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Phylogenetic analyses of DENV-3 isolated from field-caught mosquitoes in Thailand

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ABSTRACT

Dengue virus serotype 3 (DENV-3) can cause all forms of dengue diseases and is a predominant serotype in many countries. This serotype is classified into five genotypes: I–V. Genotypes I–III have widely spread throughout the world, whereas genotypes IV and V are rare. Despite the impact on the spread of dengue diseases, only a few studies have reported the characteristics of DENV present in mosquito vectors. Hence, this study aimed to identify DENV-3 genotypes and reveal genetic variation of this virus presented in field-caught mosquitoes collected from endemic areas in Thailand during 2011–2015. First, we examined the effectiveness of the *E* gene sequence on DENV-3 genotyping, with results supporting the use of this gene for genotype identification. Then, we sequenced this gene in ten DENV-3 strains isolated from mosquitoes. The results showed that eight and two samples were genotypes III and V, respectively, and that they are closely related to DENV-3 isolated from Southeast and East Asian samples. The translated *E* gene sequences showed 25 unique amino acid (AA) residues located at 23 positions. Eight out of 25 residues have different chemical properties compared to the conserved AAs that are distributed across the three domains functioning in virus-host interaction. Hence, our study reports the first DENV-3 genotype V in Thailand, with these viruses potentially influencing both the disease severity and epidemic potential of DENV-3.

1. Introduction

Dengue disease is an important arboviral disease that is widely observed in urban areas both in tropical and sub-tropical countries (Regis et al., 2014). People living in over 100 countries are at risk of Dengue virus (DENV) infection (WHO, 2017). Over 3.2 million dengue cases across the Americas, Southeast Asia and Western Pacific were reported in 2015 and the numbers of dengue cases have continued to increase (WHO, 2017). In Asia, there are several dengue cases detected in Singapore after a lapse of many years and many outbreaks in Laos have been reported (WHO, 2017). Besides, dengue incidence in China has also shown to increase since 2014 (WHO, 2017). Moreover, not only dengue cases in endemic areas have been enhancing but the disease has also spread into new areas. In 2010, there was the first local transmission of the disease in France and Croatia (WHO, 2017). In 2012, several dengue cases were reported in Portugal mainland and other 10 European countries (WHO, 2017).

The disease can be classified according to the severities of symptoms; dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF patients generally present with mild symptoms, while DHF and DSS patients suffer from severe symptoms (WHO, 2017). These diseases are caused by DENV, which can be classified into four serotypes; DENV-1, DENV-2, DENV-3 and DENV-4 (Gubler, 1998). Disease severity is associated with various causes including secondary infection of different DENV serotypes (Karyanti et al., 2014).

Each DENV serotype has been further classified into various genotypes based on its genetic variation, which are mostly associated with geographic distribution (Jarman et al., 2008; Kanakaratne et al., 2009; Khan et al., 2013; Twiddy et al., 2002, 2003). Some studies reported an association between genetic variation and disease severity. For example, DENV-2 Southeast Asian genotype was found to be associated with DHF, while the American genotype associated with DF (Cologna and Rico-Hesse, 2003). DENV-3 genotype III strains belonging to the clade IIIA have been found to be associated with the spread of DF, while the strains belonging to the same genotype but a different clade (IIIB) coincided with a DHF outbreak in Sri Lanka (Hanley et al., 2008). Hence, genetic variation of DENV potentially determines disease severity and distribution of dengue diseases.

DENV genotypes have been determined based on phylogenetic trees

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reconstructed based on nucleotide sequences of a junction between genes, a particular gene or a complete coding region of the DENV genome (Domingo et al., 2006; Messer et al., 2003; Yamashita et al., 2013). The *E/NS1* gene junction, and the *E*, *NS3* and *NS5* gene sequences have been applied to build a phylogenetic tree for DENV genotype identification (Aquino et al., 2008; Barrero and Mistchenko, 2008; Idrees and Ashfaq, 2012; Rico-Hesse, 1990, 2007; Weaver and Vasilakis, 2009). Among these DENV genome regions, the *E* gene sequence has been widely used for DENV genotype identification (Guo et al., 2015; Hapuarachchi et al., 2016; Huang et al., 2012).

Southeast Asia is one of the most places in the world where dengue morbidity rate is very high and the incidence of dengue diseases has been constantly reported (WHO, 2017). In Thailand, all four DENV serotypes have been circulated and DENV-3 is one of the most prevalent serotypes which had predominated in the country during 1992–2000, 2010 and 2014 (Messina et al., 2014; MoPH, 2016; Murray et al., 2013). This serotype is classified into five genotypes; I–V (Monteil et al., 2016; Yamashita et al., 2013). Genotype III is generally the most prevalent, and has been widely spread throughout the world (Hanley et al., 2008; Kanakaratne et al., 2009; Yamashita et al., 2013). However, this genotype is rarely observed in Thailand, with only five samples being detected in 2010 and 2013 (Huang et al., 2012; Monteil et al., 2016). Typically, genotypes I and II have been circulating in Thailand, with the latter predominating in this country since 1973 (Huang et al., 2012; Klungthong et al., 2008; Wittke et al., 2002).

Dengue incidence in Thailand has been reported by Bureau of Epidemiology in the Department of Disease Control, Ministry of Public Health since 1971 (MoPH, 2016). During 1971–2015, dengue outbreaks had occurred several times and the highest morbidity rate was found in 1987 (325.13 per 100,000 population) followed by those in 2013 (239.51), 2001 (224.43), 2015 (223.28) and 1998 (221.42), respectively (MoPH, 2016). In 2013 and 2015, the dengue outbreaks caused 154,773 and 146,082 dengue cases which 67,105 and 59,088 cases were DHF/DSS patients (MoPH, 2016).

Despite the impact on the spread of dengue diseases, the characteristics of DENV present in field-caught mosquitoes have rarely been reported. Hence, this study aimed to identify genotypes and disclose genetic variation of DENV-3 present in field-caught *Aedes aegypti* mosquitoes collected from endemic areas in Thailand during 2011–2015. Information regarding DENV genotype, the evolutionary relationship between our samples and previously reported samples and the consequence of replacement mutations in the *E* gene are reported in this study. These data would be useful for designing a dengue prevention and control strategy, especially in Thailand.

2. Material and methods

2.1. Collection of Aedes aegypti mosquitoes, RNA extraction and DENV detection

Aedes aegypti mosquitoes were caught using hand nets from endemic areas in Thailand during 2011–2015. They were kept in test tubes and frozen at −20 °C for 30 min then species and sex of the mosquitoes were identified based on their morphology (Huang and Rueda, 2014). Single or 3–10 mosquitoes were transferred into 1.5-mL tubes containing RNA*later* RNA stabilization reagent (Qaigen, Germany) and subsequently kept in −80 °C until used. For pooled samples, only mosquitoes collected in the same date and the same area were pooled together.

Legs and wings of each female *Aedes aegypti* mosquito were removed and RNA was extracted from each mosquito sample using an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Then, the first-strand cDNA was generated using the SuperScript® III First-Strand synthesis system (Invitrogen™, USA) with dengue-specific primers (Chin-inmanu et al., 2012). DENV in the field-caught mosquitoes was detected following the method of Lanciotti et al. (1992). The positive samples were further applied for serotype identification using a nested-PCR method (Lanciotti et al., 1992). The cDNAs of all DENV-3 positive samples were used in further experiments.

2.2. Amplification and sequencing of whole DENV genome and complete E gene

An entire DENV genome of a single mosquito sample named LRI18-15 was amplified using two primer pairs to produce two overlapped amplicons; 5′UTR-*NS4A* (approximately 6600 bp) and *NS4A*-3′UTR (approximately 4300 bp) fragments (Chin-inmanu et al., 2012). The complete *E* gene of all ten DENV-3 samples (eight single and two pooled mosquito samples) including LRI18-15 were amplified using two serotype-specific primers composed of a forward primer named EGENE3-SS and a reverse primer named EGENE/NS1-RR (Domingo et al., 2006). Each amplicon was visualized using 1.5% agarose gel electrophoresis and purified using a GF-1 AmbiClean Kit (Vivantis, Malaysia). The purified amplicons of the sample LRI18-15 were used for constructing a paired-end library and subsequently sequenced using Illumina Hiseq (Beijing Genomics Institute, China). For the complete *E* gene, each purified amplicon was sequenced by the Sanger sequencing method (Macrogen, South Korea).

2.3. Sequence analysis

The quality of paired-end reads obtained from Illumina Hiseq was evaluated by FastQC (Andrews, 2010). Low quality bases were removed by Trimmomatic (Bolger et al., 2014). All qualified paired-end reads were mapped to a DENV-3 reference sequence (NC_001475.2) using BWA (Li and Durbin, 2009) and a consensus sequence was calculated by Samtools (Li, 2011; Li and Durbin, 2009). The consensus sequence was annotated by performing local pairwise alignment with the reference DENV-3 sequence (NC_001475.2) using MAFFT version 7 (Katoh et al., 2002).

The complete *E* gene sequences of ten samples including the LRI18-15 were obtained by the Sanger sequencing method; then, they were edited and assembled using SeqTrace version 0.9.0 (Stucky, 2012). These complete sequences were annotated by comparing to GenBank database sequences using blastn. Regarding the sample LRI18-15, the *E* gene sequence extracted from the complete coding sequence was aligned to the complete *E* gene sequence obtained from Sanger sequencing in order to verify the consensus sequence generated from next-generation sequencing data.

Both the nearly complete genome and the complete *E* gene sequences obtained from this study were submitted to the GenBank database at the National Center for Biotechnology Information (NCBI) and their accession numbers are shown in Table 1.

2.4. Phylogenetic analysis

In order to examine the reliability of using complete *E* gene sequences to identify DENV-3 genotypes, each gene tree that was calculated from each gene in the DENV-3 genome were compared to a supermatrix tree that was calculated from a complete coding sequence of the DENV-3 genome. We firstly collected 850 complete DENV-3 genome sequences, with their genotypes having been defined from the GenBank database. These database sequences are composed of four DENV-3 genotypes: I, II, III, and V. Sequences of each gene were extracted from these database sequences and our DENV-3 complete sequence (LRI18-15). Multiple sequence alignment was performed for each gene using MAFFT version 7 (Katoh et al., 2002). The aligned sequences of every gene were concatenated to generate an alignment of complete coding sequences of DENV-3. The aligned sequences were subsequently applied to perform a model test and a phylogenetic tree calculation using functions in MEGA7 (Kumar et al., 2016). In the case of these 851 sequences, both supermatrix and gene trees were calculated based on the

Table 1
Information of DENV-3 isolated from field-caught mosquitoes (*Aedes aegypti*) in the present study.

Sample	Collection place	Collection date	DENV region	Accession no.
BKK21-11	Bangkok	June 2011	*E* gene	KY234180[a]
NBI17-11	Nonthaburi	June 2011	*E* gene	KY234181[a]
SKA1-12	Songkhla	December 2012	*E* gene	KY234172[b]
SKA5-12	Songkhla	December 2012	*E* gene	KY234173[b]
LRI2-15	Lop Buri	September 2015	*E* gene	KY234174[b]
LRI8-15	Lop Buri	September 2015	Complete coding region	MF142763[b]
BKK6-15	Bangkok	October 2015	*E* gene	KY234176[b]
BKK12-15	Bangkok	October 2015	*E* gene	KY234177[b]
BKK7-15	Bangkok	October 2015	*E* gene	KY234178[b]
BKK13-15	Bangkok	October 2015	*E* gene	KY234179[b]

[a] DENV-3 isolated from a pooled sample containing five mosquitoes.
[b] DENV-3 isolated from a single mosquito.

neighbor-joining method with the TN93 + G model (Tamura and Nei, 1993) and 1000 bootstrap replicates. Finally, each gene tree was manually compared to the supermatrix tree.

A total of 1690 complete *E* gene sequences of DENV-3 genotypes I, II, III, IV and V were retrieved from the GenBank database. These database sequences and our ten complete *E* gene sequences were aligned using MAFFT version 7 (Katoh et al., 2002). The aligned sequences were applied for model selection and phylogenetic tree reconstruction using functions in MEGA7 (Kumar et al., 2016). The phylogenetic tree of this large dataset was built by the neighbor-joining (NJ) method with the TN93 + G model and 1000 bootstrap replicates.

The evolutionary relationship between our DENV-3 samples and previously reported samples was verified by phylogenetic analysis of 145 complete *E* gene sequences; 10 sequences obtained from this study and 135 database sequences selected from the neighbor-joining tree of 1700 sequences. These database sequences were selected because they are closely related to our samples. The phylogenetic trees of this small dataset were drawn based on maximum likelihood (ML) and Bayesian methods with the GTR + G model. The ML tree was constructed using raxmlGUI v. 1.31 and the optimal tree was selected by the ML + rapid bootstrap method with 1000 bootstrap replicates (Stamatakis, 2014). The Bayesian tree was constructed using MrBayes v. 3.2.6 with 1,000,000 generations (Huelsenbeck and Ronquist, 2001).

2.5. Amino acid sequences analysis

Nucleotide sequences of 1700 complete *E* gene sequences were translated into amino acid sequences based on a standard codon table using Aliview (Larsson, 2014). Polymorphic sites were deduced from the amino acid sequence alignment. Only amino acid changes in our samples were reported in this study.

3. Results

Ten DENV-3 strains were isolated from ten samples of field-caught *Aedes aegypti* mosquitoes that were collected from dengue epidemic areas in Thailand during 2011–2015. A nearly complete DENV-3 genome sequence containing partial UTRs and complete coding sequence was obtained from the sample named LRI8-15 collected from Lop Buri province. The complete *E* gene sequence was obtained from all DENV-3 positive samples (Table 1).

3.1. Evaluating the efficiency of using a complete E gene sequence for genotype identification

One DENV-3 complete coding sequence isolated in this study and 850 complete coding sequences retrieved from GenBank were applied to reconstruct evolutionary relationships among genotypes I, II, III and V. The phylogenetic tree of the complete coding sequences, the supermatrix tree, showed four monophyletic clades corresponding to four genotypes included in this analysis. All these four clades were strongly supported by bootstrap values (Fig. 1). Genotypes I, II and V formed a monophyletic clade, while genotype III was separated from other genotypes. The genotype III clade could be separated into three subclades: the Asia, the Asia/Africa and the America/Africa subclade. Our DENV-3 sample named LRI8-15 was clustered into the Asia subclade and closely related to an Indian clinical sample isolated in 2005 (JQ922556). This supermatrix tree suggested that our sample (LRI8-15) belongs to genotype III.

The trees of each gene sequence, the gene trees, were compared to the supermatrix tree in order to examine the ability of each gene for identifying genotype and revealing relationships among genotypes. As illustrated in Fig. 1, every gene tree showed four monophyletic clades corresponding well with four genotypes. As in the supermatrix tree, these clades were strongly supported by bootstrap analysis. The comparisons between each gene tree and the supermatrix tree demonstrated that five gene trees generated from complete *C, E, NS2A, NS3* and *NS5* gene sequences showed the same topology as the supermatrix tree, with our sample (LRI8-15) in these five trees also being closely related to the Indian sample isolated in 2005 (JQ922556). Among these five gene trees, the trees of the complete *NS3* and *E* genes showed four clades of four genotypes with high bootstrap supports (95–100%). Hence, complete sequence of the *NS3* and *E* genes could be applied for genotype identification and representing the evolutionary relationships among genotypes of DENV-3.

3.2. Identifying DENV-3 based on the complete E gene sequence

The phylogenetic tree of the 1700 complete *E* gene sequences – 1690 database sequences and ten newly isolated sequences – was constructed using a neighbor-joining method. This large gene tree presented five monophyletic clades corresponding well with five genotypes: I, II, III, IV and V, included in this analysis (Fig. S1). These five clades were strongly supported by bootstrap analysis. Among ten DENV-3 isolated in this study, eight isolates: SKA1-12, SKA5-12, LRI2-15, LRI8-15, BKK6-15, BKK7-15, BKK12-15 and BKK13-15, were assigned to genotype III, while two isolates named BKK21-11 and NBI17-11 were assigned to genotype V. To verify the relationship of our ten samples and other DENV-3 previously reported samples, 135 isolates shown to be closely related to our samples in the large NJ tree of 1700 complete *E* gene sequences were applied to construct phylogenetic trees using maximum likelihood (ML) and Bayesian methods. Because both methods provided trees with the same topology, both bootstrap supports in the ML tree and posterior probability values in the Bayesian tree were reported on a single gene tree as shown in Fig. 2. This phylogenetic tree showed two monophyletic clades of genotypes III and V containing eight and two samples isolated in this study, respectively. All eight samples belonging to genotype III were clustered together into a monophyletic clade and equally related to other samples isolated during 2012–2013 in Asia including Thailand. The eight isolates were separated into three groups: Songkla, Lop Buri and Bangkok (BKK). In the first two groups, the complete *E* gene sequences of the members in each group were identical. In contrast, the sequences of all four members in the BKK group were difference and they were further separated into two sub-groups; BKK12-15/BKK13-15 and BKK6-15/BKK7-15 with high bootstrap supports. Two samples – BKK12-15 and BKK13-15–were of relatively long-branch length, indicating the relatively high genetic variation in these two samples. Our two samples belonging to

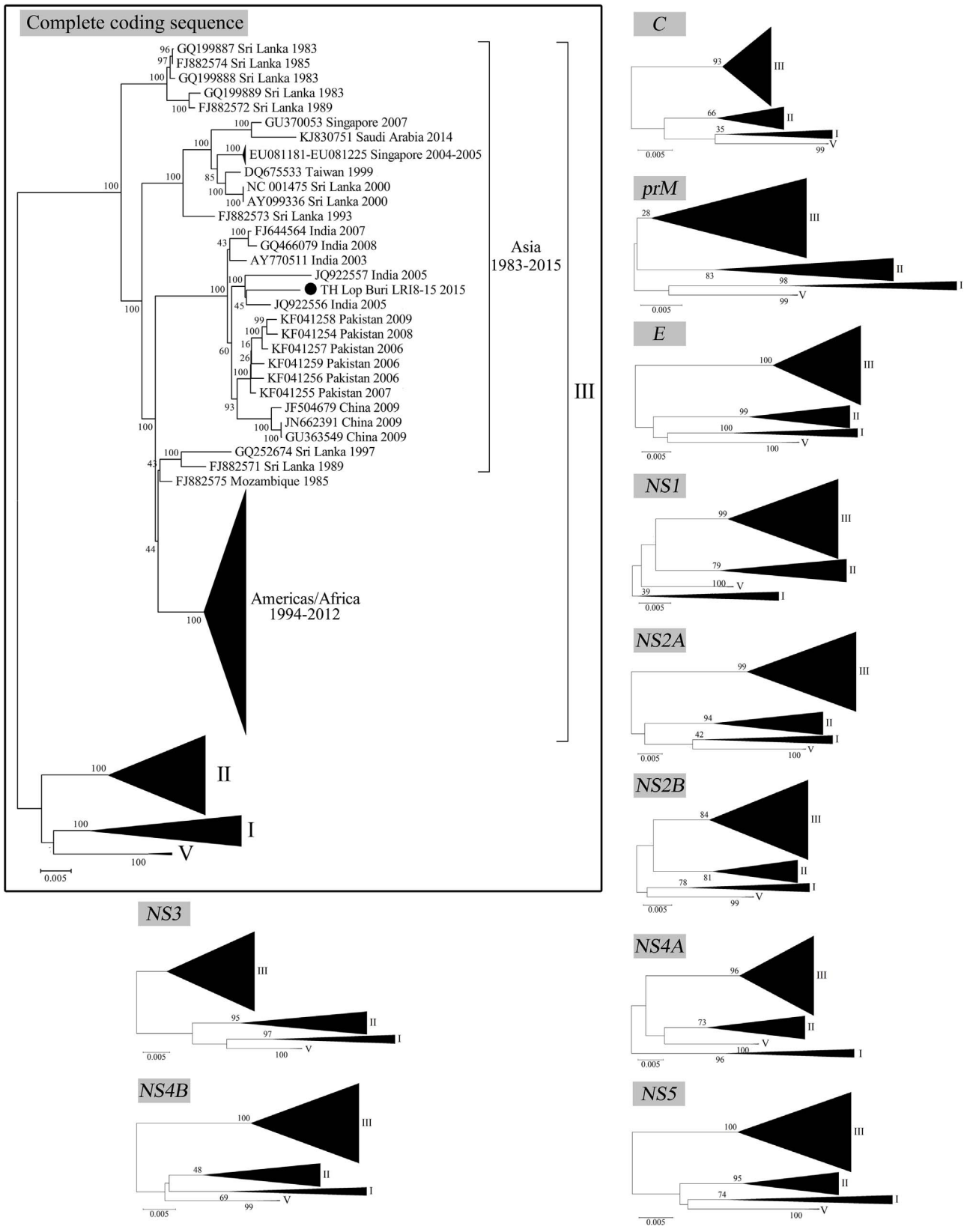

Fig. 1. Phylogenetic trees reconstructed from complete coding sequences and separated protein-coding genes: *NS3, NS4B, C, prM, E, NS1, NS2A, NS2B, NS4A* and *NS5* gene, of 851 samples. These trees were calculated using neighbor-joining method with the TN93 + G model. The complete coding sequence of our sample named LRI8-15 was represented by a black dot and the other 850 sequences were retrieved from GenBank database. The number on each branch represents a bootstrap support value calculated from 1000 replicates. Branch length represents the number of substitutions per site. Roman numbers: I–III and V, denote DENV-3 genotypes I–III and V, respectively.

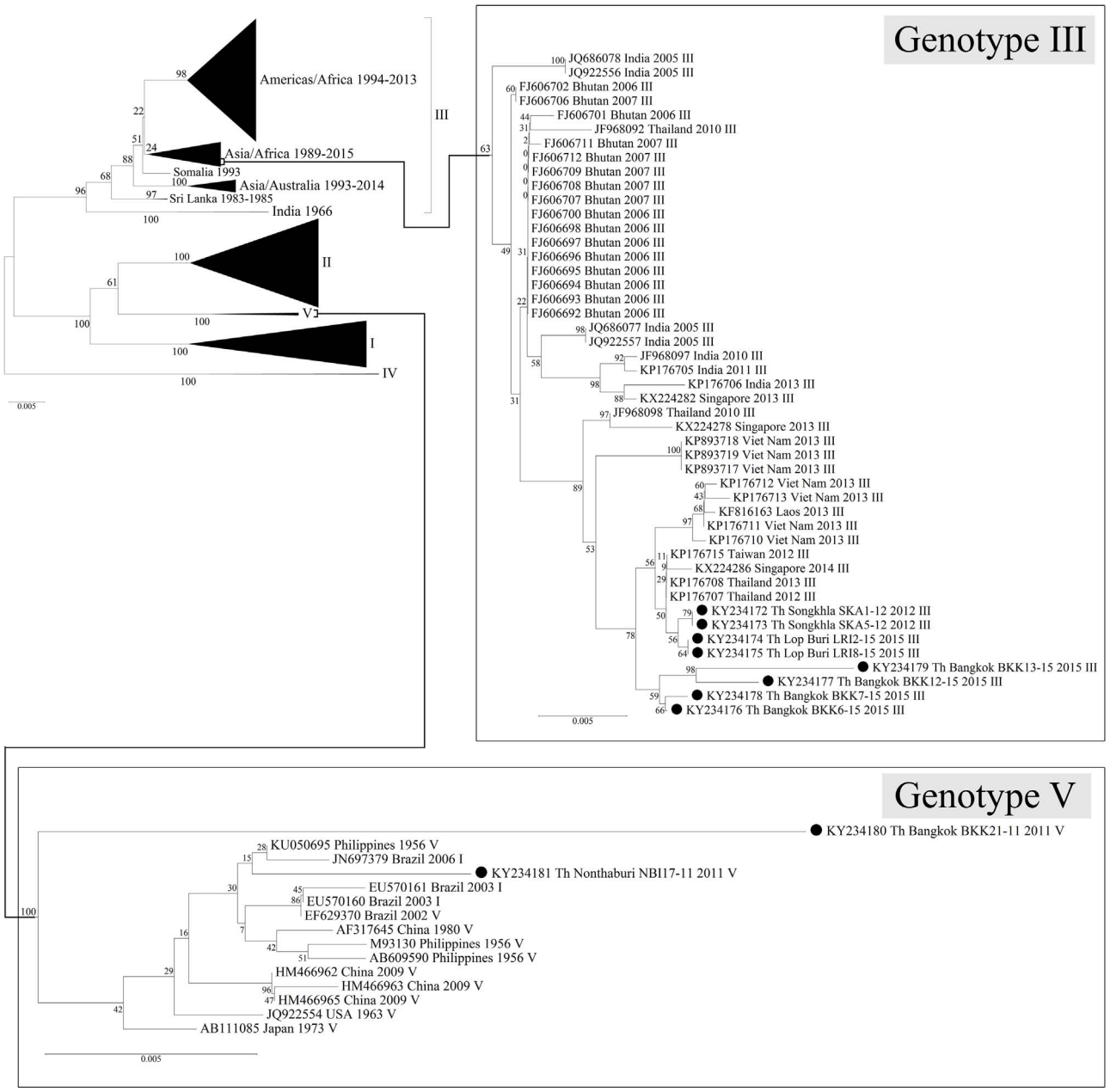

Fig. 2. A phylogenetic tree reconstructed from the complete *E* gene sequences of 145 samples carrying genotype III and V including our 10 samples represented by black dots. This tree was calculated using Maximum likelihood and Bayesian methods with the GTR + G model. The numbers presented above and below the branch represent a bootstrap support value calculated from 1000 replicates and a posterior probability value. Branch length represents the number of substitutions per site. Roman numbers: I–III and V, denote DENV-3 genotypes I–III and V, respectively.

genotype V were also clustered together in this gene tree and equally related to the first DENV-3 genotype V isolated in the Philippines in 1956 (KU050695). These three isolates were distinguished from other members of DENV-3 genotype V (Fig. 2). The character-based phylogenetic tree suggested the close relationship between our samples and previously reported samples isolated in Asia.

3.3. Observing the amino acid changes in DENV-3 isolated from field-caught mosquitoes

Based on the alignment of translated complete *E* gene sequences that included all genotypes, the pattern of amino acid (AA) changes observed in our samples was deduced as shown in Table 2. A total of 23 positions in our samples were shown to be different from 1690 previously reported sequences and generated 25 unique AA residues; 14 and 11 residues in genotype III and V samples, respectively. Six and four amino acids observed in our genotype III and V samples, respectively, were the consequence of alteration between hydrophobic and hydrophilic amino acids. These 25 AA changes are distributed across three domains of the envelope protein.

Regarding genotype III, amino acid changes were observed only in the samples collected from Bangkok named BKK7-15, BKK12-15 and BKK13-15. One amino acid change was observed in BKK7-15 and this change (E338V) alters the conserved hydrophilic AA to hydrophobic AA in domain III of the envelope protein. Many AA changes were observed in BKK12-15 and BKK13-15; however, no shared AA change was observed. Six AA changes cause alteration in AA properties from hydrophilic to hydrophobic or vice versa.

Regarding genotype V, three out of eleven AA changes: C105F, L107F and K232R, are shared between our two genotype V samples.

Table 2
Amino acid substitutions observed in the translated complete *E* gene sequences of DENV-3 collected in the study compared to those of all five genotypes retrieved from the GenBank database.

Domain	Genotype Sample No. sequences/position	I Multiple 264	II Multiple 503	III Multiple 906	III SKA1 – 12 [a] 1	III SKA5 – 12 [a] 1	III LRI2 – 15 [a] 1	III LRI8 – 15 [a] 1	III BKK6 – 15 [a] 1	III BKK12 – 15 [a] 1	III BKK7 – 15[a] 1	III BKK13 – 152 [a] 1	IV Multiple 4	V Multiple 13	V BKK21 – 11 [a] 1	V NBI17 – 11 [a] 1
II	105	C	C	C	C	C	C	C	C	C	C	C	C	C	F	F
II	107	L	L	L	L	L	L	L	L	L	L	L	L	L	F	F
II	112	S	I,F,S	S	S	S	S	S	S	S	S	S	S	S	T	S
II	116	C	C	C	C	C	C	C	C	C	C	C	C	C	F	C
II	117	A,T	A	A	A	A	A	A	A	A	A	A	A	A	P	G
II	125	I	G,I,R	I,V	I	I	I	I	I	I	I	I	I	I	K	I
II	126	E	E	E	E	E	E	E	E	E	E	E	E	E	G	E
II	132	H,Y	H,T,Y	H,Y	Y	Y	Y	Y	Y	Y	Y	Y	H	H	L	H
I	133	E	E	E	E	E	E	E	E	E	E	D	E	E	E	E
I	138	T	T	T	T	T	T	T	T	T	T	P	T	T	N	T
I	154	E	D,E	E,G	E	E	E	E	E	E	E	A	E,K	E	E	E
I	155	A,M,T	T	T	T	T	T	T	T	T	T	P	T	T	T	T
I	162	I	I	I	I	I	I	I	I	I	I	K	I	I	I	I
I	165	Q	Q	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	Q
I	171	A	A,T	A,V	A	A	A	A	A	A	A	P	V	A,V	A	A
I	172	I,V	I,T,V	I,T,V	I	I	I	I	I	I	I	F	I,T	I	I	I
I	173	L	L	L	L	L	L	L	L	L	L	W	L	L,S	L	L
I	174	P	P,L	P	P	P	P	P	P	R	P	L	P	P	P	P
I	177	G	G	G	G	G	G	G	G	E	G	G	G	G	G	G
II	232	K	K	K	K	K	K	K	K	K	K	K	K	K	R	R
III	338	E	E	E	E	E	E	E	E	E	V	E	E	E	E	E
III	379	G	G	G	G	G	G	G	G	G	G	R	G	G	G	G
III	431	G	G	G	G	G	G	G	G	A	G	G	G	G	G	G

[a] DENV-3 strains in this study.

These three residues are located in the domain II of the envelope protein and C105F is the alteration from hydrophilic to hydrophobic AA. Nine out of ten AA changes observed in BKK21-11 were located in the domain II of the envelope protein. Two AA changes at C116F and G126E in BKK21-11 cause alteration in AA properties from hydrophilic to hydrophobic.

4. Discussion

Genetic determinant of DENV is one of the most important factors involved in severity of dengue disease (Bäck and Lundkvist, 2013). The replacement of low virulent genotype/strain by high virulent counterparts has been associated with DHF outbreaks in many countries because the high virulent strains can replicate and disseminate in mosquitoes and humans faster than low virulent strains (Anderson and Rico-Hesse, 2006; Hanley et al., 2008; Kanakaratne et al., 2009). Hence, genotype identification and relationship among strains belonging to the same genotype would be useful for predicting disease incidences in the endemic area. In this study, we focused on genotypes of DENV-3 isolated from field-caught mosquitoes. The comparisons between the gene trees and the supermatrix tree showed that the gene tree constructed from the complete *E* gene could well represent the supermatrix tree. The phylogenetic tree constructed based on the complete *E* gene sequences showed that our ten samples are composed of eight samples of DENV-3 genotype III and two samples of DENV-3 genotype V. This is the first report of this genotype in Thailand. All of these samples are closely related to previous samples isolated in Asia. Twenty five unique AAs were observed in our samples. Ten hydrophobic-hydrophilic amino acid switches including eight changes on conserved sites could alter the effectiveness of dengue infection. These results suggested that all four DENV-3 genotypes have been circulating in Thailand and the genetic variation of this serotype is higher than previously known, which potentially affects dengue disease incidence in this country.

By comparing each gene tree to the supermatrix tree of complete coding sequences, the gene trees of the complete *NS3* and *E* gene could well represent the supermatrix tree (Fig. 1), thus suggesting the use of these gene sequences for genotype identification. This result further supports the use of the complete *E* gene sequences to represent evolutionary relationships among DENV genotypes (Amarilla et al., 2009; Jiang et al., 2012; Lee et al., 2012; Luo et al., 2013; Monteil et al., 2016). The branching pattern of our gene tree built based on these 851 complete *E* gene sequences corresponded well with the previous study by Jiang et al. (2012), supporting the monophyletic clade of each genotype and the monophyletic group of genotypes I, II and V (Jiang et al., 2012). Hence, the complete *E* gene sequence can be applied for identifying genotype and revealing evolutionary relationships among genotypes.

Based on the phylogenetic trees of complete *E* gene sequences (Fig. S1 and Fig. 2), our ten DENV-3 samples are composed of eight samples belonging to genotype III and two samples belonging to genotype V. The DENV-3 genotype III in Thailand was recently observed in 2010 (Huang et al., 2012), while no observation of DENV-3 genotype V in Thailand has been reported. We observed no mosquito sample carrying DENV-3 genotype II that are the predominant genotype circulating in Thailand since 1973 (Attatippaholkun et al., 1998; Yamashita et al., 2013). In addition, no DENV-3 genotype I, which was first observed in Thailand in 1988 (Wittke et al., 2002), was observed in our mosquito samples. The observation of only two genotypes in our study could not eliminate the possibility that the other two genotypes are still circulating in Thailand; thus, we suggested that at least two genotypes of DENV-3 were circulating in Thailand during 2011–2015.

Regarding genotype III, our eight samples could be separated into six strains: Songkhla, Lop Buri, BKK6-15, BKK7-15, BKK12-15 and BKK13-15, because the complete *E* gene sequences of SKA1-12 and LRI2-15 are identical to that of SKA5-12 and LRI8-15, respectively. Based on the ML/Bayesian tree, these samples formed a monophyletic clade and were closely related to DENV-3 genotype III strains from neighboring countries: Singapore, Viet Nam and Laos isolated in 2013 and 2014 as well as ones from Thailand isolated in 2012 and 2013 (Guo et al., 2015; Hapuarachchi et al., 2016; Monteil et al., 2016; Phu et al., 2015). As shown in Fig. 2, our samples formed a monophyletic clade within a clade that was composed of DENV-3 isolates from Southeast Asian countries, especially Thailand's neighbors. This pattern indicated either that our samples represent new lineages of DENV-3 genotype III that were recently evolved or that our samples represent mosquito-specific lineages (Sessions et al., 2015). The close relationship between our samples and samples from neighboring countries raised the possibility that our samples, especially the ones isolated in 2015, have recently been imported from these countries. This scenario is supported by (1) increasing numbers of people travelling across this region (Messer et al., 2003; Shi et al., 2016) and (2) the previous study by Huang et al. (2012) presenting that the first case of DENV-3 genotype III in Thailand in 2010 was imported from Taiwan during 2008–2010.

Regarding genotype V, our two DENV-3 samples: BKK21-11 and NBI17-11, isolated from mosquito samples collected from two separated areas in Thailand formed a monophyletic clade (Fig. 3). The presence of the clearly separated clade, comprised of only our genotype V samples, might suggest either that our samples represent new lineages of DENV-3 genotype V or that these samples represent lineages that could only be evolved in mosquito vectors (Patil et al., 2012).

Besides phylogenetic analyses, amino acid changes generated by replacement mutations in the *E* gene were determined. The envelope protein encoded by the *E* gene is responsible for host cell and immune recognition. The antigen presented by this protein can bind either to the host cell receptor for viral entry or to the host antibody for viral neutralization (Idrees and Ashfaq, 2012). This protein is composed of domains I–III, a stem region and transmembrane anchor and the positions of these components on the DENV-3 envelope protein have been reported (Amarilla et al., 2009; Modis, 2013; Poggianella et al., 2015). As shown in Table 2, 25 unique AAs located in 23 positions across three domains were observed in our samples. Thirteen AAs are located on the conserved positions where only one AA residue is present in 1690 previously isolated samples. Previous reports showed that amino acid substitutions in the envelope protein are associated with the severity of dengue disease (Leitmeyer et al., 1999). Due to the fact that alteration of amino acids in this protein could affect the effectiveness of viral-host cell or viral-host immune interaction, the replacement mutations observed in our samples could profoundly affect function of the envelope protein and consequently affect epidemic potential and disease severity.

Regarding genotype III, no common amino acid change was observed, thus the clustering of our eight samples would be driven by common synonymous mutations. This explanation could also be applied to explain the cluster of BKK6-15 and BKK7-15 that AA changes were observed only in BKK7-15. AA changes observed in BKK7-15, BKK12-15 and BKK13-15 are distributed only in domains I and III. A specific function or the impact of the envelope protein domain I has yet been reported. The domain III of this protein plays a role in binding to the host cell receptor or host antibodies (Ab), with Abs against E protein playing an important role in both protection from and enhancement of dengue infection (Lai et al., 2008; Poggianella et al., 2015). A previous report on DENV-2 showed that the AA difference at position 390, located in the domain III, is related to different viral replication rate in the cell line of *Aedes aegypti*; the replication rate of the Southeast Asian genotype carrying asparagine (N) at this position was approximately 3-fold faster than the American genotypes carrying aspartic acid (D) at this position (Cologna and Rico-Hesse, 2003; Diallo et al., 2005). The higher replication rate of the Southeast Asian genotype would generate higher viral load per cell, causing an increase in viremia and enhancing the inflammatory response in humans. The faster replication rate of the DENV-2 Southeast Asian genotype would, at least partially, explain why this DENV-2 genotype is more virulent than the American genotype

(bib0035Bäck and Lundkvist, 2013). In addition, our three DENV-3 strains were collected in October 2015 which dengue incidence in that year had increased dramatically during August–November (MoPH, 2016). Moreover, in 2015, dengue outbreak had also occurred in Thailand which the morbidity rate was 223.28 per 100,000 populations and high DHF/DSS cases (59,088 patients) had been reported (MoPH, 2016). Interestingly, in the same year, Bangkok was ranked in the top ten area of the highest morbidity rate which the rate was 460.81 per 100,000 populations (MoPH, 2016). Hence, the AA changes observed in our genotype III samples could affect either the viral-host interaction or the viral replication rate and consequently cause dengue outbreak.

Regarding genotype V, three common AA changes between our genotype V samples were observed. All these common AA variants are located at the conserved sites and one of them (C105F) caused alteration from hydrophilic to hydrophobic AA. These common AA changes could, at least partially, drive the clustering between our genotype V samples. Interestingly, nine out of ten positions presenting AA changes in our samples are located in the domain II of the envelope protein and five AA changes in these positions cause alteration in amino acid properties. In general, domain II initializes viral-host cell membrane fusion and dimerization of the envelope protein (Stiasny and Heinz 2006). Moreover, this domain contains subgroup cross-reactive epitopes which are involved in the enhancement of disease severity during secondary infection of heterologous serotype by an increase of DENV-infected cells (Lai et al., 2008). Thus, the AA changes in this domain could affect the fusion process and enhancement of dengue disease (Lai et al., 2008). Previously, E proteins containing a single change of AA in the fusion loop (AA residues 98–109) of domain II at 101, 107 or 108 AA residues showed significant reduction of binding activity to sera of patients with primary infection, suggesting that cross-reactive anti-E antibodies recognized these fusion residues (Lai et al., 2008). However, the reduction of E-binding activity of DENV to a monoclonal antibody was also observed in E protein containing non-synonymous mutations in a non-fusion loop of domain II (Lai et al., 2008). In our study, nine non-synonymous mutations of BKK21-11 and NBI17-11 were located in domain II, with two of them residing in the fusion loop while the other seven were located outside the fusion loop. Taken together, the results suggest that the AA changes observed in our genotype V samples potentially determine disease severity because these changes could affect the affinity of DENV to bind to host antibodies and the ability of the virus to gain entry into the host cell.

5. Conclusions

Our study provides the first evidence of the circulation of DENV-3 genotype V in Thailand. Due to the fact that both DENV-3 genotypes III and V were observed in this study, four out of five genotypes of this serotype have circulated in this country. Nucleotide sequence analysis supported the use of the complete *E* gene sequence for genotype identification and presented a distinctively high genetic variation of our samples; however, all of our samples are closely related to Southeast Asian samples. The analysis on translated nucleotide sequences showed that ten out of 25 AA changes cause hydrophobic-hydrophilic AA switches at the conserved sites of the envelope protein. These changes potentially have a profound effect on the efficiency of viral infection or host immune response, which may consequently affect DENV epidemic potential and disease severity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2017.10.021.

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