A NEW DENSOVIRUS ISOLATED FROM THE MOSQUITO *TOXORHYNCHITES SPLENDENS* (WIEDEMANN) (DIPTERA:CULICIDAE)

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Abstract. A new densovirus was isolated and characterized in laboratory strains of *Toxorhynchites splendens*. The virus was detected by polymerase chain reaction (PCR) from mosquitoes reared in our laboratory. PCR fragments from each mosquito were compared by single strand conformation polymorphism (SSCP) assay and found to be indistinguishable. Thus, it is likely the densoviruses from these mosquitoes contain homologous nucleotide sequences. The PCR fragment corresponding to a 451 bp densovirus structural gene segment from each of 5 mosquitoes had 100% identical nucleotide sequences. Phylogenetic analysis of the structural gene sequence suggests the newly isolated densovirus is more closely related to *Aedes aegypti* densovirus (*Aae*DNV) than to *Aedes albopictus* densovirus (*Aal*DNV). Analysis of offspring and predated larvae suggests that vertical and horizontal transmission are responsible for chronic infections in this laboratory strain of *Toxorhynchites splendens*. The virion DNA is 4.2 kb in size, is closely related to, but distinct from, known densoviruses in the genera *Brevidensovirus* and *Contravirus*. The virus is tentatively named *Toxorhynchites splendens* densovirus (*Ts*DNV).

INTRODUCTION

Densoviruses (DNV) are small non-enveloped, single-stranded DNA viruses that belong to the family Parvoviridae (Kurstak, 1972; Bachmann *et al,* 1975; Tijssen *et al,* 1976; Siegl *et al,* 1985). This family is currently subdivided into two subfamilies, the Parvovirinae and the Densovirinae. Viruses in the former subfamily infect vertebrates while the latter infects invertebrates (mainly insects). All densoviruses have narrow host ranges, infecting only closely related insects and causing fatal disease in their host larvae. Within the Densovirinae, three genera are known: *Densovirus* (infecting cockroaches), *Iteravirus* (infecting silk worms) and *Brevidensovirus* or *Contravirus* (infecting mosquitoes). The members of the genus *Densovirus*, *Junonia coenia* densovirus (*Jc*DNV) and *Galleria mellonella* densovirus (*Gm*DNV), have a genome size of

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6 kb, while a member of the genus *Iteravirus*, *Bombyx mori* densovirus (*Bm*DNV), possesses a genome of 5.2 kb (Jourdan *et al,* 1990; Bando *et al,* 1992,1995; Dumas *et al,* 1992).

The genomes of the members of both *Densovirus* and *Iteravirus* genera contain inverted terminal repeats and can separately encapsidate (equally) plus or minus a single-stranded DNA in their virions (Jourdan *et al,* 1990; Bando *et al,* 1990,1995; Dumas *et al,* 1992). The genus *Brevidensovirus* or *Contravirus* contains *Aedes aegypti* densovirus (*Ae*DNV or *Aa*e DNV) and *Aedes albopictus* parvovirus or *Aedes albopictus* densovirus (*Aa*PV or *Aal*DNV). *Aedes aegypti* densovirus was originally isolated from a laboratory colony of *Ae. aegypti* larvae (Lebedeva *et al,* 1973). This virus is infectious to mosquito larvae of the genera *Aedes*, *Culex* and *Culiseta* when it is introduced into the water in which they are reared. The virus can infect all stages, including larvae, pupae and adults of both sexes. *Ae. albopictus* densovirus was isolated from an *Ae. albopictus* cell line (C6/36) and was discovered during experiments designed to determine the pathogenicity of the Mosquito African Virus (MAV) for *Ae. aegypti* larvae (Jousset *et al,* 1993). The genomes of both viruses have been cloned and sequenced (Lebedeva *et al,* 1973; Afanasiev *et al,* 1991; Jousset *et al,* 1993; Boublik *et al,* 1994a). Both are small icosahedral, non-enveloped viral particles of 18-20 nm in diameter containing a single-stranded linear DNA of 4 kb and mostly minus polarity (Afanasiev *et al,* 1991; Jousset *et al,* 1993; Boublik *et al,* 1994a,b; Chen *et al,* 2004). These two densoviruses share about 77.3% similarity in nucleotide sequences over the whole genome (Boublik *et al,* 1994a). Both have palindromic structures that can form stable hairpin structures at both termini. These structures are believed to be involved in DNA replication, excision from plasmids and integration into host cell DNA (Afanasiev *et al,* 1991, 1994; Boublik *et al,* 1994a). Most of their genome is encoded in 3 open reading frames (ORF) on the plus strand with the left and mid ORFs coding for non-structural (NS) proteins and the right ORF coding for structural proteins (Boublik *et al,* 1994a). In *Aae*DNV, there is one extra ORF encoded on the minus strand, but it codes for a polypeptide of no known function (Afanasiev *et al,* 1991).

Apart from these two well characterized densoviruses, several densoviruses have also been demonstrated in cell lines of several mosquito species, including *Culex theileri*, *Haemagogus equinus* and *Toxorhynchites amboinensis* (O'Neill *et al,* 1995; Paterson *et al*, 2005), indigenous *Ae. aegypti* mosquitoes (Kittayapong *et al,* 1999) and a laboratory strain of *Cx. pipiens* larvae (Jousset *et al,* 2000). We describe here a novel densovirus from laboratory strains of the mosquito *Tx. splendens.* This mosquito was used routinely in the laboratory for dengue virus isolation by intrathoracic injection from patient's serum.

MATERIALS AND METHODS

Mosquitoes

Tx. splendens mosquitoes were field-collected from rural Thai locations and identified according to the pictorial key described by Huang (1977). They were maintained in the laboratory for serveral generations at a temperature of 28ºC. They were fed daily with larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. Adults were kept in large mesh cages (60 x 60 x 60 cm) and given 10% sugar solution and 10% multivitamin syrup soaked in cotton pads as food. No blood meals were required. Mating took place while in flight, with the pairs usually falling downward during copulation. Oviposition began approximately one week after emergence.

Aedes albopictus densovirus (*Aal*DNV)

Ae. albopictus densovirus was kindly provided by Dr Pattamaporn Kittayapong, Depart-

ment of Biology, Faculty of Science, Mahidol University (Burivong *et al,* 2004). This virus was maintained by culturing in densovirus-free C6/36 cells in 5 ml Leibovitz's medium (L-15) containing 10% FBS in T-25 tissue culture flasks for 7 days at 28ºC. Culture supernatants were collected in aliquots for isolation of DNA and used as positive control DNA templates for PCR reactions.

DNA isolation for polymerase chain reaction (PCR)

Adult mosquitoes. DNA from *Tx. splendens* mosquitoes was isolated using DNAzol reagent (GibcoBRL, New York, USA). Briefly, a mosquito was homogenized with a hand held glass homogenizer in a 1.5-ml microcentrifuge tube containing 300 μl of Leibovitz's medium (L-15) and 1% fetal bovine serum (FBS). Then the homogenate was centrifuged at 11,600*g* for 20 minutes at 4ºC. One hundred microliters of supernatant was added to 250 μl of DNAzol solution followed by gentle mixing, then allowed to stand at room temperature for 5 minutes before further centrifugation again at 11,600*g* for 20 minutes at 4ºC. The DNA was precipitated by adding 125 μl of cold absolute ethanol and mixing thoroughly, then allowed to stand at room temperature for 5 minutes. The DNA pellet was then collected by centrifugation at 11,600*g* for 20 minutes at 4ºC and washed twice with 500 μl of 70% ethanol before being dried and dissolved in 10 μl of distilled water for use as a template for PCR reactions.

Larvae. DNA from a pool of 5 larvae of *Ae. aegypti* or *Cx. quinquefasciatus,* and a larva of *Tx. splendens,* was isolated using DNAzol reagent (GibcoBRL, New York, USA) as described above. Finally, 5 μl of undiluted or 1:10 diluted DNA was used as a template for PCR.

*Aal*DNV culture supernatant. One hundred microliters of *Aal*DNV culture supernatant was added to 250 μl of DNAzol solution and the DNA was purified as described in the protocol above. Finally the DNA was dissolved in 10 μl of distilled water and 5 μl was used for PCR.

Amplification of densovirus DNA using PCR

Primer design. The primers used for amplification were the forward primer (5′ AAC AAG ACA GAG ACT GCT AAC 3′, or residues 2968 to 2988) and the reverse primer (5′ GCA TTC TTG GAT ATG ATG TTC T 3′, or residues 3422 to 3401). This primer pair was selected from conserved sequences of the structural gene of both the *Aal*DNV and *Aae*DNV genomes and was expected to be specific for PCR amplification of a densovirus DNA fragment. The nucleotide positions cited corresponded to the sequence of *Aae*DNV (GenBank Accession No. M37899). All primers were synthesized by the Bioservice Unit, National Science and Technology Development Agency, Thailand.

DNA amplification. PCR was performed in 25 μl of reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 10 pmol of primers, 0.2 mM dNTPs, 1 unit of *Taq* DNA polymerase (Promega, Madison, USA) and 5 μl of purified DNA extracted from mosquito samples. The reaction mixture was initially denatured at 94ºC for 5 minutes and subjected to 30 cycles with the cycling profile of denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute. Finally, the PCR products were detected by electrophoresis of 5 ml reaction mixture in 2% agarose gel (FMC Bioproducts, Rockland, USA) and examination of the ethidium bromide-stained gel under a UV transilluminator. In the case of larvae, reamplification was performed by adding 1 μl of primary PCR product into 24 μl of freshly prepared reaction mixture. Then the tubes were taken to the thermal cycler and the same procedure was followed as used for the primary PCR.

Single strand conformation polymorphism (SSCP)

A slightly modified SSCP analysis (Orita

et al, 1989; Bannai *et al*, 1994) was performed to analyze the heterogeneity of densovirus isolated from the mosquitoes. Specifically, 1 μl of amplified PCR product was mixed with 7 μl of denaturing solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. The mixtures were denatured at 95ºC for 5 minutes and immediately cooled on ice before 1-5 μl of the mixture was applied on 10% polyacrylamide gel (acrylamide:bisacrylamide = 49:1) containing 5% glycerol in 45 mM Tris-borate (pH 8.0)/ 1 mM EDTA buffer at 4ºC or room temperature. The electrophoresis was performed at 20 mA for 4-6 hours in a minigel electrophoresis apparatus with a constant temperature control system (AE-6410, ATTO, Tokyo, Japan). Single-strand DNA fragments in the gel were visualized by silver staining (Daiichi Pure Chemicals, Tokyo, Japan).

Nucleotide sequencing

Nucleotide sequencing was performed using a BigDye™ Terminator Cycle Sequencing kit (PE-Applied Biosystems, CA, USA) according to the supplier's protocol with an ABI PRISM 310 Genetic Analyzer. Briefly, the PCR fragments were first purified using a QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany) and 40 ng of purified fragments were used as the template for cycle sequencing using Ampli Taq DNA polymerase. The sequencing was performed on both strands of DNA fragments.

Phylogenetic analysis of densovirus genomic fragments

To characterize the genome of the densovirus from *Tx. splendens* mosquitoes, nucleotide sequences derived from PCR fragments were used for analysis. The resulting sequences were then aligned with other densovirus sequences deposited in the GenBank database, reported by O'Neill *et al* (1995) and assessed by employing PHYLIP package version 3.57c. Maximum likelihood analysis was used to calculate genetic distances using the DNADIST program. Phylogenetic trees were constructed using UPGMA algorithms available in the NEIGHBOUR program. The reliability of different phylogenetic groupings was evaluated by using bootstrap analysis (1000 bootstrap replications) using SEQBOOT and CONSENSE programs.

Cultivation of densovirus in mosquito cell lines

A source mosquito was homogenized in 1 ml of Leibovitz's medium (L-15) containing 1% fetal bovine serum (FBS) and 10% tryptose phosphate broth (TPB). The homogenate was filtered through a 0.2 μ Millipore membrane and then applied to a culture of densovirus-free C6/36 cells and a culture of *Tx. splendens* (TRA284) cells in T-25 tissue culture flasks at room temperature overnight before further cultivaiton in 5 ml L-15 containing 10% FBS for another 6 days at 28ºC. Culture supernatants were collected in aliquots for further virus identification and analysis.

Isolation of viral DNA from mosquitoes and *Aal*DNV culture supernatant

Isolation of viral DNA was performed according to Molitor *et al* (1984) with some modification. Pools of 60 mosquitoes were homogenized in 30 ml of Leibovitz's medium (L-15) containing 1% fetal bovine serum (FBS) and centrifuged at 10,600*g* for 10 minutes. The supernatant was collected and then centrifuged at 114,000*g* using a 60 Ti rotor (Beckman, USA) for 4 hours at 4ºC. Viral pellets were resuspended in 50 mM Tris-HCl, pH 8.0, 25 mM EDTA and 20 μg/ml RNase A and incubated at 37ºC for 30 minutes. After incubation, suspensions were mixed with 0.5% SDS, 0.4 mg/ml proteinase K and incubated further at 65ºC for 2 hours. After protein digestion, suspensions were extracted twice by vortexing for 5 minutes in the presence of 0.15 M NaCl and 1 volume of saturated phenol. The virion DNA was precipitated twice with the addition of 2 volumes of absolute ethanol and kept at -70ºC for 20 minutes before centrifu-

gation at 11,290*g* for 20 minutes. Virion DNA was resuspended in 50 μl of sterile distilled water and stored at -70ºC until used. Three microliters of DNA was applied for agarose gel electrophoresis to determine the size of the DNA. In the case of purification of *Aal*DNV DNA, 400 ml of infected culture supernatant was clarified at 10,600*g* for 10 minutes using a JA 14 rotor (Beckman, USA) to remove cell debris before further processing as described earlier. Finally the DNA was dissolved in 50 μl of distilled water and 1 μl of DNA was also applied in the same agarose gel electrophoresis.

RESULTS

Detection of densovirus in mosquitoes and larvae

Two pools of *Tx. splendens* mosquitoes randomly collected from pools of mosquitoes reared in our laboratory were studied for the presence of densoviruses. The two pools were collected 2 years apart. Mosquitoes from the first pool (n=27) and from the second pool (n=20) were individually analysed by PCR. All mosquitoes gave a similar amplicon of 451 bp although the electrophoresis band intensity differed (Fig 1). Amplicon size was the same as that of the positive control DNA template isolated from *Aal*DNV. No amplicons were detected in the negative controls. By SSCP analysis, only a single pattern of single-stranded DNA was obtained from *Tx. splendens* mosquitoes in non-denaturing polyacrylamide gel electrophoresis (Fig 2). The pattern was quite different from that of the *Aal*DNV samples. To examine the possibility of densovirus transmission in *Tx. splendens* mosquitoes, 20 pools of 5 *Ae. aegypti* or *Cx. quinquefasciatus* larvae and 50 *Tx. splendens* larvae were collected and analysed by PCR. A positive PCR was observed in 15 pools of *Ae. aegypti*, 10 pools of *Cx. quinquefasciatus* and 50 *Tx. splendens* larvae after re-amplification. All the PCR products proved to

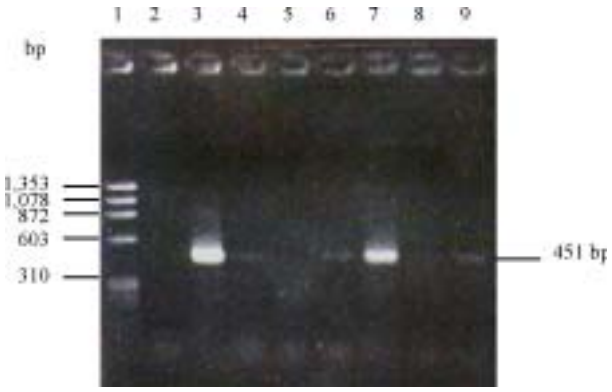

Fig 1–PCR amplification of a densovirus specific amplicon using DNA template isolated from *Toxorhynchites splendens* mosquitoes. The expected amplified product was approximately 451 bp. Lane 1: *Hae* III digested ΦX174 DNA marker. Lanes 2 and 3 are a negative reagent blank and *Aal*DNV DNA positive control, respectively. Lanes 4-9: PCR product from mosquito template DNA. The results shown are representative examples from 27 mosquitoes that showed similar PCR products.

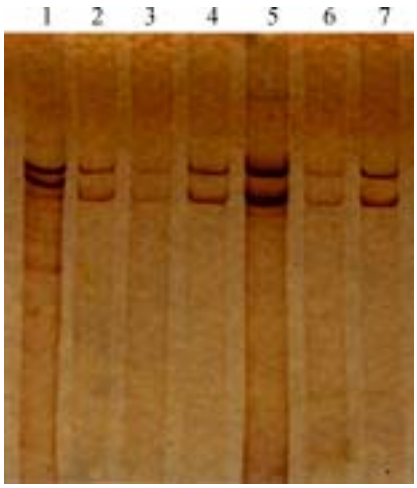

Fig 2–Single strand conformation polymorphism (SSCP) analysis of amplified PCR products from *Toxorhynchites splendens* mosquitoes. Lane 1 is PCR-SSCP from *Aal*DNV DNA is shown in lane 3 in Fig 1. Lanes 2-7 are PCR-SSCP from related mosquito specimens shown in lanes 4-9 in Fig 1. These again serve as representatives for the samples from 27 mosquitoes.

be homogeneous by SSCP (data not shown). Two representative PCR products from each species were shown to have identical DNA sequences.

Isolation of virion DNA from mosquitoes

Virion DNA isolated from the pool of *Tx. splendens* was shown to be 4.2 kb, which was the same size as the DNA isolated from *Aal*DNV. This closely resembled the size of densoviruses in the genus *Brevidensovirus* or *Contravirus* (Fig 3). Two other major bands of very small size (less than 500 bp) were also observed.

Nucleotide sequencing of the PCR fragment from *Tx. splendens*

The nucleotide sequence of 412 bp from the central region of the PCR product (excluding the primer sequences at each end of the 451 bp amplicon) were aligned with the mosquito densovirus sequences obtained from the GenBank database (Fig 4). There were 53 nucleotide substitutions when compared with *Aae*DNV and 78 nucleotide substitutions, 4 deletions and 1 insertion when compared with *Aal*DNV. This translates as 87.1% and 79.9% similarity in nucleotide sequences, respectively.

Phylogenetic analysis

A 301-bp portion of the nucleotide sequence of the amplified PCR product from *Tx. splendens* was compared with matching regions of other densoviruses obtained from the GenBank database and other published data. A phylogenetic tree generated from 1,000 replicates indicated that the new virus was more closely related to *Aedes aegypti* densovirus (*Aae*DNV) than to other insect densoviruses observed in cultured cell lines (Fig 5).

Cultivation of densovirus in cell lines

Attempted culture of the densovirus in mosquito cell lines using inoculum prepared from *Tx. splendens* homogenates was performed in both *Ae. albopictus* (C6/36) cells and *Tx. amboinensis* (TRA284) cells. Serial passage was confirmed by PCR using extracts from culture supernatant as the template. The PCR results were positive for only the first 4 passages of cultivation and negative thereafter (data not shown).

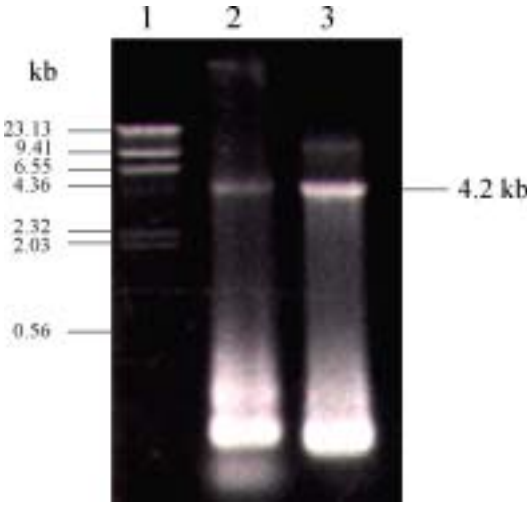

Fig 3–Agarose gel electrophoresis of densovirus DNA isolated from *Toxorhynchites splendens* mosquitoes. Viral DNA was isolated from a pool of 60 *Toxorhynchites splendens* mosquitoes and 3 μl of the DNA solution was subjected to 1% agarose gel electrophoresis. Lane 1: *Hind* III digested λ DNA marker. Lane 2 shows the 4.2 kb DNA band from the pooled mosquito extract. Lane 3 is the 4.2 kb DNA band from *Aal*DNV-infected culture supernatant which served as a size reference for DNA isolated from viruses of the genus *Brevidensovirus* or *Contravirus*. The 2 bands of small DNA fragments at around 500 bp have not yet been identified but may be residual host DNA remaining in the viral DNA preparation.

DISCUSSION

Densoviruses have been identified and isolated from mosquito larvae and cell lines for many years (Lebedeva *et al,* 1973; Buchatsky, 1989; Boublik *et al,* 1994b; O'Neill *et al,* 1995; Burivong *et al,* 2004; Chen *et al,* 2004; Paterson *et al,* 2005). Some densovi-

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TsDNV       AGGAACAACA AACCAAACAA CATGGGACTT TGAAACAAGT CAAAACATGT   3038
AaeDNV      G..G..... ........T. .......... .......... ..........
AalDNV      G......... ..T....... .T........ C......... ..........

            TCATCGCAGA TGCAGACAGA GAACCAGAAA ACTTCAACTT AACAACAGCA   3088
            .......... .......... .......... .T........ GG........
            .......... C......... .....G.... .T......C. .GA.....TT

            GCAGCAACTG GACCACTTGC ACAGCAAACA GCACAAACAT TACTTTTCAA   3138
            .......... .......... ...A...... A.........C ....A.....
            ........C. .....T.A.. ...A...... A.......... ....A.....

            TGCAAACAAT GACAGATATA CAAAATATGA ATTACCACAA AGAAACCAAT   3188
            .........C .......... .......... .......... ........G.
            .T.TC..... ........C. .......C.. .C........ ..G.......

            ATACAAGAGA AATTGACTTC CAACAACTAA CAAACAATTA TATGTGGAAA   3238
            .......... .TA....... ........T. ....T..C.. C.........
            .......G.. ...CA..... ..G...T... ....T..C.. ........G.

            CCATTGGACA TCAGCGCTGC AACAAACTTT AGAAAATTAA TCCCAATGGC   3288
            ...ACA.... .T........ .G........ ....G...G. ..........
            .....A..T. .....AAA.A ..........C ...TCCC... .T......T.

            AGAAGGAATA TACACAACAT CCAATGCTAC AAGTGATATG ACCGAATTAA   3338
            G......G.. ..T......A .AGCG..A.. ..C.A.A... G.A.......
            .......G.. ..T....A.. .GG-A-GA.. TT.A..C-AA ..G.....C.

            CACAACAAAA TACAACATGG GCCACATCAG GAAAAACAAC CCAAGCAACA   3388
            ..G....... AT..GT..AT ..AGG..... .C........ AG.....T..
                                                          C
                                                          |
            ..T.TG...C ........AT .....G.... ..GC....-A .G.G.A...C

            CTATTCAGAA AT   3400
            .......... ..
            ........G. .C
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Fig 4–Nucleotide sequence of the PCR amplicon from the new densovirus isolated from *Toxorhynchites splendens* mosquitoes (*Ts*DNV). Shown is the 412 bp nucleotide sequence of the 451 bp amplicon with the primer sequences removed from each end. It is aligned with sequences of *Aae*DNV (M 37899) and the *Aal*DNV (X 74945). Only the nucleotide residues of *Aae*DNV and *Aal*DNV that are different from *Ts*DNV sequence are shown. The nucleotide position numbers of the *Aae*DNV gene are presented at the right side. Dots indicate positions where sequences are the same as in *Ts*DNV. Nuceleotide deletions and insertions are represented as dashes and as base letters over the sequence, respectively. The nucleotide sequence of *Ts*DNV shown is the representative sequence of 5 separate amplicons derived by PCR from different individual mosquitoes.

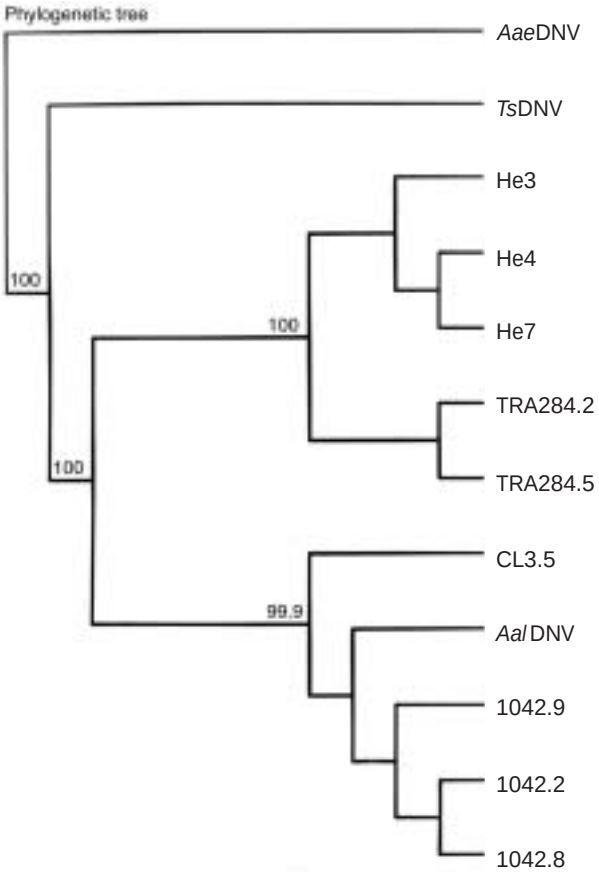

Fig 5–Phylogenetic tree analysis of *Ts*DNV and other densoviruses found from mosquitoes. The phylogenetic tree is based on a comparison of a 301-bp portion of the sequence shown in Fig 4. It was analyzed using PHYLIP package version 3.57c and shows the relationship of *Ts*DNV to other described densovirises. The sources of densoviruses were *Aae*DNV (M 37899) and *Aal*DNV (X 74945) from the GenBank database and others from mosquito densoviruses from cell lines including clones 1042.2, 1042.8 and 1042.9 from *Aedes aegypti* cell line 1042; CL3.5 from *Culex theileri* cell line CL3; He3, He4 and He7 from *Haemagogus equinus* cell lines; and TRA284.2 and TRA284.5 from *Toxorhynchites amboinensis* cell lines (O'Neill *et al,* 1995). Numbers above the branches refer to the percentage of bootstrap values for 1000 replicates and only the percentage over 50% is indicated.

ruses have been described as widespread in mosquito cell lines distributed in several laboratories (Boublik *et al,* 1994b; O'Neill *et al,* 1995; Chen *et al,* 2004; Paterson *et al,* 2005), suggesting that chronic infection is a common phenomenon that results from contamination by culture manipulation and by exchanges between laboratories. Densoviruses have been reported in laboratory strains of *Cx. pipiens* (Jousset *et al,* 2000) and in indigenous *Ae. aegypti*, *Ae. albopictus* and *An. minimus S.L.* from Thailand (Kittayapong *et al,* 1999; Rwegoshora *et al,* 2000).

The virus we describe here was found unexpectedly in laboratory strains of *Tx. splendens.* This mosquito colony had been used extensively for isolation of dengue virus by inoculation with serum from hospital patients as a standard method for routine virologic confirmation of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) cases (Rosen and Gubler, 1974; Gubler *et al,* 1979; Sumarmo *et al,* 1983; Vaughn *et al,* 1997). The virus was discovered using primers based on conserved densovirus nucleic acid sequences and its uniformity demonstrated by SSCP. Thus, these techniques might be adopted as appropriate techniques in broader screening for the prevalence of densoviruses in indigenous mosquitoes.

Our original inclination was to suspect that *Tx. splendens* had become infected with a densovirus derived from experimental cell lines. However, results from DNA analysis showed that this was not the case, since its DNA sequence differed mostly from the viruses associated with cell lines. It was closer to sequences of viruses derived from captured mosquitoes, suggesting that it came with the original *Tx. splendens* isolate or from contact with other mosquitoes. Indeed, the *Tx. splendens* colony was maintained by feeding with larvae of *Ae. aegypti* or *Cx. quinquefasciatus* and it is possible that they could have been the source of the original infection.

The fact that the virus in all the sampled mosquitoes was identical and that positive PCR results were uniformly obtained within the laboratory for a span of 2 years, suggests the virus had been maintained in the laboratory by horizontal, venereal or vertical transmission throughout the period. Although *Tx. splendens* was found to be infected by predated *Ae. aegypti* larvae in the laboratory, these two mosquito species are also found together in the same breeding places in nature. Unfortunately, the new densovirus did not replicate well in the mosquito cell lines used. It may require mosquito larvae or adults for good replication or it may require primary cultures derived from a specific mosquito. This phenomenon was described previously for its failure to cultivate *Cx. pipiens* densovirus beyond the second passage in *Ae. albopictus* (C6/36), *An. gambiae*, *Cx. quinquefasciatus*, *Cx. tarsalis*, *Drosophila melanogaster* dipteran cells and *Spodoptera littoralis* (SPC SL52) lepidopteran cells (Jousset *et al,* 2000). As far as we could determine, this densovirus did not have any pathological effect on its host during observation period of 2 years. This phenomenon may be similar to that of infectious hypodermal and hematopoietic necrosis virus (IHHNV) that can survive and replicate in some species and varieties of shrimp without noticeable harmful effect (Shike *et al,* 2000).

Many arthropod-borne viral diseases, such as dengue virus, Japanese encephalitis virus, West Nile virus and St. Louis encephalitis virus are transmitted by mosquitoes and cause serious illness in humans (Gubler, 1996; Rigau-Perez *et al,* 1998). Thus, control of these diseases has relied on several intervention strategies that focus on infected mosquitoes. Densoviruses are expected to offer possibilities as potential tools for biological control of arthropod-borne diseases based on experiments performed by several investigators (Belloncik, 1990; Kimmick *et al,* 1998; Afanasiev *et al,* 1999; Allen-Miura *et al,* 1999; Carlson *et al,* 2006). Before this goal can be achieved however, much more work is needed on the molecular biology, physiology and pathology of these viruses in their hosts.

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