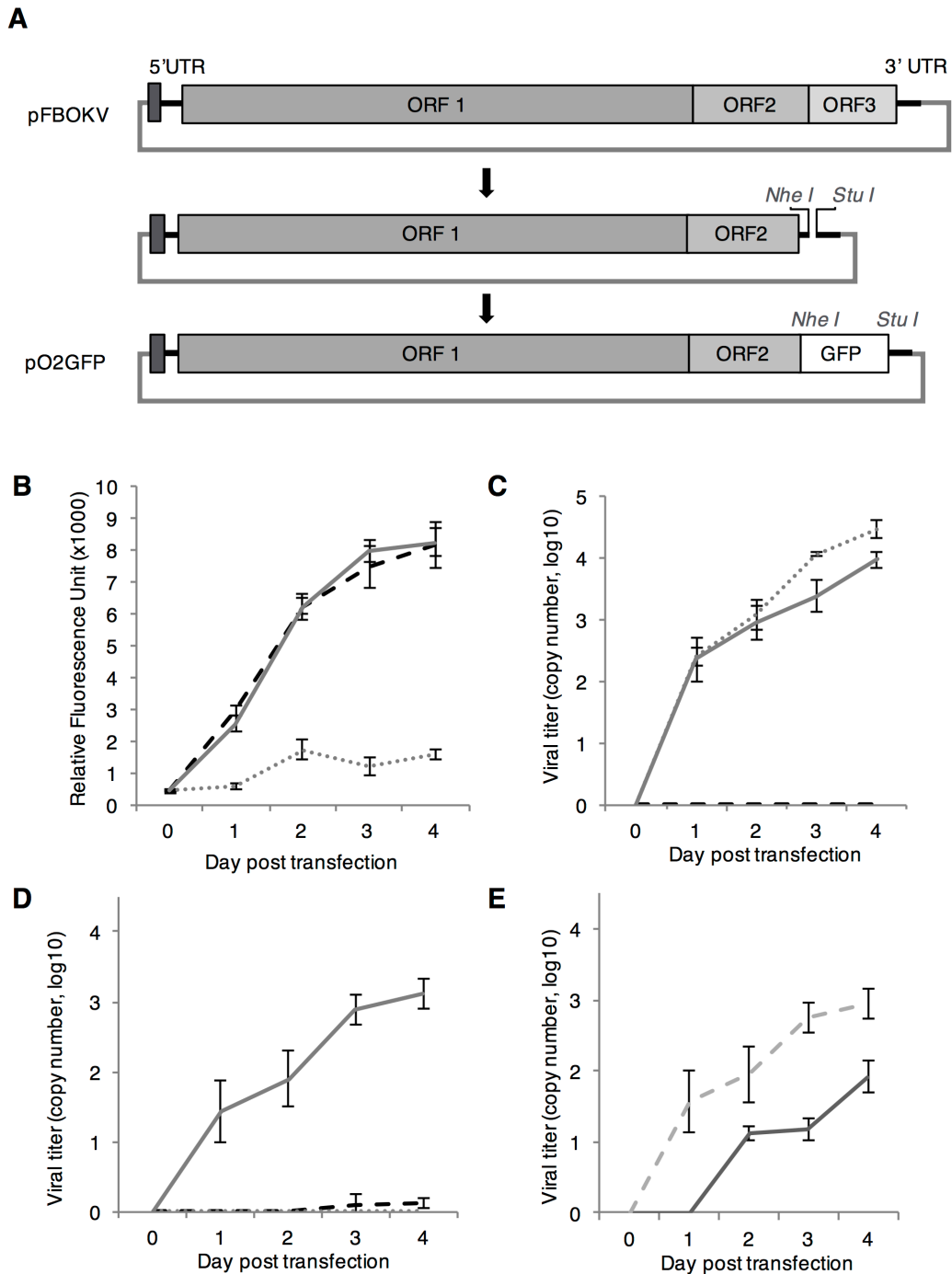


Figure 10. Characterization of RNA derived from pO2GFP, a pFBOKV derivative carrying GFP instead of ORF3. A, A schematic representation of construction of pO2GFP. B, GFP expression in C6/36 cells transfected with pO2GFP-derived RNA. GFP fluorescence in transfected cells in 24 well plates was measured using a plate reader. C and D, Profiles of viral RNA amount in culture medium after transfection of C6/36 cells with pO2GFP-derived RNA. RNA quantification was performed using primers to anneal 3' untranslated region (C) and GFP region (D). From B to D, Black dashed, grey dotted, and grey solid lines indicate transfection with

pO2GFP-derived RNA, with pFBOKV-derived RNA, and co-transfection with both RNA, respectively. E, Differential RNA accumulation in culture medium from co-transfected C6/36 cells. RNA quantification was performed using primers to anneal ORF3 (solid line) or GFP (dashed line). For transfection only with pO2GFP- or pFBOKV-derived RNA, 2 μ g of RNA was used. For co-transfection with pO2GFP- and pFBOKV-derived RNA, 1 μ g of each RNA was combined and used. Dashed and solid lines indicate estimated copy numbers for pFBOKV- and pO2GFP-derived RNA in culture medium, respectively. Each data point represents the mean copy number and standard deviation obtained from triplicates.

2.3.4. Co-transfection with pO2GFP- and pFBOKV-derived RNA rescued defects in progeny virus production

RNA derived from pO2GFP could not accomplish a process(es) during production of progeny viruses, presumably due to lack of P22, putative structural protein encoded in ORF3. In order to test the possibility that ORF3 was required to produce progeny viruses, C6/36 cells were transfected with RNA derived from pO2GFP together with RNA derived from pFBOKV. As negative control, RNA from pFBOKV was transfected into C6/36 cells, resulted in no detectable GFP fluorescence in the cells (Fig. 11C). The co-transfected and the cells transfected with only pO2GFP showed comparable GFP expression (Fig. 11 A and B). The co-transfected cells produced a detectable amount of progeny viruses carrying GFP in cell medium (Fig. 10E). Infectivity of these progeny viruses generated in co-transfected cells was examined by inoculation of cells with the culture medium. Cells inoculated with culture medium from co-transfected cell culture showed GFP fluorescence (Fig. 11E) while those inoculated with the medium of the cells transfected with only pO2GFP did not (Fig. 11D). However, number of the cells with GFP fluorescence was not increased during the observation until four days after inoculation (data not shown).

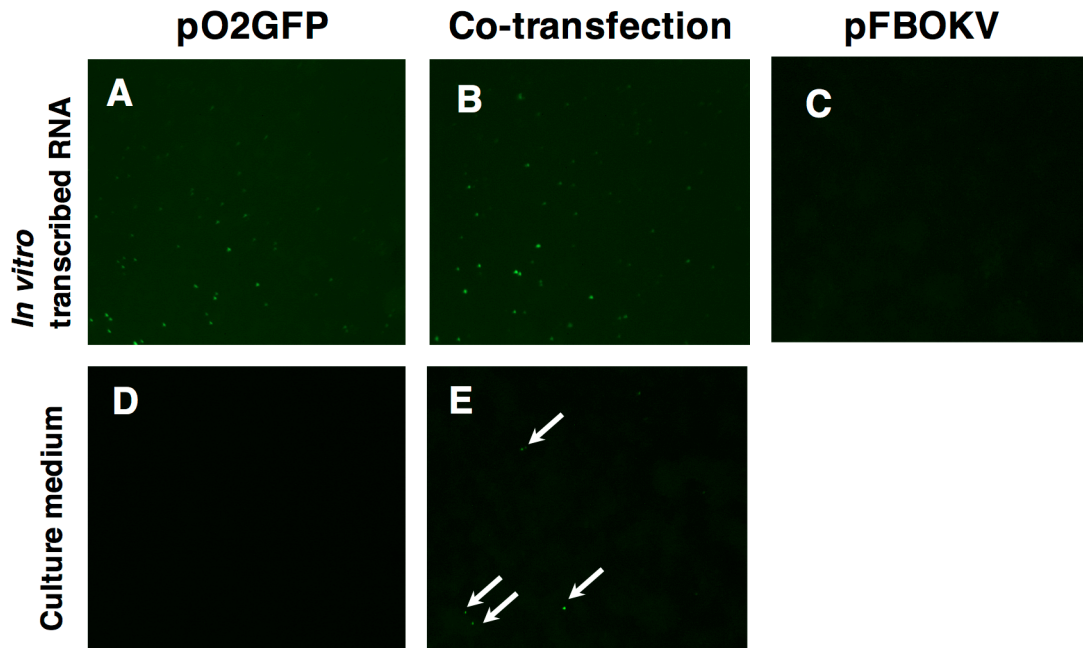


Figure 11. GFP expression and infectivity of pO2GFP-derived RNA or progeny viruses. GFP expression in C6/36 cells with indicated treatments was visualized under fluorescent microscope 2 days after transfection or inoculation. A, C6/36 cells transfected only with pO2GFP-derived RNA; B, C6/36 cells co-transfected with pO2GFP- and pFBOKV-derived RNA; C, C6/36 cells transfected only with pFBOKV-derived RNA; D, C6/36 cells inoculated with culture medium from cells transfected with pO2GFP-derived RNA; E, C6/36 cells inoculated with culture medium from cells co-transfected with pO2GFP- and pFBOKV-derived RNA. The amount of transfected RNA was identical as in Fig. 3. For infection, 100 μ l of culture medium of transfected cells 4 d.p.t. was used. Arrows in E indicate cells expressing GFP.

2.4. DISCUSSION

In this study, we successfully constructed an OKV infectious cDNA clone. The pFBOKV cDNA clone was generated after serial ligation of five sub-cloned genomic fragments (1.6, 1.8, 2.7, 1.5, and 2.1 kb cDNA fragments) (Fig. 8). A larger number of cDNA fragments was used for constructing full-length cDNA clone in this study than the numbers of fragments used in some other studies generating infectious cDNA clones of less than 10 kb in length (Yamshchikov *et al.*, 2001; Isawa *et al.*, 2012; Gorchakov *et al.*, 2014; Ishikawa *et al.*, 2015), since PCR amplification of longer sequences of OKV resulted in generation of unexpected nucleotide mutations frequently, that could result from the fidelity of Taq DNA polymerase (McInerney *et al.*, 2014) and/or sequence characteristics itself. As a result, full-length OKV cDNA clone without any single nucleotide mutation was successfully constructed and is infectious (Fig. 8 and 9).

A derivative of OKV infectious cDNA clone successfully expressed GFP by itself and produced progeny viruses by co-transfection with the wild-type viral RNA. Infectivity of the progeny viruses was low, presumably due to lack of multiple components necessary for proper virion structure and/or initiation of infection cycle. P22, a putative membrane-bound protein encoded by ORF3 that was replaced with GFP coding sequence in pO2GFP, was likely to be supplied by co-transfection with the wild-type viral RNA for pO2GFP-derived molecules that were being packaged during infection cycle. We assume that supplement of P22 is responsible for improvement of production of pO2GFP-derived progeny viruses in culture medium (Fig. 10E). However, these progeny viruses remained to be poorly infective to C3/36 cells or failed to express GFP in the

cells by passage of the culture medium in which the recombinant and wild-type progeny viruses co-existed (Fig. 11E). This implies that supplement of P22 by the wild-type virus as a helper virus was not sufficient to produce fully infective pO2GFP-derived progeny viruses and/or pO2GFP-derived progeny viruses that could compete with the wild-type progeny viruses. It is possible to speculate many possible causes of the poor infectivity or low fitness of pO2GFP-derived progeny viruses other than P22 protein. These include existence of uncharacterized genetic elements in ORF3 sequence that support efficient progress of infection processes such as virus RNA replication, gene expression, and morphogenesis. Considering these possibilities, a rational approach to generate more efficient OKV-based expression vector is designing a genome that drives expression of a foreign gene under control of an exogenous genetic element without removing ORF3. Such OKV derivative allows for OKV expression vector design that avoids removal of unidentified genetic elements in ORF3 as well as P22 and use of a helper virus that may compete with the recombinant virus (Liljestrom and Garoff, 1991; Flotte *et al.*, 1995; Xiao *et al.*, 1998). Nonetheless, this study is the first report of the construction of a negevirus-based expression vector capable of expressing a foreign gene, and has suggested strategies to generate improved negevirus-based expression vectors.

This study also has provided biological insights into OKV biology. The RNA likely replicated in the transfected cells without P22. Although we cannot rule out possibility that P22 supports efficient genome replication, the transfection only with pO2GFP-derived RNA and the co-transfection showed similar profiles on GFP expression (Fig. 10B). This observation suggested that

equivalent amount of positive-sense viral RNA derived from pO2GFP in these two cases. Considering the ability of pO2GFP-derived RNA to increase the viral titer in the co-transfected cells (Fig. 10C to E), it is reasonable to conclude that viral replications occurred in both cases. Therefore, P22 of OKV, and presumably other negevirus, is not necessary for replication but required for production of infectious progeny viruses.

Structures of negeviral RNA remained to be characterized. Production of progeny viruses from RNA transcribed *in vitro* with or without cap analogue differed only at early time point in our experiment (Fig. 9A) and previous reports (Gorchakov *et al.*, 2014). The delay of progeny virus production, i.e. viral growth shown cap-dependent enhancement of initial infection of the *in vitro* transcribed RNA. This enhancement appeared not to be required after propagation of progeny viruses was initiated. The previous study argued that 5' untranslated region contained internal ribosomal entry site (Gorchakov *et al.*, 2014). We rather suspect that OKV RNA is capped from the result shown in Fig. 9 and previous observation in chapter 1 Fig. 4 that the decapping treatment of viral RNA was required in 5' RACE of the RNA (Kawakami *et al.*, 2015), but would wait for further biochemical analysis to reach a conclusion.

2.5 CONCLUSION

In Chapter 1, a novel negevirus was isolated in Japan, designated as OKV, its genomic characterization was carried out. OKV belongs to one of the two phylogenetic clades of negevirus. The characterization of OKV as a negevirus isolated from mosquito at larval in subarctic area had open access for deeper understanding on diversity of negevirus.

In Chapter 2, successful construction of an infectious cDNA clone of OKV and its derivative capable of expressing a foreign gene, together with biological insights of OKV obtained in the course of the present work had provided strategies to establish a negevirus expression vector system in future studies.

Taken together, the identification, molecular characterization, and application of OKV in this study would become foundation for further elucidation on the nature of negevirus as well as for implication with control of mosquito-borne diseases including the vector ecology and the vector control strategy.

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