COMPARISON OF MOSQUITO DENSOVIRUSES: TWO CLADES OF VIRUSES ISOLATED FROM INDIGENOUS MOSQUITOES

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Abstract. We analyzed the phylogenetic tree of densoviruses isolated from indigenous mosquitoes and mosquito cell lines. Our findings suggest two distinct clades of densovirus. The viruses in the first clade were isolated from an indigenous mosquito which had the *Aedes aegypti* densovirus (*Aae*DNV) as a representative virus. The other clade of viruses was isolated from mosquito indigenous cell line which had the *Aedes albopictus* densovirus (*Aal*DNV) as the representative virus. The origin of the two clades of DNVs is unclear but the phylogenetic trees were significantly different from each other. The two major densoviruses, *Aae*DNV and *Aal*DNV, that infect mosquitoes that are known to carry viruses responsible for dengue hemorrhagic fever and yellow fever. Understanding the evolution of these two clades of densoviruses is important for studying the distribution of these viruses in mosquito cell lines and the information gained may be applied to understanding other viruses in various mosquito cell lines.

Keywords: densovirus, mosquito, cell line

INTRODUCTION

The family Parvoviridae is composed of two subfamilies: Parvovirinae and Densovirinae. Parvovirinae viruses infect vertebrates while Densovirinae viruses infect invertebrates, mostly insects (Kurstak and Small, 1972; Bachmann *et al*, 1975; Siegl *et al*, 1985). Densovirinae is composed of three genera: *Densovirus* (infecting cockroaches), *Iteravirus* (infecting silk worms) and *Brevidensovirus* or *Contravirus* (infecting mosquitoes). The latter genus is comprised of the *Aedes aegypti* densovirus (*Aae*DNV) and the *Aedes albopictus* densovirus (*Aal*DNV), as well as several other densoviruses that have been reported in various mosquito species, including *Culex pipiens, Toxorhynchites splendens* and *Haemagogus equinus* (O'Neill *et al*, 1995; Pattanakitsakul *et al*, 2007; Zhai *et al*, 2008).

Densovirus is small, non-enveloped icosahedral virus 18-20 nm in diameter containing single-stranded DNA of 4.0-4.2 kb (Afanasiev *et al*, 1991; Jousset *et al*, 1993; Boublik *et al*, 1994). The genome contains palindromic structures at both termini

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which can form stable hairpin structures and has been suggested to play a role in DNA replication (Afanasiev *et al*, 1991, 1994; Boublik *et al*, 1994). The DNV genome encodes for 3 open reading frames (ORF) on the plus strand with the left and mid ORFs encoding for non-structural proteins (NS) and the right ORF encodes for structural proteins. However, the *Aae*DNV, has an extra ORF in the minus strand of the genome that encodes for a protein of unknown function. In the mosquito, the DNV genome can be encapsidated mostly in the minus strand and is packaged in their virions (Afanasiev *et al*, 1991; Boublik *et al*, 1994).

*Aae*DNV has been suggested as a model for developing biological control of mosquitoes because of the small genome size and the ease of cloning and propagation in expression vectors (Jousset *et al*, 1990; Dumas *et al*, 1992; Giraud *et al*, 1992). The *Aae*DNV is specific for mosquitoes and has not been reported to be harmful to humans.

We report here a phylogenetic tree analysis of the available reported mosquito DNVs.

MATERIALS AND METHODS

Phylogenetic analysis of the mosquito DNV genome

The nucleotide sequences of the densoviruses (DNVs) derived from various mosquitoes (*Aedes, Culex, Haemagogus, Toxorhynchites*) and a mosquito cell line (C6/36) was obtained from the GenBank database and other references. These DNV sequences were for: *Aal*DNV-1 (X 74945), *Aal*DNV-2 (AY 095351), *Aal*DNV-3 (AY 310877), *Aae*DNV (M 37899), *He*DNV (AY 605055) and *Cpp*DNV (EF 579771) (from GenBank) and *Aal*DNV-4 from Sangdee and Pattanakitsakul, (2012). Multiple sequence alignment (MUSCLE) was used for sequence comparisons, and molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods (MEGA5) were employed to assess these sequences and to calculate the evolutionary distances. The phylogenetic trees were constructed using the Neighbor-Joining method with 1000 bootstrap replications. The evolutionary distances were compared using the Kimura 2-parameter method and are reported as of the number of base substitutions per site.

Preparation of densovirus stock in the *Aedes albopictus* cell line (C6/36)

The densovirus stock was prepared in a T-75 flask by infecting 10^7 C6/36 cells with 5 ml of viral supernate, which had been maintained in our laboratory and kept at -86°C. The stock was kept at room temperature overnight before further cultivation by adding 10 ml of fresh Leibowiz's medium (L-15) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) and 10% tryptose phosphate broth (TPB). This was maintained for 7 days at 28°C. After 7 days the supernatant was collected for western blot analysis of the antigenicity of the DNV.

SDS-PAGE and western blot analysis

DNV-infected C6/36 cells ($1x10^5$) were prepared in the same way as the preparation of the virus stock. Seven days post-infection with the DNV the infected cells were suspended in 100 l PBS and used for analysis concentration of 10% SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane (0.45 m, Sartorius, Goettingen, Germany) using a semi-dry blotter (Amersham Biosciences, Sandiego, CA) then a current (0.8 mA/cm^2) was applied for 1 hour. The membrane was blocked with 5% skimmed

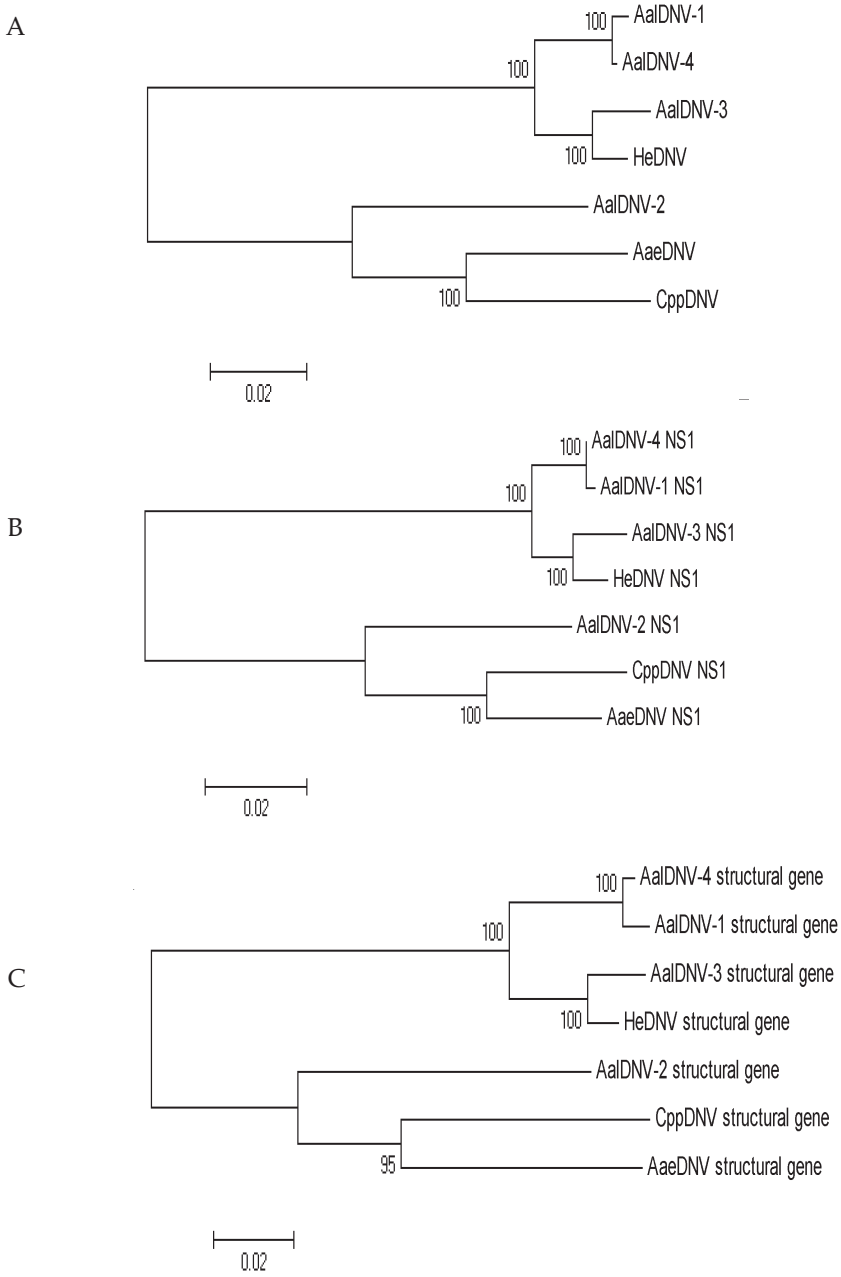

Fig 1–Phylogenetic tree analysis of mosquito densoviruses (DNVs). The phylogenetic tree is based on a comparison of a 3.9-kb sequence of *Aal*DNV-4 between nucleotide 113 and 4025 (A) or a non-structural gene between nucleotide 290 and 2707 (B) or a structural gene between nucleotide 2575 and 3666 (C). It was analyzed with the Molecular Evolutionary Genetic Analysis program using maximum likelihood, evolutionary distance, and maximum parsimony methods. The sources of the DNVs were *Aal*DNV-1 (X 74945), *Aal*DNV-2 (AY 095351), *Aal*DNV-3 (AY 310877), *Aae*DNV (M 37899), *He*DNV (AY 605055) and *Cpp*DNV (EF 579771) from GenBank and the *Aal*DNV-4 (Sangdee and Pattanakitsakul, 2012). Numbers above the branches refer to the percentage of bootstrap values for 1,000 replicates.

Table 1
Source and origin of densovirus sequence.

Abbreviation	Full name	Genome size (kb)	Source	GenBank accession number	References
*Aal*DNV-1	*Ae. albopictus* densovirus 1	4.2	C6/36 cell line	X 74945	Boublik *et al*,1994
*Aal*DNV-2	*Ae. albopictus* densovirus 2	4.1	C6/36 cell line	AY 095351	Chen *et al*, 2004
*Aal*DNV-3	*Ae. albopictus* densovirus 3	4.1	C6/36 cell line	AY 310877	Paterson *et al*, 2005
*Aal*DNV-4	*Ae. albopictus* densovirus 4	4.2	C6/36 cell line	-	Sangdee and Pattanakitsakul, 2012
*He*DNV	*Haemagogus equinus* densovirus	4.0	GML-HE-12 cell line	AY 605055	O'Neill *et al*, 1995 Paterson *et al*, 2005
*Aae*DNV	*Ae. aegypti* densovirus	4.0	*Ae. aegypti* larvae	M 37899	Afanasiev *et al*, 1991
CppDNV	*Culex pipiens pallens* densovirus	3.3[a]	*Culex pipiens quinquefasciatus* mosquito	EF 579771	Zhai *et al*, 2008

[a]The genome size was reported only coding region of non-structural and structural genes.

milk in PBS for 1 hour at room temperature, then incubated with rabbit polyclonal antibodies against the structural protein of *Aal*DNV at a dilution of 1:100 in blocking buffer for 2 hours at room temperature as described previously (Sangdee and Pattanakitsakul, 2012). The nitrocellulose membrane was then incubated with swine anti-rabbit immunoglobulin conjugated with horseradish peroxidase at a dilution of 1:1,000 for 1 hour at room temperature. The chromogenic substrate of 0.4% 3, 3' diaminobenzidine (Sigma, St Louis, MO) was added to develop the enzymatic reaction for 5 minutes. The reaction was stopped by briefly rinsing the membrane in distilled water. A positive reaction appeared as a dark brown color on the membrane.

RESULTS

Phylogenetic analysis of mosquito densoviruses

To determine whether the mosquito DNVs were obtained by the isolation of different host indigenous mosquitoes and mosquito cell lines, the phylogenetic tree was analyzed using the alignment of the whole sequence: non-structural and structural sequences of the DNVs. The sources and origin of these DNVs are shown in Table 1. The 3 phylogenetic analyses had similar patterns that could be categorized into two clades (Fig 1 A-C). The first clade consisted of *Aal*DNV-1, *Aal*DNV-4, *Aal*DNV-3 and *He*DNV, and the second clade consisted of *Aal*DNV-2, *Aae*DNV and *Cpp*DNV (Fig 1 A-C). We used *Aal*DNV as a

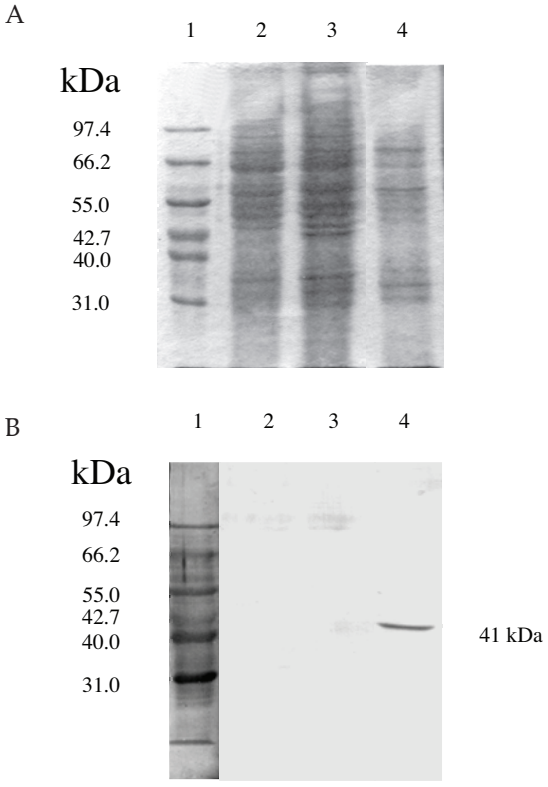

Fig 2–Western blot analysis of the *Aal*DNV structural protein. *Aae*DNV- and *Aal*DNV-infected C6/36 cell lysates were analyzed with 10% SDS-PAGE and proteins were stained with Coomassie blue (A) or reacted with rabbit immune serum against a 41 kDa structural protein of *Aal*DNV (B). Lane 1 is a mid-range standard protein marker. Lanes 2-4 were mock-, *Aae*DNV-, and *Aal*DNV-infected C6/36 cell lysates, respectively.

representative of virus in the first clade which was isolated from the mosquito cell line, and *Aae*DNV was representative of the second clade, which was isolated mostly from indigenous mosquitoes.

Determination of reactivity against structural proteins of the *Aal*DNV-infected C6/36 cell line

To determine whether the rabbit immune serum against the structural protein of *Aal*DNV can cross-react with another clade of densovirus, C6/36 cells infected with either *Aal*DNV or *Aae*DNV were processed for western blot analysis using rabbit antiserum against the structural protein of *AalDNV* (Sangdee and Pattanakitsakul, 2012). A major protein band at 41 kDa was observed only in the C6/36 cells infected with *Aal*DNV, but no protein was found in the mock and *Aae*DNV-infected cells (Fig 2).

DISCUSSION

The mosquito DNV is interesting for its potential to be used as a vector to introduce foreign genes into a mosquito, to express foreign proteins in mosquito cells and to target the product to the desired cell compartment (Afanasiev *et al*, 1994, 1999; Allen-Miura *et al*, 1999). This virus has also been used for vector control due to its compact size, relative ease of handling and genetic manipulation. Since the report of the first DNV in 1973 (Lebedeva *et al*, 1973), various DNVs have been described and isolated from different mosquitoes and cell lines by investigators worldwide (Afanasiev *et al*, 1991; Boublik *et al*, 1994; O'Neill *et al*, 1995; Chen *et al*, 2004; Paterson *et al*, 2005; Zhai *et al*, 2008; Sivaram *et al*, 2010; Sangdee and Pattanakitsakul, 2012). This virus is known only to infect mosquitoes and has not been reported to infect humans. DNVs have distinct amino acid sequences specific to the host infected (Roekring *et al*, 2002). Mosquito DNVs are helpful for examining the evolution patterns of microorganisms. The genome of DNVs is comprised of non-structural and structural regions that can be categorized into two distinct clades of virus depending on their origin of isolation, except for *Aal*DNV-2 which is similar to *Aae*DNV.

The similarity between *Aal*DNV-2 and *Aae*DNV not only includes nucleotide sequence homology but also similar genome organization (Paterson *et al*, 2005). Members within the *Aal*DNV clade had more than 95% homology, whereas those within the *Aae*DNV clade had 80% homology (Chen *et al*, 2004). *Aal*DNV-2 is suspected of having a common shared ancestor with *Aae*DNV rather than *Aal*DNV (Chen *et al*, 2004; Paterson *et al*, 2005). Another possibility is that this virus adapted to selective pressure and underwent independent evolution in chronically infected C6/36 cells, which caused it to become similar to *Aae*DNV. Of the *Aal*DNVs studied, great similarity was found between *Aal*DNV-1 and *Aal*DNV-4 (Boublik *et al*, 1994; Sangdee and Pattanakitsakul, 2012). Similarity was also seen between *Aal*DNV-3 and *He*DNV. *Aal*DNV-3 was isolated from C6/36 mosquito cells while *He*DNV was isolated from the GML-HE-12 cell line (Table 1). The C6/36 cell line is commonly used in laboratories to propagate flaviviruses, such as dengue virus and Japanese encephalitis virus while GML-HE-12 is used in some laboratories (O'Neill *et al* 1995). The second clade virus is isolated from *Ae. aegypti* larvae, *Culex pipiens quinquefasciatus* mosquitoes and the C6/36 mosquito cell line. The *Aal*DNV-2 may be unique *Ae. albopictus* DNV, distinct from other *Aal*DNVs. The sizes of the DNVs genomes are not much different from each other except the *Cpp*DNV, which is slightly shorter than other DNVs; investigators have reported only non-structural and structural genomes, except for the non-coding region and hair-pin terminal structures (Zhai *et al*, 2008). The alignment of the non-structural sequence of DNVs which encode the 199-aa conserved region characteristic of the NTP-binding and helicase domains still has the same clade grouping (Boublik *et al*, 1994) (Fig 1B *vs* Fig 1A and Fig 1C). Western blot analysis also provided results of these two different clades of the *Aal*DNV and *Aae*DNV; no cross-reactive 41 kDa protein was observed when reacted with rabbit immune serum against the structural protein of *Aal*DNV. These results also suggest structural proteins of DNVs are less conserved between the different clades. The non-structural protein 1 (NS1) has been described as a more conserved region than structural protein region because this protein plays an important role in viral replication (Afanasiev *et al*, 1991).

*Aal*DNV has been demonstrated in C6/36 cells along with other viruses, *ie*, dengue or Japanese encephalitis viruses (Burivong *et al*, 2004; Kanthong *et al*, 2008, 2010). Phylogenetic studies of these mosquito DNVs may provide more information about the evolution of DNVs in indigenous mosquitoes and mosquito cell lines. This study suggests the presence of viruses in cell lines worldwide in laboratories. The DNV may become an important virus to explore molecular cloning, expression of foreign proteins and manipulation of genetically modified mosquitoes and could be applied to future vector control programs.

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