## RESEARCH NOTE

# PHYLOGENETIC ANALYSIS REVEALS GENETIC VARIATIONS OF DENSOVIRUS ISOLATED FROM FIELD MOSQUITOES IN BANGKOK AND SURROUNDING REGIONS

Kobporn Boonnak*, Aroonroong Suttitheptumrong, Ubonwan Jotekratok and Sa-nga Pattanakitsakul

Division of Molecular Medicine, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

**Abstract.** Screening for densoviruses (DNVs) from *Aedes*, *Culex* and *Toxorhynchites* mosquitoes collected in Bangkok and surrounding regions identified two clades of *Aedes* DNV; *Ae*. *aegypti* DNV (*Aae*DNV) and *Ae*. *albopictus* DNV (*Aal*DNV) by PCR-restriction fragment length polymorphism (PCR-RFLP). From nucleotide sequencing and phylogenetic analysis of PCR amplicons of a fragment of DNV capsid gene, these DNVs were shown to be new DNV genetic variations similar to *Aae*DNV. Isolation and identification of densoviruses from indigenous field mosquitoes reside in natural habitat should be helpful in monitoring the distribution of DNVs in important mosquitoes, especially *Ae*. *aegypti* and *Ae*. *albopictus*, vector for dengue and yellow fever viruses.

**Keywords:** densovirus, mosquito, nucleotide sequence, phylogenetic tree

## INTRODUCTION

*Brevidensoviruses* or *Contravirus* of the subfamily of invertebrate Densovirinae of Parvoviridae family consists of *Aedes aegypti* densovirus (*Aae*DNV) and *Aedes albopictus* densovirus (*Aal*DNV) (Kurstak and Small, 1972; Bachmann *et al*, 1975; Siegl *et al*, 1985). Currently, several types of DNVs have been reported in a variety of mosquito species and cell lines, such as *Aedes aegypti, Culex pipiens, Toxorhynchites splendens* and *Haemagogus equinus* (O'Neill *et al*, 1995; Pattanakitsakul *et al*, 2007; Zhai *et al*, 2008; Sivaram *et al*, 2009).

DNV is the smallest non enveloped and icosahedral viral particle containing 4.0-4.2 kb single-stranded plus or minus DNA genome (Afanasiev *et al*, 1991; Jousset *et al*, 1993; Boublik *et al*, 1994). The presence of a palindromic hairpin structure

Correspondence: Dr Sa-nga Pattanakitsakul, Division of Molecular Medicine, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, 4th Fl, SiMR Bldg, Wanglang Road, Bangkok 10700, Thailand.
Tel: +66 (0) 2419 2755; Fax: +66 (0) 2411 0169
E-mail: sanga.pat@mahidol.ac.th; sispn@yahoo.com
*Present address: Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.

at both termini of the DNV genome is a unique characteristic and plays a critical role in virus DNA replication (Afanasiev *et al*, 1991; 1994; Boublik *et al*, 1994). There are three open reading frames (ORF) in DNV plus DNA strand encoding two non-structural proteins, NS1 and NS2, and a structural protein, except for *Aae*DNV, which has an extra ORF in the minus DNA strand encoding a protein of unknown function. A plus or minus strand of DNV genome is encasidated separately in the virion (Afanasiev *et al*, 1991; Boublik *et al*, 1994). NS1 and NS2 of mosquito DNV play roles in viral replication and production of virion (Azarkh *et al*, 2008). *Aedes* DNVs have been extensively studied because these viruses infect *Ae. aegypti* and *Ae. albopictus*, which are important vectors of dengue viruses that cause dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in humans (Hayes and Gubler, 1992; Rigau-Perez *et al*, 1998; Rodriguez-Tan and Weir, 1998).

The application of molecular biology techniques for gene manipulation of insect DNV clones is currently being used for the development of biological control of insects because of the small size of the virus, which facilitates transfection of expression vectors in insects (Jousset *et al*, 1990; Dumas *et al*, 1992; Giraud *et al*, 1992; Carlson *et al*, 2006; Jiang *et al*, 2007). Recent studies of *Aedes* DNV have shown it to be more pathogenic to *Ae. albopictus* cell line (C6/36 cells) and there has been no report to date describing it causing any serious disease to humans (Paterson *et al*, 2005; Wei *et al*, 2006). Moreover, this virus has been proven to be able to be maintained as a persistent co-infection with other viruses, such as dengue or chikungunya virus in C6/36 cell lines (Kanthong *et al*, 2008, 2010; Sivaram *et al*, 2010). On account of its small size and infection of only insects, DNV has been suggested as a potential biological agent for control of mosquitoes and other insects.

We report in this study the prevalence of DNVs in field mosquitoes and larvae and determined their identities by nucleotide sequencing and phylogenetic analysis.

## MATERIALS AND METHODS

### Mosquito specimens

Larvae and adult *Ae. aegypti*, *Cx. quinquifasciatus* and *Tx. splendens* were field collected from Bangkok, Thailand and rural locations about 120 km surrounding Bangkok and identified according to the pictorial key guidelines (Huang, 1977). The samples were kept at -80ºC freezer until used.

### *Aal*DNV DNA

*Aal*DNV DNA used in the study as a positive control for PCR consisted of a partial full length genome of 3.7 kb (nucleotide number 351-4025) inserted in pUC18 plasmid vector (Sangdee and Pattanakitsakul, 2012).

### PCR amplification of DNV from mosquito specimens

Each adult mosquito or pool of 5 mosquito larvae in 0.1 ml of 1% fetal bovine serum (FBS) and Leibovitz's (L-15) (1% FBS/L-15) medium were homogenized using a glass homogenizer. Then 0.4 ml aliquot of 1% FBS/L-15 medium was added and 0.1 ml of this mixture was used for DNA extraction by incubating with 0.25 ml of DNAzol (GibcoBRL, Grand Island, NY) for 5 minutes at room temperature. The solution was centrifuged at 10,600*g* for 10 minutes at 4ºC and the supernatant was added to 125 ml of absolute ethanol for 5 minutes at room temperature. Following centrifugation at 11,290*g* for

10 minutes at 4ºC, the DNA pellet was washed twice with 70% ethanol, air-dried and dissolved in 100 µl of distilled water. PCR amplification was conducted in 25-µl volume containing 5 µl of DNA solution, 1X PCR buffer, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer pair [forward primer: 5′ AACAAGAC AGAGACTGCTAAC 3′ (nt 2997-3017) and reverse primer: 5′ GCATTCTTGG ATATGATGTTCT 3′ (nt 3448-3427)], and 0.5 U *Taq* DNA polymerase (Promega, Madison, WI). Thermocycling (using Thermal Cycler 2400; Applied Biosystems, Foster City, CA) conditions were as follows: 94ºC for 5 minutes; 30 cycles of 94ºC for 30 seconds, 50ºC for 30 seconds and 72ºC for 30 seconds; and a final step at 72ºC for 5 minutes.

**Analysis of *Aedes* DNV by restriction fragment length polymorphism (RFLP)**

In order to analyze the polymorphism of *Aedes* DNV, amplicons were subjected to RFLP assay (Jotekratok *et al*, 2014). A 5-µl aliquot of the PCR solution was digested with 5 U *Eco*R V (New England Biolabs, Ipswich, MA) at 37ºC for 1.5 hours and the DNA fragments were separated by 5% polyacrylamide gel-electrophoresis (PAGE) at 150 volts for 40 minutes, stained with ethidium bromide and visualized under an UV transilluminator (Spectronics, West Miami, FL).

**Nucleotide sequencing and phylogenetic analysis**

Nucleotide sequencing was performed on both strands using BigDye™ Terminators Cycle Sequencing kit (PE-Applied Biosystems, Foster City, CA) and ABI PRISM 310 Genetic Analyzer according to the supplier's protocol. Nucleotide sequences were processed using Data Collection and Sequence Navigator softwares (PE-Applied Biosystems).

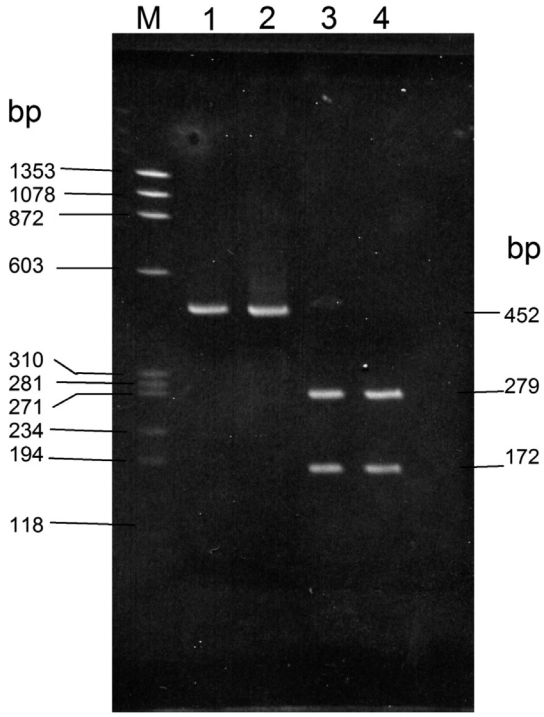


Fig 1–PCR-RFLP analysis of DNV from mosquito larvae. PCR amplicons from DNV capsid gene were digested with *Eco*R V and analyzed by 5% polyacrylamide gel-electrophoresis. Lane 1, from *Culex quienquefasciatus*; lane 2, *Ts*DNV; lane 3, *Aal*DNV; lane 4, recombinant *Aal*DNV clone.

The sequences were aligned with other DNV sequences deposited in GenBank database (O'Neill *et al*, 1995; Pattanakitsakul *et al*, 2007) employing PHYLIP package version 3.57c (University of Washington, Seattle). Maximum likelihood was determined using DNADIST program (University of Washington, Seattle) to calculate genetic distances. Phylogenetic trees were constructed using UPGMA algorithms available in the NEIGHBOUR program (University of Washington, Seattle). Bootstrap analysis (1,000 replications) using SEQBOOT and CONSENSE programs (University of

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AaeDNV      GGGGACAACA AACCAAACTA CATGGGACTT TGAAACAAGT CAAAACATGT   3038
MMU00       ...A..G..G .......... .......... .......... ......T...
MMU02       A..A...... ........A. .......... .......... ..........
AalDNV      ...A...... ..T.....A. .T........ C......... ..........

            TCATCGCAGA TGCAGACAGA GAACCAGAAA ATTTCAACTT GGCAACAGCA   3088
            .......... .......... .......... .C...GGAT. A...T.....
            .......... .......... ..C....... .C......C. AA........
            .......... C......... .....G.... ........C. A.A.....TT

            GCAGCAACTG GACCACTTGC ACAACAAACA ACACAAACAC TACTATTCAA   3138
            .......... .T........ T......... .......... ..........
            .......... .......... ...G...... G........T ....T.....
            ........C. .....T.A.. .......... .........T ..........

            TGCAAACAAC GACAGATATA CAAAATATGA ATTACCACAA AGAAACCAGT   3188
            AAA.C..... ........C. .......... .......... .....TA.A.
            .........T .......... .......... .......... ........A.
            .T.TC....T ........C. .......C.. .C........ ..G.....A.

            ATACAAGAGA ATATGACTTC CAACAACTTA CAAATAACTA CATGTGGAAA   3238
            .......G.. .ATC...... ...A....A. ....C..... T.........
            .......... .AT....... ........A. .G..C..T.. T.........
            .......G.. .ATCA..... ..G...T.A. ....T..C.. T.......G.

            CCAACAGACA TTAGCGCTGC AGCAAACTTT AGAAGATTGA TCCCAATGGC   3288
            .....G.... .A.A..AA.A ..T....... .....G..A. ........T.
            ...TTG.... .CG....... .A........ ....A...A. ..........
            ...TT...T. .C...AAA.A .A.......C ...TCCC.A. .T......T.
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Fig 2–Nucleotide sequence analysis of 300-bp PCR amplicon spanning nt 2989-3288 of capsid gene of DNV isolated from *Culex quinquefasciatus* and *Tx. splendens* compared to *Aae*DNV and *Aal*DNV.

Washington, Seattle) was employed to evaluate the reliability of different phylogenetic groupings.

## RESULTS

Among 110 adult *Ae. aegypti* (singly) and 130 mosquito larvae (70 *Cx. quinquefasciatus*, 50 *Ae. aegypti* and 10 *Tx. splendens* (in groups of 5) were identified for DNV infection by PCR amplification of a 451-bp fragment of the virus genome. DNV amplicons were observed in 1 pool of *Cx. quinquefasciatus* and 1 pool of *Tx. splendens* larvae (Fig 1), while pools of others larvae and adult mosquitoes were DNV negative (data not shown). The prevalence rate of DNV in these mosquitoes tested was 0.8%. RFLP analysis revealed a pattern of *Aae*DNV clade because there is no *Eco*R V site in the capsid gene of *Aae*DNV, while there is an *Eco*R V site in *Aal*DNV clade (Fig 1).

Nucleotide sequence analysis of DNV amplicons isolated from *Tx. splendens* (MMU00) and *Cx. quinquefasciatus* (MMU02) larvae showed, as expected, more similarity to *Aae*DNV than *Aal*DNV. There were 39 and 29 nucleotide mismatches of MMU00 and MMU02, respectively when compared to *Aae*DNV (GenBank accession no. M 37899 and 56 and

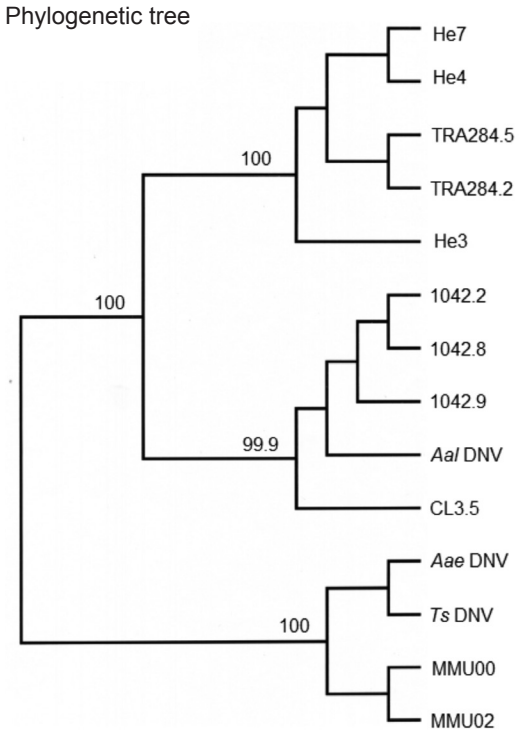


Fig 3–Phylogenetic tree of DNVs isolated from *Culex quinquefasciatus* (MMU02) and *Tx. splendens* (MMU00) larvae compared to other DNVs. DNV sources were *Aae*DNV (M 37899), *Aal*DNV (X 74945) and *Ts*DNV (AF 395903) from GenBank database and other DNVs from cell lines including clones 1042.2, 1042.8 and 1042.9 from *Ae. aegypti* cell line 1042; CL3.5 from *Cx. theileri* cell line CL3; He3, He4 and He7 from *Haemagogus equinus* cell lines; and TRA284.2 and TRA284.5 from *Tx. amboinensis* cell lines (O'Neill *et al*, 1995).

48 nucleotide mismatches, respectively compared to *Aal*DNV (GenBank accession no. X 74945) (Fig 2).

Phylogenetic tree constructed from the nucleotide sequence of MMU00 and MMU02 and the available sequences of mosquito DNV from GenBank and other references showed that they formed a distinct branch of the tree (Fig 3), but was still more closely related to *Aae*DNV and *Ts*DNV previously found in *Ae. aegypti* and *Tx. splendens*, respectively (Pattanakitsakul *et al*, 2007).

## DISCUSSION

The circulation of DNVs in mosquitoes in the field has been little described (Flegel, 2012; Stentiford, 2012). In the present study we used PCR-RFLP and DNA sequencing to screen for and identify DNVs infecting adult and larval mosquitoes caught in Bangkok and surrounding regions. With a prevalence of < 1%, two clades of mosquito DNV were found, which were phylogenetically closely related to *Aae*DNV and *Ts*DNV (Afanasiev *et al*, 1991; Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2013).

The low prevalence of DNV-positive mosquitoes observed and only found in larvae may be explained by the low transmission of virus in mosquitoes in their natural habitats, or that the PCR primers used did not match the genetic diversity of DNVs infecting mosquitoes in the wild. However, the prevalence of DNV detected in mosquitoes has been reported between 1.57%-4.4% (Faye *et al*, 2013; Papa *et al*, 2014).

Recently, flaviviruses, including West Nile virus and other novel flaviviruses, have been detected in *Culex* and *Aedes* mosquitoes, collected in Finland and Greece (Huhtamo *et al*, 2014; Papa *et al*, 2014). These mosquitoes are known as vectors of pathogenic viruses, such as dengue or West Nile, which can cause serious diseases in humans. As the circulation of flaviviruses and DNVs can co-exist in similar mosquitoes and locations, this may trigger a possible interaction of these viruses in mosquitoes. An *in vitro*

study of triple-infections of DNV, dengue and Japanese encephalitis viruses in C6/36 cells has been described and these viruses were maintained as persistent infection through several passages of the cells (Kanthong *et al*, 2010). Two or three of these viruses might be found in field mosquitoes, but there have been no reports of such co-infections. Only artificial feeding of different strains of each dengue serotype in *Ae. aegypti* or co-infection with DNV and dengue virus in *Ae. albopictus* have been demonstrated (Wei *et al*, 2006; Quintero-Gil *et al*, 2014). Although the route of infection by DNV in mosquitoes have not been described. However, *Junonia coenia* DNV infection in lepidopteran cell line occurs through internalization of viral particles into clathrin-coated vesicles and trafficking within early and late endosomes during viral production (Vendeville *et al*, 2009).

In summary, the present study employed PCR-based techniques and phylogenetic analysis to identify DNVs in mosquitoes in their natural environment. This approach might be applied to study the transmission dynamics of DNV infection and epidemiology in field mosquitoes. This provides information for further studies of the interaction of DNV with other viruses that may co-circulate in mosquito vectors. Understanding the interaction of these viruses in mosquitoes may lead to the development of novel strategies to control mosquito-borne arbovirus diseases.

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