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Original article

Complete genome sequences and phylogenetic analysis of dengue virus in Southern Vietnam during 2014-2015

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Abstract: ***Objective:*** Dengue is an infectious disease that causes a worldwide health and economic burden despite the efforts to eradicate the disease. From 2013 to 2015, dengue epidemic significantly increased from 33,626 to 50,205 cases in Vietnam. This study aims to determine the genotype variations of dengue virus (DENV) circulating in Southern Vietnam during 2014-2015. ***Methods:*** C6/36 cells were infected with twenty-four strains of dengue virus isolated in 2014-2015 and kept frozen. The complete nucleotide sequence of dengue virus genomes was obtained by polymerase chain reaction (PCR). The genome was sequenced in the MiSeq system and analyzed by the basic local alignment search tool (BLAST) program. Data from GeneBank was used to create the phylogenetic trees. ***Results:*** Among the 17 analyzed strains from 8 southern provinces, four (23.53%) were DENV-1, three (17.65%) were DENV-2, five (29.41%) were DENV-3, and five (29.41%) DENV-4 were isolated. Four DENV-1 isolates belong to Asia genotype. Three DENV-2 strains were concentrated in a subgroup of Asian 1 genotype. Five DENV-3 isolates were identified as belonged to Asian 2 genotype and five DENV-4 isolates were found as belong to Asia 1 genotype. There were no amino acid mutations and the transition capacity between the nucleotide among four types of DENV serotypes suggested that the probability of conversion from C to T was the highest conversion rate. ***Conclusions:*** These DENV isolates were genetically close to other previous strains isolated from Vietnam and its neighboring countries, including Thailand, China, Cambodia, and Singapore, Brazil, Sri Lanka due to dynamic transmission.

Keywords: Dengue outbreak; Southern Vietnam; genome sequence; phylogenetic tree; diversity.

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1. INTRODUCTION

Dengue virus (DENV) is a single-stranded *flaviviridae* RNA virus and responsible for causing the mosquito-borne infectious dengue disease mostly in tropical areas [1]. The disease manifestations range from mild dengue fever (DF) to the life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Naturally, the virus is circulating in four major serotypes (DENV-1–4) and showing about 65–70 % sequence homology with each other [2]. These serotypes show the highest mutation rates among other flaviviruses [3, 4]. This has led to the formation of different lineages and genotypes within each serotype [5]. Accordingly, DENV-1 comprises of five genotypes (I–V); DENV-2 has six genotypes (Southeast Asian/American, Asian I, Asian II, Cosmopolitan, American, and Sylvatic); DENV-3 has four genotypes (I–IV); and DENV-4 also consists of four genotypes (I, II, III, Sylvatic) [6]. Genetic changes can predominantly affect the disease burden by changing genotypes and subsequent changes in disease virility. An example of that is the changes of DHF rates in Sri Lanka when the DENV-3 (genotype III) group A was replaced with group B viruses [7] . Another example is when the American DENV-2 was replaced with the more severe Southeast Asian DENV-2 and the associated changes following this event [8].

Vietnam is an endemic country of dengue; particularly Southern Vietnam being a hyper-endemic area with nearly ten-fold more dengue than Central Vietnam every year [9, 10]. According to the summary report of the Pasteur Institute in Ho Chi Minh City, in 2013-2015, the number of dengue cases had tended to increase from 33,626 cases in 2013 to 50,205 cases in 2015. DENV-4 has been found in each area of Vietnam (Southern, Northern, Central) with over 50 strains isolated and the data was available in GenBank [10] . In 2013, a large dengue outbreak occurred in central Vietnam resulting in 204,661 clinical cases, with DENV-4 being the most predominant serotype followed by DENV-1, DENV-3 and DENV-2, respectively [10]. Similarly, another outbreak was detected around the same period at Cat Ba Island with identified 192 cases [11]. The cases were attributed to DENV-3 serotype (genotype III), which presented a high homology with the DENV strains affecting the nearby city of Hanoi in the same year [11]. These finding suggested that the viruses were probably introduced to the islands from the mainland (likely Hanoi), hence, the prevention efforts should be focused mainly at the origin [11]. Additionally, DENV-3 (genotype III) was detected in cerebrospinal fluid of an accountant living in Hai Phong, which showed a close relationship with the aforementioned serotypes using phylogenetic analysis. Noteworthy to mention that DENV-3 (genotype III) was reported for the first time in Vietnam in 2013 while genotype II had been the one previously circulating in the country [12]. In 2011, DF epidemics were noted in Hanoi with 24 outbreak points hitting eight districts with serotypes DENV-1 and DENV-2 detected in human samples [13]. Back to 1998, a widespread epidemic occurred of DHF with the incidence rate of 438.98 cases per 100,000 population, representing a 152.4% increase compared to 1997 epidemic [9]. Although most prevalent serotype was DENV-3, other serotypes were noted as well but in lower percentages [9]. A recent study presented that DENV-1 strains in Northern Vietnam were most closely related to the dengue virus outbreak in Cambodia from 2006 to 2008 [14].

In this study, we aimed to identify a complete genome sequence analysis for all four types of DENV strains circulating in Southern Vietnam during 2014-2015. The identification of recent serotypes and genotypes of the strains will contribute in predicting the transmissibility and virulence of the virus responsible for epidemic.

2. MATERIALS AND METHOD

2.1. Growth and identification of the dengue viruses

We employed *Aedes albopictus* (C6/36) cell line to grow viruses. C6/36 cells were cultured in 25cm^2 flask (Nunc, Cat.No. 163371) with Dulbecco's Modified Eagle Medium (Gibco, Cat.12100-061) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Cat.10084-168) for growth at 28°C. Twenty-four strains of dengue viruses isolated in 2014-2015 and kept in −80°C storage were used to infect the cell lines. After the incubation period of 7 days at 28°C, infected cells were harvested and identified by direct fluorescent assay (DFA) as well as immunofluorescence assay (IFA) with the following monoclonal antibody: anti-Flavin conjugated fluorescein isothiocyanate (FITC), anti-dengue virus serotype 1-4 and anti-mouse IgG conjugated FITC (Sigma, F5262). The information about monoclonal antibody was provided by US CDC: DEN.1 Hawaii (D2-1F1-3); DEN.2 NGC (3H5-1-21); DEN.3 H87 (5D4-11-24); and DEN.4 H241 (1H10-6-7).

Study samples were collected by Centers for Disease Control (CDC) in 20 provinces in the Southern Region. The agency responsible for isolation and sequencing is the Pasteur Institute in Ho Chi Minh City, Vietnam.

2.2. Collecting whole-genome of dengue viruses

RNA from dengue viruses of which serotypes had been identified (1 to 4) was extracted using the QIAampR Viral RNA Mini Kit (Qiagen 52906). Full genome of the virus strains was identified using the Superscript III Reverse Transcriptase kit (Invitrogen, Cat. 18080-051) and the Platinum Taq DNA polymerase (Invitrogen, Cat. 10966-018). Brieflt, we used Superscript III RT and random primer to synthesize cDNA. Then, we performed PCR to collect full genome by Plantinium Taq DNA polymerase (Invitrogen, Cat. 10966-018) with primer in **Supplementary Table S1**. Specifically, for the Superscript III Reverse Transcriptase kit, the reaction mixture recipe was 1 µL random primer, 1 µL dNTP, 3 µL diethyl pyrocarbonate (DEPC)-treated water, 2 µL buffer 10X, 4 µL Mg^{2+}, 2 µL DDT, 1 µL RI, 1 µL Enzyme Reverse Transcriptase and 5 µL RNA target with the following thermal cycle: 25ºC for 10 minutes, followed by 50ºC for 50 minutes, then 85ºC for 5 minutes, and finally 4ºC for 60 minutes. For Plantinium Taq DNA polymerase (Invitrogen, Cat. 10966-018), the reaction mixture recipe was 2.5 µL Buffer 10X, 1 µL Mg^{2+}, 0.5 µL dNTP, 0.5 µL for each forward primer and reverse primer, 0.1 µL enzyme, 17.4 µL DEPC and 2.5 µL cDNA. DNA polymerase activation was followed by 35 amplification cycles of 30 seconds at 94°C, then 30 seconds at 55°C and 3 minutes at 72°C.

The genome virus was purified using the QIAquick ® PCR Purification (Qiagen 21480) and QIAquick ® Gel Extraction kit (Qiagen 28704). DNA fragments from 100bp to 10kb were purified from primers, nucleotides, polymerases,

and salts by the QIAquick spin column under the effect of centrifugation.

2.3. Sequencing and analysis

DNA concentration was quantified by Qubit fluorometer. The fluorescent dyes were associated with specific target molecules. Calibration curve was used to generate the quantitation results. Later, the DNA concentration was diluted to 0.2 ng/µL, then the DNA was ligated and indexed by PCR reaction with the following reaction mixture: 25 µL DNA, 15 µLNPM, 5 µL Index 1 and 5 µL Index 2. The thermal cycle was 72ºC for 3 minutes, followed by 95ºC for 30 seconds, then 12 amplification cycles of 10 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C, then 72ºC for 5 minutes and finally 4ºC for 60 minutes.

After being attached with the index, the products were purified by AMPure XP beads kit (Beckman Coulter A63880). The genome of dengue viruses was sequenced using the Next-Generation Sequencing (NGS)-Illumina method in MiSeq system. Briefly, the DNA adhered to the adapter would be attached to the flowcell. Each small segment on the flowcell was synthesized to form a cluster to amplify the read signal. Each run provided a line of A, T, G, or C, which would be attached to the blocker. The signal from the attached unit would be received, analyzed and processed [15]. Finally, the sequencing results were analyzed by BLAST program [16]. The phylogenetic analyses and estimates of transition capacity between DENV nucleotides were conducted using MEGA7 version 7 (http://www.megasoftware.net/). The sequences were purified by CLC Genomics Workbench 10.1.1 software and aligned by the maximum likelihood estimation method with bootstrap analysis of 100 repetitions and reliability value greater than 70%. The analysis begins with a specialized tree created from the input data. The original tree branches were swapped until the tree with the highest likelihood score was obtained [17]. All reference sequences of different geographical regions were retrieved from GeneBank and used for genome analysis to create phylogenetic trees (**Supplementary Table S2**).

3. RESULTS

3.1. Dengue virus isolation

Table 1. Results of virus isolation

ID	Strains	Year	Region	DFA	IFA	Serotype
1	ĐT-HT-281/14 D1(1F1)	2014	Dong Thap	(-)		
2	VT-HT-00518/14 D1(1F1)	2014	Ba Ria-Vung Tau	3+	3+	DENV-1
3	BD-DNT-5527/14 D1(1F1)	2014	Binh Duong	3+	3+	DENV-1
4	ST-HT-1191/15 D1(1F1)	2015	Soc Trang	2+	3+	DENV-1
5	BT-HT-8442/15 D1(1F1)	2015	Ben Tre	(-)		
6	TN-HT-9062/15 D1(1F1)	2015	Tay Ninh	2+	2+	DENV-1
7	VT-HT-4573/14 D2	2014	Ba Ria-Vung Tau	3+	3+	DENV-2
8	VT-HT-4577/14 D2	2014	Ba Ria-Vung Tau	3+	3+	DENV-2
9	BD-HT-4598/14 D2	2014	Binh Duong	3+	3+	DENV-2
10	HCM-M-1226/15 D2	2015	Ho Chi Minh	4+	3+	DENV-2
11	ĐN-HT-4581/15 D2	2015	Dong Nai	3+	3+	DENV-2
12	ĐN-HT-6763/15 D2	2015	Dong Nai	3+	3+	DENV-2
13	BD-HT-258/14 D3	2014	Binh Duong	3+	3+	DENV-3
14	TN-HT-6073/14 D3	2014	Tay Ninh	(-)		
15	VT-HT-6426/14 D3	2014	Ba Ria-Vung Tau	4+	3+	DENV-3
16	ĐN-HT-1070/15 D3	2015	Dong Nai	2+	2+	DENV-3
17	BD-HT-5786/15 D3	2015	Binh Duong	(+)	(+)	DENV-3
18	ĐT-HT-6970/15 D3	2015	Dong Thap	2+	2+	DENV-3
19	BD-HT-3268/14 D4	2014	Binh Duong	4+	3+	DENV-4
20	VT-HT-3837/14 D4	2014	Ba Ria-Vung Tau	2+	2+	DENV-4
21	ĐN-HT-7366/14 D4	2014	Dong Nai	(-)		
22	LA-HT-1128/15 D4	2015	Long An	3+	3+	DENV-4
23	BD-HT-5297/15 D4	2015	Binh Duong	(+)	(+)	DENV-4
24	BD-HT-7053/15 D4	2015	Binh Duong	2+	3+	DENV-4

The results of virus isolation were presented in **Table 1**. Of 24 strains, we identified 20 positive strains, including 4 strains of DENV-1, 6 strains of DENV-2, 5 strains of DENV-3 and 5 strains of DENV-4. The results of IFA under fluorescence microscope were presented in **Fig. 1.** The positive samples after virus isolation were sequenced. The DENV genome was about 11kb in length. From 20 sequenced samples, after purification by CLC Genomics Workbench, 17

strains had appropriate quality for sequencing and further genotype analysis (**Supplementary Table S2**). Of these 17 DENV isolates, 4 (23.53%) were DENV-1, 3 (17.65%) were DENV-2, 5 (29.41%) were DENV-3, and 5 (29.41%) DENV-4 was identified in our study. Most of them distributed across the Southern Vietnam regions including Dong Thap, Ba Ria-Vung Tau, Binh Duong, Soc Trang, Ben Tre, Tay Ninh, Ho Chi Minh City, Dong Nai (**Table 1**).

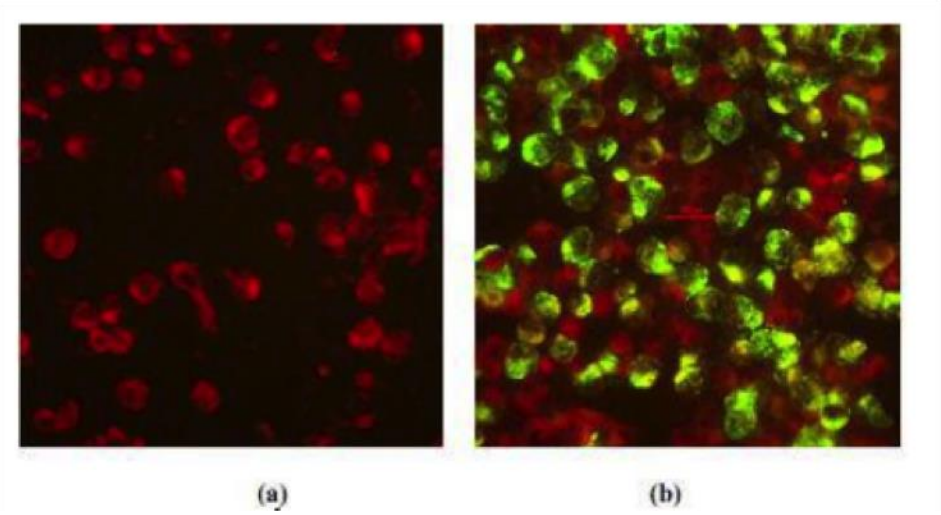

Figure 1. Negative (a) and positive (b) results of immunofluorescence assay (IFA) under fluorescence microscope observation. IFA is an indirect immunofluorescence reaction for determination DENV type. The positivity level is assessed based on the percentage of fluorescence cells

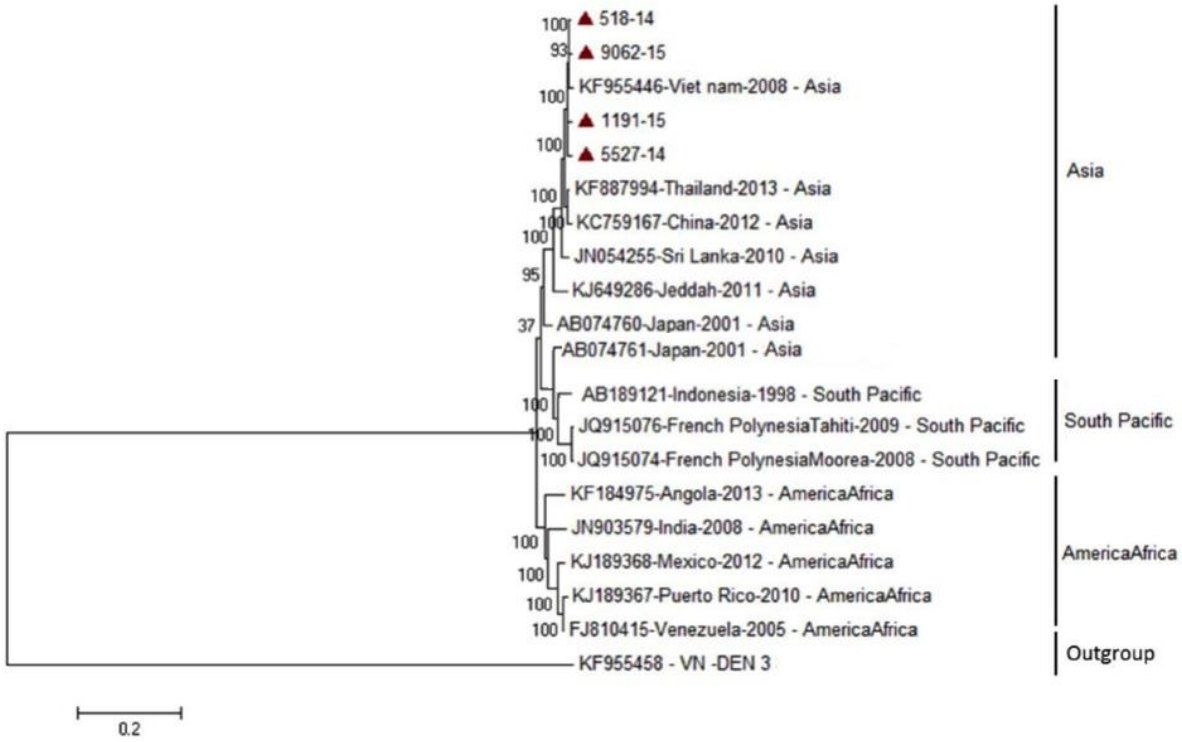

Figure 2. Phylogenetic tree of genome DENV-1 sequences. Each geographical strain was abbreviated by its accession number followed by country, year of isolation and their region. Four DENV-1 isolates from our study were labeled as ▲

3.2. Nucleotide sequence and phylogenetic analysis

Phylogenetic tree revealed that DENV-1 strains were divided into three main genotypes according to the geographic area, including Asia, South Pacific and America/Africa, in which four genome sequences of DENV-1 isolates belong to small subgroup of Asia genotype and genetically close to previous strains from Vietnam, Thailand, China and Sri Lanka (**Fig. 2**). For DENV-2, the phylogenetic tree showed three DENV-2 isolates were belong to Asian 1 group and genetically closest to the Thailand strain (**Fig. 3**).

Regarding DENV-3, the phylogenetic analysis reported that five strains isolated from our study were identified to group of Asian 2 genotype and genetically close to previous

strains from Vietnam, Cambodia, Thailand and Singapore (**Fig. 4**). For DENV-4, the phylogenetic analysis demonstrated that five DENV-4 strains isolated from our study were identified as Asia 1 genotype. DENV-4 isolates were genetically close to other previous isolates from Thailand and Brazil (**Fig. 5**).

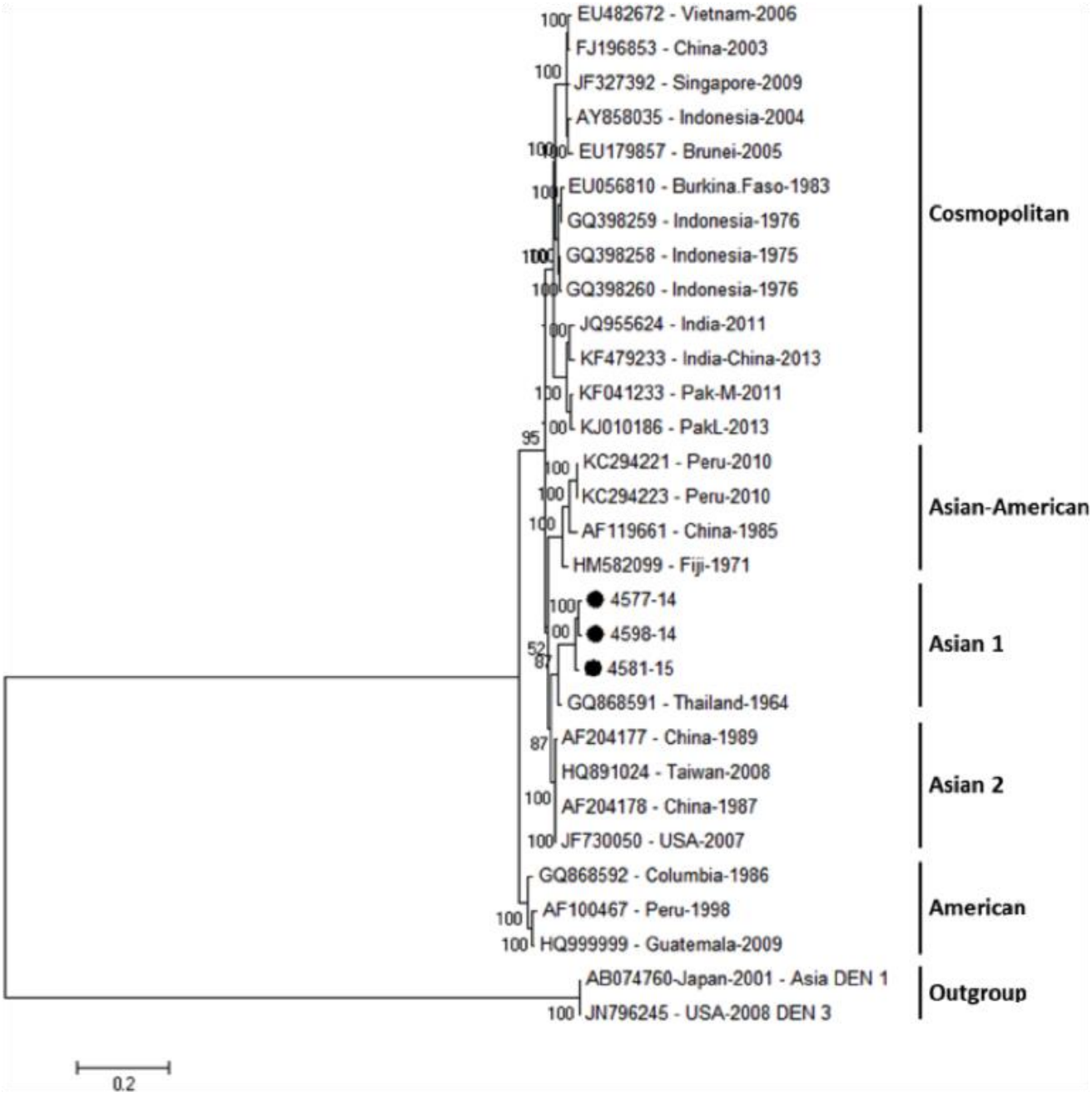

Figure 3. Phylogenetic tree of genome DENV-2 sequences. Each geographical strain was abbreviated by its accession number followed by country and year of isolation. Three DENV-2 isolates from our study were shown as •

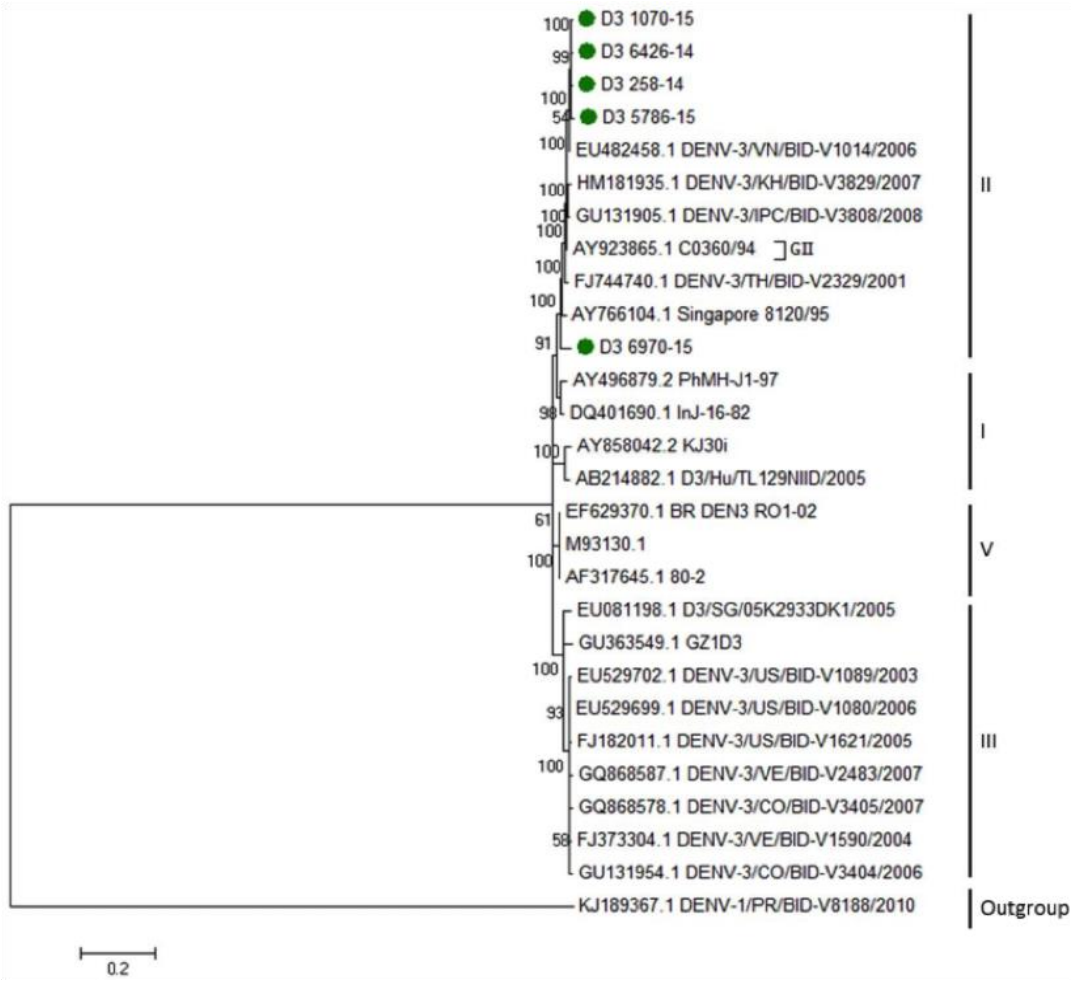

Figure 4. Phylogenetic tree of genome DENV-3 sequences. Each geographical strain was abbreviated by its accession number followed by country, year of isolation and their region. The genotypes were denoted as Asian 1 (I), Asian 2 (II), American (III), America/Asian (IV) and Cosmopolitan (V). Five DENV-3 isolates from our study were labeled as •

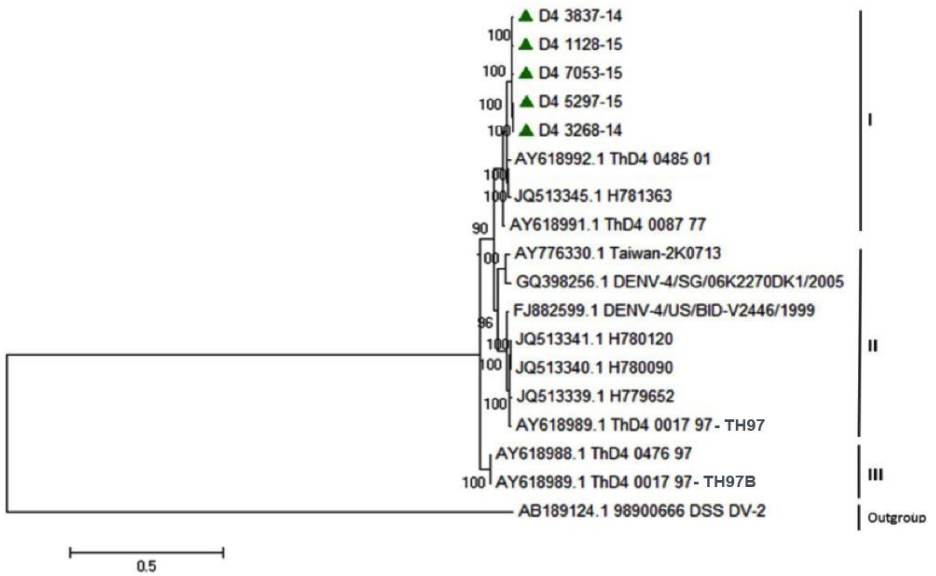

Figure 5. Phylogenetic tree of genome DENV-4 sequences. Each geographical strain was abbreviated by its accession number followed by country, year of isolation and their region. The genotypes were denoted as Asian 1 (I), Asian 2 (II), American (III), America/Asian (IV) and Cosmopolitan (V). Five DENV-4 isolates from our study were marked as ▲

3.3. The transition capacity among nucleotide

The transition capacity among nucleotides of four DENV was presented in **Table 2**. The result of analysis of DENV-1 showed that the highest ratio mutation was from C to T at 31.99% and the lowest ratio mutation was from G to C at 0.67%. Similarly, in DENV-2, the ratio mutation C to T was highest at 27.54% meanwhile from A to C was lowest at 1.61%. In DENV-3, the highest ratio mutation C to T was 33.87% whereas from G to C was 0.92 % and in DENV-4, the ratio mutation C to T was highest at 29.87%, while G to T was 0.5 %, respectively.

Table 2. Predicted transition ability among nucleotide between DENV-1, DENV-2, DENV-3 and DENV-4 sequences, respectively

	DENV-1	DENV-2	DENV-3	DENV-4
A to T	1.68	1.65	0.97	1.88
A to C	1.46	1.61	0.92	1.04
A to G	11.82	13.01	11.10	14.08
T to A	2.49	2.59	1.45	2.65
T to C	30.38	26.88	32.12	28.16
T to G	0.96	1.99	1.16	0.6
C to A	2.27	2.59	1.45	1.56
C to T	31.99	27.54	33.87	29.87
C to G	0.83	1.99	1.16	1.64
G to A	14.65	16.87	13.92	16.72
G to T	0.81	1.65	0.97	0.50
G to C	0.67	16.1	0.92	1.30

Overall, the result of analysis revealed that the probability of conversion from C to T was the highest conversion rate among all four types of DENV-1, -2, -3, -4 serotypes. For comparison at the level of amino acids, the percentage difference in each pair of amino acid sequences between the four strains ranged from 0 to 1.03% (**Supplementary Table S3**).

4. DISCUSSION

In this study, we identified 17 complete DENV genome sequences, including 4 strains of DENV-1, 3 strains of DENV-2, 5 strains of DENV-3 and 5 strains of DENV-4 from patients with dengue during 2014-2015 in Southern Vietnam. Based on maximum likelihood method, the phylogenetic trees were constructed to detect any changes in the circulating strains of dengue in Southern Vietnam. Maximum likelihood method was considered as the most suitable fit for phylogenetic trees with large number of taxa and long sequence strings for viral phylogeny with its added bootstrap technique and high accuracy that we used to determine the branching patterns [18]. This information will help health authorities identify and take the necessary measure to prevent another epidemic.

DENV-1 was divided into the following genotypes: sylvatic/Malaysia, America/Africa, South Pacific, Asia, and Thailand [18]. Our phylogenetic analysis of four strains of DENV-1 revealed that the circulating strains belong mainly to Asian lineage and Thailand group. It was also related to both South Pacific and America/Africa. This demonstrates the diversity of the strains isolated from our cases. This was consisted with a study performed in Taiwan where they isolated DENV-1 from travelers returning from Vietnam. They observed this diversity in the strains of circulating genotypes in Vietnam [19]. Another study isolated dengue virus from 81 Vietnamese patients, in which, Rabaa *et al.* found that the circulating DENV-1 lineage in Southern Vietnam had come from Cambodia which contracted this lineage from Thailand [20]. This explained our tree results, which has shown genetic proximity to Thailand strains. Another study supported our finding that the DENV-1 strains were mainly distributed from Thailand and Indonesia [21]. Moreover, Mizuno *et al.* also proved that Vietnamese strain was the origin for strains circulating in China, Cambodia and Malaysia. Strains circulating in Japan and Korea were also found to be from South East Asia [22]. For its relation to Americas/African strain, a phylogenetic analysis revealed that the circulating strains in Caribbean and Americas was introduced from South Asia, mainly Thailand, which may explain the phylogenetic relationship between the strains [21].

Identification of the phylogenetic relations to other genotypes of DENV-1 will predict the incidence and severity of DENV-1 epidemic in Southern Vietnam [23]. It is well-known that American/African genotype has higher likelihood to cause DHF and more severe form of disease [18]. These strains also had a high potential to cause an epidemic [18, 23]. Similarly, DENV-1 also reached the dominant serotype in the outbreak of Myanmar in 2013, in which, the primary DENV infection remained at high level among the severe dengue cases [24].

In addition, the analysis revealed that three isolates of DENV-2 sequences in Vietnam were concentrated in a subgroup of the Asian genotype which along with the 1964 Thailand strain, are called Asian 1. A phylogenetic analysis of 273 strains of Asian lineage was divided into two groups; one comprised all strains from Thailand and China strains isolated during 1985–2001 and the other included Vietnam, Thailand and Cambodia from 2001 to 2008 [25]. In our analysis, our strains were related to older strains of the 1964 Thailand strain. It was demonstrated that the parent strain of modern strains in Vietnam is from Thailand [25]. It was also found that the strains in nearby regions are from Thailand. These findings were also supported by findings from a study in Central Vietnam during the outbreak of 2010-2012 [26]. Moreover, our results were consistent with another study that studied the evolution of DENV-2 in Vietnam [27]. They found that Asian I lineage had totally replaced the predominant Asian/American lineage since 2003 [27]. This was highly important since it was found that Asian strains were more related to development of sever forms of disease unlike other strains [23, 28]. It was able to have persistent high blood viremia in dengue patients. It was also found that genotypes of DENV-2 had high transmissibility, signifying its epidemics [18, 23]. A most recent study reported that DENV-2 isolated from the 2017 dengue outbreak in Northern Vietnam originated from India in 2006 [29].

Other two serotypes extracted from our patients were DENV-3 and DENV-4. DENV-3 isolates from our study were found as genetically close to previous strains from Vietnam, Cambodia, Thailand and Singapore. This finding was in agreement with results from Podder *et al.* [30] and Shu *et al.* [31] on the E gene region. Phylogenetic analysis of DENV-3 using E gene revealed four subtypes based on geographical distribution including Americas, Indian subcontinent,

Thailand and Southeast Asia/South Pacific [18]. This classification was useful to identify the virulence and epidemic potential for the circulating serotype across the years in each country. The year of 1990 was considered as the cut-off year for the circulating DENV-3 in America, before 1990, only American genotype was the one circulating with low incidence of new cases and only DF was diagnosed [32, 33]. Unlike after 1990, Southeast Asian serotypes were associated with high epidemic and more diagnosis of DHF; Indian type was also found after 1990 in Americas and Asia and was correlated to severity of the disease [34, 35].

In case of DENV-4, the rate of incidence of epidemic from this serotype was less predominant than other serotypes imposing an obstacle for its classification into well-established genotypes [18]. Besides, our DENV-4 isolates were proved to genetically close to other previous isolates from Thailand and Brazil. This is consistent with the hypothesis that the presence of the Americas is due to the spread of these strains from Asia to America [36]. After the discovery of full sequence of E gene, only three genotypes were reported with only one type found to be circulating along many continents. Still, more efforts are needed to classify this serotype [36, 37].

The result of analysis revealed that the probability of conversion from C to T was the highest conversion rate among all four types of DENV-1, -2, -3, -4 serotypes. Based on the wobble theory [38], the transition from C to T was more likely to cause significant mutations on the protein surface such as Ser transfer to Phe and Leu, or Leu to Pro. These findings could play a key role in the genetic evolution. In addition, the percentage difference in each pair of amino acid sequences between the four strains ranged from 0 to 1.03% (**Supplementary Table S3**). However, the degree of amino acid sequence difference is always lower than that at the nucleotide level, indicating that most mutations occur in the third-base of the coding codon. There is almost no change at the amino acid level, so this result shows that most of these isolated viruses are in the stage of accumulation of mutations.

Limitations

Apparent limitations of the present study include that we analyzed a relatively small numbers of specimens. In addition, there was a small number of isolates in each serotype, which might not give a complete representation of the circulating DENV in Southern Vietnam.

Conclusion

In conclusion, our study describes, for the first time, the genetic diversity of all four serotypes of DENV in Southern Vietnam during 2014-2015. We demonstrated that DENV is hyperendemic in Southern Vietnam, rendering co-infection between serotypes and genotypes possible. We observed the co-circulation of multiple genotypes within DENV-1 to DENV-4; four DENV-1 isolates belong to Asia genotype, three DENV-2 strains were concentrated in a subgroup of Asian 1 genotype, five DENV-3 isolates were identified as belonged to Asian 2 genotype and five DENV-4 isolates were found as belong to Asia 1 genotype. These findings highlighted the geographic distribution and dynamic transmission of DENV strains occurred in Southern Vietnam. The transition capacity between the nucleotide among all four types of DENV-1, 2, 3, 4 serotypes also suggested that the probability of conversion from C to T was the highest conversion rate, which implied genetic evolution of DENV strains.

LIST OF ABBREVIATIONS

DENV: dengue virus; PCR: polymerase chain reaction; BLAST: basic local alignment search tool; IFA: immunofluorescence assay; DF: dengue fever; DHF: dengue hemorrhagic fever; DSS: dengue shock syndrome; FBS: Fetal Bovine Serum; DFA: direct fluorescent assay; FITC: fluorescein isothiocyanate; CDC: Centers for Disease Control; DEPC: diethyl pyrocarbonate.

ETHICAL STATEMENT

Ethical approval for this study was provided on September 2014 by the Institutional Review Board of the Ministry of Health, Vietnam (3711/QD-BYT). Informed consent was obtained from patients prior to the collection of samples (**Supplementary Figure S1**).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Experiments were done by HPT, BCT and NHQ. Data analysis and its interpretation were done by LT, NHQ. All authors contributed to results interpretation, manuscript writing and give approval of the final version. All authors critically revised and approved the final manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1. The primers for RT-PCR. The primer sets are self-developed

No.	Primer name	Location		Sequence (5' - 3')	Length of products (in base pairs)
		From	To		
1	D1,1F	21	44	CGACAAGAACAGTTTCGAATCGGA	2179
2	D1,4R	2182	2199	AAGTCCCATGCGGTGTCT	
3	D1,5F	1918	1937	AACAGATGCACCATGCAAGA	1938
4	D1,8R	3836	3855	AGTCCATCCCCCAGCTCCTC	
5	D1,9F	3672	3690	ACAGGATGGGGATGGGAAC	1257
6	D1,11R	4910	4928	ATCCAGATGTGCCGGGTTT	
7	D1,12F	4801	4820	AGTGCAGGTGATTGCTGTTG	1659
8	D1,14R	6440	6459	AGGTTGTCCAAGGCATTCTG	
9	D1,15F	6306	6325	GGCTGGATGCCAGAACATAC	1490
10	D1,17R	7820	7838	ATGACCAGCCACCTCTTCC	
11	D1,18F	7691	7710	GAAGCCAAAGAGGGACTGAA	1490
12	D1,20R	9161	9180	CGGCTGTGTCATCTGCATAC	
13	D1,21F	9091	9110	TGGAAGGAGAAGGACTCCAC	1536
14	D1.23R	10607	10626	GGTCTCTCCCAGCGTCAATA	
1	D2,1F	87	110	CAGATCTCTGATGAATAACCAACG	1737
8	D2,4R	1803	1823	CCTTTGAGCTGTAGCTTGTCC	
9	D2,5F	1714	1733	ATGCACACAGCACTCACAGG	2033
16	D2,8R	3727	3746	CCAGCTGCAAAAGTTGGTCT	
17	D2,9F	3605	3626	TGACATTGATCACAGGGAACAT	1847
24	D2,12R	5432	5451	AGCTGCCTCACCCATCTCTA	
25	D2,13F	5372	5391	ACGAAGCCCATTTCACAGAC	1964
32	D2,16R	7316	7335	TTGTCCCAACTGCTTTTCAA	
33	D2,17F	7243	7262	GGCATCATGAAAAACCCAAC	2011
40	D2,20R	9233	9253	TGTGTTCTCCTTCCATGTGGT	
41	D2,21F	9166	9183	GATGACACCGCAGGATGG	1304
46	D2,23R	10450	10469	GCGTACAGCTTCCATGGTTT	
1	D3,1F	12	31	TACGTGGACCGACAAGAACA	1698
8	D3,4R	1690	1709	GCATTGCTCCCTCTTGCGAT	
9	D3,5F	1577	1597	CCTCTACCATGGACATCAGGA	1839

No.	Primer name	Location		Sequence (5' - 3')	Length of products (in base pairs)
		From	To		
16	D3,8R	3397	3415	TTCCATGCCATACCAGCAG	
17	D3,9F	3326	3346	GGGAAGTTGATACACGAATGG	1788
24	D3,12R	5094	5113	CTTTCCTGACCCAGGATGAA	
25	D3,13F	4958	4978	CTGTATGGCAATGGAGTGGTT	1985
32	D3,16R	6923	6942	TACAATGTCCAGGCTGATGC	
33	D3,17F	6787	6807	GCATACTTACATTGGCTGCAA	1760
40	D3,20R	8527	8546	TGAGGAGTTTCACGACTCCA	
41	D3,21F	8371	8390	AACACCCAACATGGATGTCA	1541
46	D3,23R	9892	9911	CTGCTGAACATATGGCGTTG	
1	D4,1F	137	156	CAATATGCTGAAACGCGAGA	1985
8	D4,4R	2101	2121	CTTTCCTGAACCAATGGAGTG	
9	D4,5F	2005	2024	CTTTTGCTGAGaATACCAAC	1816
16	D4,8R	3801	3820	GAATTGAAAGCACCGTTGTC	
17	D4,9F	3711	3731	GCAGTGTTCAAGATGTCACCA	1858
24	D4,12R	5549	5568	CGAACCCTGTGTTCCATGAC	
25	D4,13F	5461	5480	TCATGACTGCAACCCCTCCT	2008
32	D4,16R	7449	7468	ATGGTCGTGTTCCAAAACCT	
33	D4,17F	7231	7250	CTGCTGGGATCATGAAGAAC	1768
40	D4,20R	8979	8998	AGCCACATGTACCAGATTGC	
41	D4,21F	8815	8834	GATGGACATCAGCCAGTGAA	1422
46	D4,23R	10215	10236	CGCTGTATCTTTTCATGACTGG	

Supplementary Table S2. List of all strains used for genotype analysis

No.	Genbank No.	Strains	Year	Geographic origin	Genotype	Label
1	AB074760.1	Mochizuki	2001	Japan	Asia	JA01
2	JN054255.1	DV1_SL_2010b	2010	Sri Lanka	Asia	SL10
3	KC759167.1	DF1203	2012	China	Asia	CH12
4	KF887994.1	DENV-1/8/Thailand/01/2013	2013	Thailand	Asia	TH13
5	KF955446.1	DENV-1/VN/BID-V3909/2008	2008	Vietnam	Asia	VN08
6	KJ649286.1	DENV-1-Jeddah	2011	Saudi Arabia	Asia	SA11
7	AB074761.1	A88	2001	Japan	South Pacific	JA01A
8	AB189121.1	98901530 DFDV-1	1998	Indonesia	South Pacific	ID98
9	JQ915074.1	PF08/180908-01	2008	French-Moorea	South Pacific	FM08

10	JQ915076.1	PF09/090609-76	2009	French-Tahiti	South Pacific	FT09
11	FJ810415.1	DENV-1/VE/BID-V2253/2005	2005	Venezuela	America/Africa	VE05
12	JN903579.1	RGCB419	2008	India	America/Africa	IN08
13	KF184975.1	Angola_2013	2013	Angola	America/Africa	AN13
14	KJ189367.1	DENV-1/PR/BID-V8188/2010	2010	Puerto Rico	America/Africa	PR10
15	KJ189368.1	DENV-1/MX/BID-V8195/2012	2012	Mexico	America/Africa	ME12
16	KJ189367.1	DENV-1/PR/BID-V8188/2010	2010	Puerto Rico	America/Africa	PR10
17	KF041233.1	D2/Pakistan/2011-3/2011	2011	Pakistan	Cosmopolitan-I	PA11
18	KJ010186.1	DENV-2/PK/2013	2013	Pakistan	Cosmopolitan-I	PA13
19	JQ955624.1	Od2112	2011	India	Cosmopolitan-I	IN11
20	KF479233.1	QHD13CAIQ	2013	China	Cosmopolitan-I	CH13
21	EU056810.1	1349	1983	Burkina.Faso	Cosmopolitan-II	BF83
22	GQ398258.1	DENV-2/ID/1016DN/1975	1975	Indonesia	Cosmopolitan-II	IN75
23	GQ398259.1	DENV-2/ID/1017DN/1976	1976	Indonesia	Cosmopolitan-II	IN76
24	GQ398260.1	DENV-2/ID/1070DN/1976	1976	Indonesia	Cosmopolitan-II	IN76A
25	AY858035.2	BA05i	2004	Indonesia	Cosmopolitan-III	IN04
26	EU179857.1	DS31-291005	2005	Brunei	Cosmopolitan-III	BR05
27	EU482672.1	DENV-2/VN/BID-V735/2006	2006	Vietnam	Cosmopolitan-III	VN06
28	FJ196853.1	GD01/03	2003	China	Cosmopolitan-III	CH03
29	JF327392.1	DENV-2/SG/D2Y98P-PP1/2009	2009	Singapore	Cosmopolitan-III	SI09
30	AF100467.1	IQT1797	1998	Peru	American	PE98
31	GQ868592.1	DENV-2/CO/BID-V3358/1986	1986	Columbia	American	CO86
32	HM582099.1	D2/FJ/UH21/1971	1971	Fiji	American	FI71
33	AF119661.1	China 04	1985	China	Asian-American	CH85
34	HQ999999.1	DENV-2/GU/FDA-GUA09/2009	2009	Guatemala	Asian-American	GU09
35	KC294221.1	DENV-2/PE/IQA 2080/2010	2010	Peru	Asian-American	PE10
36	KC294223.1	DENV-2/PE/NFI1159/2010	2010	Peru	Asian-American	PE10A
37	GQ868591.1	DENV-2/TH/BID-V3357/1964	1964	Thailand	Asian-I	TH64
38	AF204177.1	44	1989	China	Asian-II	CH89
39	AF204178.1	43	1987	China	Asian-II	CH87
40	HQ891024.1	DENV-2/TW/BID-V5056/2008	2008	Taiwan	Asian-II	TA08
41	JF730050.1	DENV-2/US/BID-V5412/2007	2007	US	Asian-II	US07
42	JN796245.1	DENV-3/US/BID-V5055/2008	2008	US	II	US08
43	EU482458.1	DENV-3/VN/BID-V1014/2006	2006	Vietnam	II	VN06
44	HM181935.1	DENV-3/KH/BID-V3829/2007	2007	Cambodia	II	CA07
45	GU131905.1	DENV-3/IPC/BID-V3808/2008	2008	Cambodia	II	CA08
46	AY923865.1	C0360/94	1994	Thailand	II	TH94
47	FJ744740.1	DENV-3/TH/BID-V2329/2001	2001	Thailand	II	TH01
48	AY766104.1	Singapore 8120/95	1995	Singapore	II	SI95
49	AY496879.2	PhMH-J1-97	1997	Philippines	I	PH97
50	DQ401690.1	InJ-16-82	2006	Indonesia	I	IN06
51	AY858042.2	KJ30i	2004	Indonesia	I	IN04

52	AB214882.1	D3/Hu/TL129NIID/2005	2005	East Timor	I	TL05
53	EF629370.1	BR DEN3 RO1-02	2007	Brazil	V	BR07
54	M93130.1	H87	1990	Philippines	V	PH90
55	AF317645.1	80-2	2001	China	V	CH01
56	EU081198.1	D3/SG/05K2933DK1/2005	2005	Singapore	III	SI05
57	GU363549.1	GZ1D3	2009	China	III	CH09
58	EU529702.1	DENV-3/US/BID-V1089/2003	2003	US	III	US03
59	EU529699.1	DENV-3/US/BID-V1080/2006	2006	US	III	US06
60	FJ182011.1	DENV-3/US/BID-V1621/2005	2005	US	III	US05
61	GQ868587.1	DENV-3/VE/BID-V2483/2007	2007	Venezuela	III	VE07
62	GQ868578.1	DENV-3/CO/BID-V3405/2007	2007	Colombia	III	CO07
63	FJ373304.1	DENV-3/VE/BID-V1590/2004	2004	Venezuela	III	VE04
64	GU131954.1	DENV-3/CO/BID-V3404/2006	2006	Colombia	III	CO06
65	AY618992.1	ThD4_0485_01	2001	Thailand	I	TH01
66	JQ513345.1	H781363	2011	Brazil	I	BR11
67	AY618991.1	ThD4_0087_77	1977	Thailand	I	TH77
68	AY776330.1	Taiwan-2K0713	2004	Taiwan	II	TA04
69	GQ398256.1	DENV-4/SG/06K2270DK1/2005	2005	Singapore	II	SI05
70	FJ882599.1	DENV-4/US/BID-V2446/1999	1999	Puerto Rico	II	PR99
71	JQ513341.1	H780120	2010	Brazil	II	BR10
72	JQ513340.1	H780090	2010	Brazil	II	BR10A
73	JQ513339.1	H779652	2011	Brazil	II	BR11
74	AY618989.1	ThD4_0017_97	1997	Thailand	II	TH97
75	AY618988.1	ThD4_0476_97	1997	Thailand	III	TH97A
76	AY618989.1	ThD4_0017_97	1997	Thailand	III	TH97B
77	AB189124.1	98900666 DSS DV-2	1998	Indonesia	Outgroup	IN98
78	KF955458.1	DENV-3/VN/BID-V1876/2007	2007	Vietnam	Outgroup	VN07
79	Our sample	VT-HT-00518/14 D1(1F1)	2014	Vietnam		0518/14
80	Our sample	BD-DNT-5527/14 D1(1F1)	2014	Vietnam		5527/14
81	Our sample	ST-HT-1191/15 D1(1F1)	2015	Vietnam		1191/15
82	Our sample	TN-HT-9062/15 D1(1F1)	2015	Vietnam		9062/15
83	Our sample	VT-HT-4577/14 D2	2014	Vietnam		4577/14
84	Our sample	BD-HT-4598/14 D2	2014	Vietnam		4598/14
85	Our sample	ĐN-HT-4581/15 D2	2015	Vietnam		4581/15
86	Our sample	BD-HT-258/14 D3	2014	Vietnam		0258/14
87	Our sample	VT-HT-6426/14 D3	2014	Vietnam		6426/14
88	Our sample	ĐN-HT-1070/15 D3	2015	Vietnam		1070/15
89	Our sample	BD-HT-5786/15 D3	2015	Vietnam		5786/15
90	Our sample	ĐT-HT-6970/15 D3	2015	Vietnam		6970/15
91	Our sample	BD-HT-3268/14 D4	2014	Vietnam		3268/14
92	Our sample	VT-HT-3837/14 D4	2014	Vietnam		3837/14
93	Our sample	LA-HT-1128/15 D4	2015	Vietnam		1128/15

94	Our sample	BD-HT-5297/15D4	2015	Vietnam		5297/15
95	Our sample	BD-HT-7053/15 D4	2015	Vietnam		7053/15

Supplementary Table S3. Summary of results for percent difference in nucleotides and amino acids sequences between DENV strains.

A) DENV-1 strains.

Difference (%)	Vietnamese strains		Asia		South Pacific		America/Africa	
Sequence	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
Mean	2.6	1.03	3.55	1.38	8.01	6.54	8.4	7.2
Minimum	0.5	0	0.7	0.4	7.4	6.8	7.6	6.9
Maximum	3.5	1.8	4.6	2.1	8.9	8.3	9.2	9.9

B) DENV-2 strains.

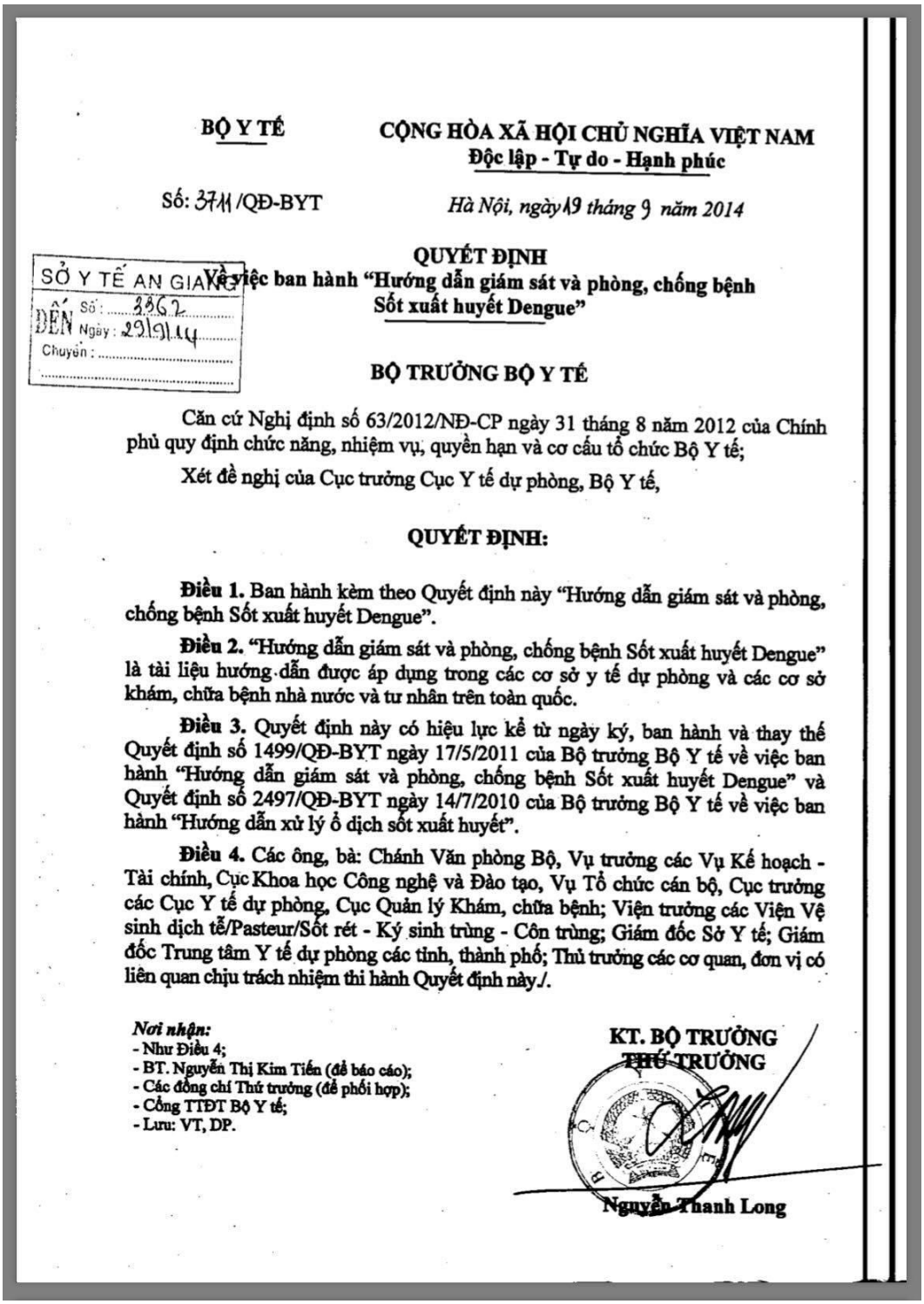
	Vietnamese strains	Asian 1	Asian 2	America	Cosmopolitan
Asian 1	1.44-5.41				
Asian 2	6.07-9.78	6.0-8.14			
America	10.22-11.46	8.61-11.08	7.51-10.61		
Cosmopolitan	8.0-11.43	6.16-10.58	7.05-11.27	9.48-12.38	
Asian-America	7.57-9.27	7.6-8.39	7.13-8.77	7.37-10.63	6.15-10.4

C) DENV-3 strains.

	Vietnamese strains	I	II	III	IV	V
Minimum	0.27	7.40	1.58	7.79	10.73	6.02
Maximum	1.86	9.22	4.39	9.76	12.92	6.96
Mean	1.31	8.45	3.12	8.53	11.70	6.35

D) DENV-4 strains.

	% difference	Minimum	Maximum
Vietnamese strains	0.54	0.542	2.331
Genotype I	5.15	3.106	5.808
Genotype II	9.538	8.633	10.36
Genotype III	11.06	10.71	11.67



BỘ Y TẾ

CỘNG HÒA XÃ HỘI CHỦ NGHĨA VIỆT NAM
Độc lập - Tự do - Hạnh phúc

Số: 3711/QĐ-BYT

Hà Nội, ngày 19 tháng 9 năm 2014

SỞ Y TẾ AN GIANG
ĐẾN Số: 3362
Ngày: 29/9/14
Chuyển:

QUYẾT ĐỊNH
Về việc ban hành "Hướng dẫn giám sát và phòng, chống bệnh Sốt xuất huyết Dengue"

BỘ TRƯỞNG BỘ Y TẾ

Căn cứ Nghị định số 63/2012/NĐ-CP ngày 31 tháng 8 năm 2012 của Chính phủ quy định chức năng, nhiệm vụ, quyền hạn và cơ cấu tổ chức Bộ Y tế;

Xét đề nghị của Cục trưởng Cục Y tế dự phòng, Bộ Y tế,

QUYẾT ĐỊNH:

Điều 1. Ban hành kèm theo Quyết định này "Hướng dẫn giám sát và phòng, chống bệnh Sốt xuất huyết Dengue".

Điều 2. "Hướng dẫn giám sát và phòng, chống bệnh Sốt xuất huyết Dengue" là tài liệu hướng dẫn được áp dụng trong các cơ sở y tế dự phòng và các cơ sở khám, chữa bệnh nhà nước và tư nhân trên toàn quốc.

Điều 3. Quyết định này có hiệu lực kể từ ngày ký, ban hành và thay thế Quyết định số 1499/QĐ-BYT ngày 17/5/2011 của Bộ trưởng Bộ Y tế về việc ban hành "Hướng dẫn giám sát và phòng, chống bệnh Sốt xuất huyết Dengue" và Quyết định số 2497/QĐ-BYT ngày 14/7/2010 của Bộ trưởng Bộ Y tế về việc ban hành "Hướng dẫn xử lý ổ dịch sốt xuất huyết".

Điều 4. Các ông, bà: Chánh Văn phòng Bộ, Vụ trưởng các Vụ Kế hoạch - Tài chính, Cục Khoa học Công nghệ và Đào tạo, Vụ Tổ chức cán bộ, Cục trưởng các Cục Y tế dự phòng, Cục Quản lý Khám, chữa bệnh; Viện trưởng các Viện Vệ sinh dịch tễ/Pasteur/Sốt rét - Ký sinh trùng - Côn trùng; Giám đốc Sở Y tế; Giám đốc Trung tâm Y tế dự phòng các tỉnh, thành phố; Thủ trưởng các cơ quan, đơn vị có liên quan chịu trách nhiệm thi hành Quyết định này./.

Nơi nhận:
- Như Điều 4;
- BT. Nguyễn Thị Kim Tiến (để báo cáo);
- Các đồng chí Thứ trưởng (để phối hợp);
- Cổng TTĐT Bộ Y tế;
- Lưu: VT, DP.

KT. BỘ TRƯỞNG
THỨ TRƯỞNG

Nguyễn Thanh Long

Supplementary Figure S1. Ethics approval