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Complete genome analysis of dengue virus type 3 isolated from the 2013 dengue outbreak in Yunnan, China

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ABSTRACT

In the past few decades, dengue has spread rapidly and is an emerging disease in China. An unexpected dengue outbreak occurred in Xishuangbanna, Yunnan, China, resulting in 1331 patients in 2013. In order to obtain the complete genome information and perform mutation and evolutionary analysis of causative agent related to this largest outbreak of dengue fever. The viruses were isolated by cell culture and evaluated by genome sequence analysis. Phylogenetic trees were then constructed by Neighbor-Joining methods (MEGA6.0), followed by analysis of nucleotide mutation and amino acid substitution. The analysis of the diversity of secondary structure for E and NS1 protein were also performed. Then selection pressures acting on the coding sequences were estimated by PAML software. The complete genome sequences of two isolated strains (YNSW1, YNSW2) were 10,710 and 10,702 nucleotides in length, respectively. Phylogenetic analysis revealed both strain were classified as genotype II of DENV-3. The results indicated that both isolated strains of Xishuangbanna in 2013 and Laos 2013 stains (KF816161.1, KF816158.1, LC147061.1, LC147059.1, KF816162.1) were most similar to Bangladesh (AY496873.2) in 2002. After comparing with the DENV-3SS (H87) 62 amino acid substitutions were identified in translated regions, and 38 amino acid substitutions were identified in translated regions compared with DENV-3 genotype II stains Bangladesh (AY496873.2). 27(YNSW1) or 28(YNSW2) single nucleotide changes were observed in structural protein sequences with 7(YNSW1) or 8(YNSW2) non-synonymous mutations compared with AY496873.2. Of them, 4 non-synonymous mutations were identified in E protein sequences with (2 in the β-sheet, 2 in the coil). Meanwhile, 117(YNSW1) or 115 (YNSW2) single nucleotide changes were observed in non-structural protein sequences with 31(YNSW1) or 30 (YNSW2) non-synonymous mutations. Particularly, 14 single nucleotide changes were observed in NS1 sequences with 4/14 non-synonymous substitutions (4 in the coil). Selection pressure analysis revealed no positive selection in the amino acid sites of the genes encoding for structural and non-structural proteins. This study may help understand the intrinsic geographical relatedness of dengue virus 3 and contributes further to research on their infectivity, pathogenicity and vaccine development.

1. Introduction

The dengue virus (DENV) is an arthropod-borne virus belonging to the *Flaviviridae* family, genus *Flavivirus*, and occurs as four antigenically related but distinct serotypes designated as DENV-1, 2, 3 and 4 (Nogueira et al., 2001). Currently, DENV-3 has five distinct genotypes (I to V) found in the following regions: Southeast Asia, the Indian subcontinent, the South Pacific, East Africa and the Americas (Aquino et al., 2009; Lanciotti et al., 1994; Wittke et al., 2002). The genome of DENV is a single-stranded RNA approximately 11 kb in length including untranslated regions (5′ UTRs, 3′ UTRs) and a single open reading frame (ORF). Translation of the single ORF generates one polyprotein

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that is cleaved by host- and virus-derived proteases to encode three structural proteins (capsid, premembrane, envelope) and seven non-structural proteins (NS1, NS2A,NS2B,NS3,NS4A,NS4B,NS5) (Lindenbach et al., 2007; Weaver and Vasilakis, 2009).

Dengue fever is the most important and rapidly spreading vector-borne viral disease in Southeast Asia, Central and South America, and Africa (Moi et al., 2010; Undurraga et al., 2013). China has seen an increase in human cases of dengue fever in recent years. Since 1978, dengue fever occurred epidemically every 4–7 years in China, commonly affecting people between the ages of 20–60 years old (Xiong and Chen, 2014; Yang et al., 2014). Dengue is an emerging disease in China, for instance, from 2009 to 2014, a total of 52749 cases of dengue fever and six deaths were reported, according to the China National Notifiable Disease Surveillance System (Chen and Liu, 2015). In 2013, more than 4500 DENV infection cases were reported in mainland China. Of these, more than 2800 cases were reported in Guangdong Province, and both DENV1 and DENV2 were reported by local CDC. Meanwhile, 37 cases were reported in Zhejiang, 36 cases in Henan, and 33 cases in Fujian (Hang Zhou et al., 2015). A total of 1331 cases were identified as DENV by dengue fever symptoms and positive DENV nonstructural protein (NS) 1 antigen detected with the Dengue Ag Rapid Test in Yunnan Province. Severe haemorrhage was seen in 28 patients, and severe plasma leakage in 27 patients (Hu et al., 2017; Lao et al., 2014). However, there were no death patients in this outbreak. Of all the DENV cases in Yunnan Province were mainly DENV serotype 3 and small amount of DENV serotype 1, 2, 4 (Hu et al., 2017). A lack of complete genome information was a major hindrance in understanding the DENV circulating in Yunnan. In this study, DENV-3 strains were isolated from patients serum samples collected during the outbreak in Yunnan Province. Two strains were selected for full-length gene sequencing followed by phylogenetic and selection pressure analyses. Further analyses of base substitutions in coding genes, amino acid mutations in structural proteins (C, prM and E) and nonstructural proteins protein molecules (NS1, NS2, NS3, NS4, NS5) were completed to better understand the DENV-3 circulating in this area.

2. Materials and methods

2.1. Ethics statement

Written consent was obtained from each participant. The study protocol was approved by the Institutional Ethics Committee (Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College) and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2000).

2.2. Virus isolation

During the dengue outbreak in the Yunnan Province in 2013, the serum samples were collected from viremic human patients at Xishuangbanna Dai Autonomous Prefecture People's Hospital (XDAPPH) in August. Ten strains were isolated from those serum samples, and two strains were selected randomly to be sequenced. These two strains were isolated from a 51 year old male patient and a 52 year old female patient. Both patients had mild symptoms and no record of going abroad. A few days before sample collection, these two patients developed symptoms of fever, with body rush and fatigue. Patient serum samples were diluted with RPMI 1640 culture medium using the ratio 1:10, and then 100 µl of the diluted serum was inoculated into 25 cm^2 bottle with a monolayer of C6/36 cells. After 2 h of adsorption, the supernatant was discarded and the cells were cultured in RPMI 1640 culture medium with 2% serum for 7d, followed by sub culturing three times. Detection of DENV type was conducted by RT-PCR (data not shown). Two isolated strains, at passage level 4 in C6/36 cells, were selected for complete genome sequencing.

2.3. Extraction of viral RNA

Viral RNA was extracted from 140 µl of dengue virus-infected culture supernatant using the QIAamp viral RNA mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Finally, RNA was eluted in 60 µl of nuclease-free water and stored at −80 °C.

2.4. Primer design

A total of 22synthetic oligo nucleotide primer pairs were designed to amplify overlapping fragments of sizes between 500 and 800 nt spanning the entire viral genome of DENV-3, based on the strain 98TW407 (GenBank accession no. DQ675528).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

A total of 22 overlapping amplicons spanning the complete genomic region were amplified using 44 primers. RT-PCR was carried out in one step with the following protocol: initial reverse transcription at 50 °C for 30 min; denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 37 s; and a final elongation step at 72 °C for 5 min. PCR products were confirmed by agarose gel electrophoresis and sent to Sangon Biotech (Shanghai, China) for sequencing.

2.6. Genome synthesis for amplification and sequencing

Total RNA was extracted from infected C6/36 cells. One-step PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China) was used to amplify the virus in 22 overlapping fragments by RT-PCR and directly sequence the amplicons.

2.7. Genetic analysis

The 22 sequences were assembled using DNASTAR version 7.0. The assembled nucleotide sequences and translated amino acid sequences were analyzed by BioEdit. The phylogenetic analysis, based on the complete envelope glycoprotein (E) protein genes, was conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (the neighbor-joining method) (Lao et al., 2014).The reference viral sequences used to construct the distinct phylogenetic branches were collected from the GenBank sequence database under the following country accession numbers: Fiji(L11422.1); Thailand (L11440.1, L11441.1, GQ868593.1); Viet Nam (EU482460.1, EU482461.1, FJ562103.1); Bangladesh (AY496873.2, DQ401691.1); China (GU363549.1); India (L11424.1); Puerto Rico (L11434.1); Indonesia (L11428.1); Tahiti(L11439.1); Malaysia (L11429.1); Samoa (L11435.1); Haiti (KT279761.2, KX702403.1); Brazil (KT794007.1); H87 (M93130.1); Viet Nam (EU482460.1, EU482461.1, FJ562103.1); Lao 2013 (KF816161.1, KF816158.1, LC147061.1, LC147059.1, LC147060.1, KF816162.1); China 2013 (KJ622198.1, KJ622191.1, KJ622195.1, KJ622194.1, KM651781.1, KF954947.1, KF954949.1, KF824903.1, KF824902.1); Solomon Islands 2013 (KU053467.1, KU053466.1); Indonesia 2013 (KF709425.1, KF709426.1); Singapore 2013 (KP685235.1, KR779787.1, KX224291.1, KX224280.1, KX224271.1); Tahiti 2013 (KU053472.1, KU053471.1); Paraguay 2013 (JF808129.1); Viet Nam 2013(KP176712.1, KP176711.1, KP176710.1, KP893717.1); Thailand 2013 (KP176108.1); India 2013 (KU216209.1); Pakistan 2013 (KM217133.1, KM217159.1).

2.8. Selection pressure analysis

To assess the selection pressure acting on individual codons of the ORF (C-prM/M, E, NS1, NS2, NS3, NS4, NS5), a dataset comprising of other close related genotypes DENV-3 (n = 20) was prepared (S8 Table). The non-synonymous to synonymous substitution rate ratio

omega (dN/dS, ω) were calculated among various lineages by CODEML in PAML 4.8.

3. Results

3.1. Base sequence analysis of the YNSW1 and YNSW2 sequences

RT-PCR amplification generated 22 specific amplicons, which were gel purified and sequenced. After editing and aligning the sequences of overlapping fragments, the complete genome sequences of the newly isolated strains (named YNSW1 and YNSW2) were determined. The complete genome sequence of the newly isolated strain YNSW1 was 10,710 nucleotides (nt) in length, with the ORF located between nucleotides 88–10260. Interestingly, the length of the other isolated strain, YNSW2, was 10,702 nt, with the ORF located between nucleotides 96–10268. The length of the two isolates was similar to that in previous reports (Zhang et al., 2014). The BioEdit analysis results identified the nucleotide composition of the YNSW1 strain as 32.18% A, 25.98% G, 21.22% U, and 20.63% C. In comparison, the nucleotide composition of the YNSW2 strain was 32.24% A, 25.94% G, 21.16% U, and 20.66% C. (Table S1) The values of A + G content in YNSW1 and YNSW2 were the same (58.19%), which was similar to the 58.14% found in the DENV-3 standard strain H87 (M93130.1) (Osatomi and Sumiyoshi, 1990) and the 58.16% found in the DENV-3 genotye II strain AY496873.2. The complete genome sequences of these two isolates were deposited into the NCBI GenBank database (http://www.ncbi.nim.nih.gov/GenBank/index.html): KR296743 and KR296744.

3.2. Phylogenetic analysis of the YNSW1 and YNSW2 sequences

The E protein genes of YNSW1 and YNSW2 were determined and analyzed by aligning their sequences with 55 other representative DENV-3 strains of diverse geographical origins retrieved from GenBank, including 38 DENV-3 strains collected in 2013 (Fig. 1). The result of phylogenetic analysis indicated that YNSW1 and YNSW2 strains were closest relative to the following strains: Laos 2013 (KF816161.1, KF816158.1, LC147061.1, LC147059.1, KF816162.1), China-Henan 2013 (KJ622198.1, KJ622191.1, KJ622195.1, KJ622194.1), China 2013 (KM651781.1, KF824902.1, KF824902.1), Bangladesh (AY496873.2, DQ496873.2), Viet Nam (EU482460.1, EU482461.1, FJ562103.1), Thailand (L11440.1), Bangladesh (AY496873.2, DQ496873.2). Thailand (L11440.1), Bangladesh (AY496873.2) and Bangladesh (DQ496873.2) strains was verified as genotype II (Islam et al., 2006). Laos (KF816161.1, KF816158.1, LC147061.1, LC147059.1, KF816162.1) were isolated in Laos in 2013, so YNSW1/YNSW2 and those Laos strains are likely to have originated in the Bangladesh.

3.3. Base substitution and amino acid mutation analysis of the YNSW1 and YNSW2 coding DNA sequences (CDS)

Base substitution mutations usually lead to alterations in coded amino acids. The Blastx-translated nucleotide sequence identity showed that YNSW1 and YNSW2 were highly matched, with an identity of 99%. The total number of amino acids in both YNSW1 and YNSW2 was 3391. As shown in Table 1, compared to genotype I H87 strain, the total number of base substitutions in YNSW1 and YNSW2 was 553 nt and 552 nt, respectively. The number of non-synonymous substitutions in both stains was 62 nt, with a rate of 11.23%. However, in comparison with genotype II strain AY496873.2. The total number of base substitutions in YNSW1 and YNSW2 was 143 nt and 144 nt. The number of non-synonymous substitutions in both stains was 38 nt, the rate of non-synonymous substitutions was 26.57% (YNSW1) and 26.39% (YNSW1).

3.3.1. Amino acid mutations in structural protein regions

In the structural protein regions of both strains, the nucleotide sequence coding for C-prM/M-E was 2319 nt in length and codes for a 773 amino acid sequence. Therein, the length of the capsid, pre-membrane, membrane and envelope amino acid sequences in both strains was 114, 91, 75 and 493, respectively. As identified in Table S1, compared to the DENV-3 standard strain H87, the total number of base substitution mutations in the C-prM/M-E region was 137 (YNSW2:138), and the number of non-synonymous substitutions was 15. Compared with AY496873.2, the total number of base substitution mutations in this region was 27 (YNSW2: 28) the number of non-synonymous substitutions was 7 (YNSW2: 8). Consequently, the rate of amino acid substitutions in the structural protein regions of the C-prM/M-E proteins was 1.94% compared to H87, and 0.91% (YNSW2:8: 1.03%) compared to AY496873.2 (Table 1 and Fig. 2).

There are three structural domains, DI (amino acids (aa) 1–52, 134–191, 280–295), DII (aa 53–133, 192–279), DIII (aa 296–394), and the C-terminal region (aa 394–495), which compose the E protein[16]. Compared with those two stains, separately, there were some single nucleotide changes identified in the E protein sequence; Of them, 12 non-synonymous mutations in H87, 4 non-synonymous mutations in AY496873.2; Compared with H87, three non-synonymous mutations were observed in the sequences encoding the β-sheet (140:I → T; 164:S → P; 270:T → N), and 7 non-synonymous mutations were observed in the sequences encoding the coil (154:E → D; 169:A → V; 225:K → E; 291:K → E; 391:R → K; 479:A → V; 479:A → V; 489:V → T). Only one non-synonymous mutation was observed in the sequences encoding the α-helix (447:S → G) compared with H87. However, compared with AY496873.2, there was no non-synonymous mutation was observed in the sequences encoding the α-helix, two non-synonymous mutation in β-sheet (160: V → A; I → V (YNSW2: I)), and two non-synonymous mutation in coil (81: T → I; 124: P → S). The detected mutations are summarized in Table S3 and Fig. 2.

As Fig. 2 indicates, within Domain I of the E protein, a T to C substitution at position 1330 changes the amino acid S (Serine) to P (Proline), which changes the polarity. In Domain II of the E protein, base substitution mutations of a A changed to G at positions 1513 and 1515 were found, and this substitution changed the amino acid K (Lysine) to E (Glutamic acid), which modified the acid-base properties of the protein from alkali to acid. Compared with AY496873.2, base substitution mutations of a C changed to T at positions 1082 was found, this substitution changed the amino acid T (Threonine) to I (Isoleucine), which changes the polarity; And base substitution mutations of a C changed to T at positions 1210 was found, this substitution changed the amino acid P (Proline) to S (Serine), which changes the polarity and the change of polarity could make the protein becomes lyophobic. In Domain III of the E protein, the amino acid transition of V (Valine) to T (Threonine) was due to a base mutation at position G2305A, T2306C, and C2307T, which creates a hydrophilic area.

3.3.2. Amino acid mutations in non-structural protein regions

In non-structural protein regions, the length of the NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 sequence in both strains was 7854 nt. Compared with H87, there were 49 single nucleotide changes identified in the NS1 region, including 14 non-synonymous substitutions. But only 4 non-synonymous substitutions were found in comparison with AY496873.2. (Fig. 2 and S4 Table) Compared with H87, 3 non-synonymous mutations were observed in the sequences encoding the β-sheet (83:D → N; 217:L → F; 350:A → V), 7 non-synonymous mutations were observed in the sequences encoding the coil (94:T → I; 103:T → A; 127:T → I; 139: S → N; 188: L → T; 198: V → I; 288:S → T), and 2 non-synonymous mutations were observed in the sequences encoding the α-helix (42: V → L; 120: L → K). The base substitution C2600T modified amino acid T (Threonine) to I (Isoleucine), which causes a polarity reversal from polar to nonpolar (in the coil). At amino acid location 188, L (Leucine) changed to T (Threonine), which was caused by the base substitution of T (Thymine) to C (Cytosine), and also caused a polarity reversal from polar to nonpolar (in the coil). Compared with AY496873.2, all 4 non-

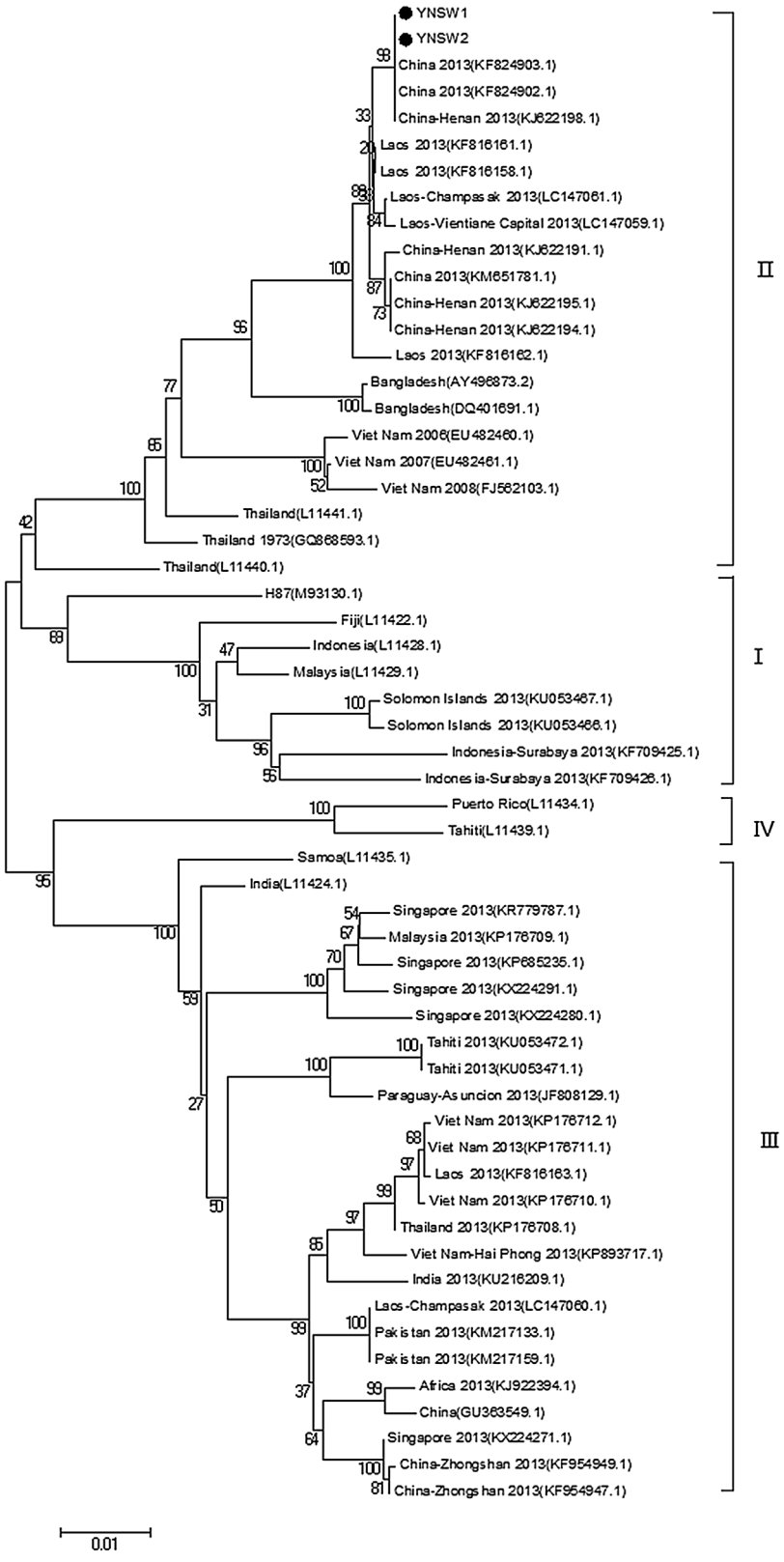

Fig. 1. A phylogenetic tree showing the genome sequence analysis of YNSW1, YNSW2 and other DENV-3 strains retrieved from the NCBI GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches.

synonymous mutations were observed in the sequences encoding the coil (103: T → A; 125: I → T; 128: T → I; 290: D → N). At amino acid location 103 and 128, T (Threonine) changed to A (Alanine) or I (Isoleucine), which caused a polarity reversal from polar to nonpolar. At amino acid location 125, I (Isoleucine) changed to T (Threonine), which caused a polarity reversal from nonpolar to polar. At amino acid location 290, D (Aspartic acid) changed to N (Asparagine), which caused acidic change to neutral.

Compared with H87, there were 64 base mutations found in the NS2A-NS2B region, and eight were non-synonymous substitutions. However, there were 13 base mutations found in this region, and 4 were non-synonymous substitutions compared with AY496873.2. Compared with H87, the V (Valine) to M (Methionine) change was caused by the base substitution G3496C, which led to a polarity reversal

Table 1
Amino acid substitutions in the translated regions.

	C-prM/M-E	NS1	NS2A/B	NS3	NS4A/B	NS5	Total	
Base substitutions	137[a]	49	64	103	60	138[b]	552[a]	YNSW1(YNSW2)VS H87
Base substitution rate	5.96%	4.63%	6.13%	5.55%	5.03%	5.11%	5.43%	
AA substitutions	15	12	8	12	4	11	62	
AA substitution rate	1.94%	3.41%	2.30%	1.94%	1.01%	1.22%	1.83%	
Base substitutions	27[a]	14	13	34	12	42[b]	143[a]	YNSW1(YNSW2)VS AY496873.2
Base substitution rate	1.16% (1.21%)	1.33%	1.25%	1.83%	1.01%	1.30% (1.36%)	1.41%	
AA substitutions	7[a]	4	4	6[a]	4	11[b]	38	
AA substitution rate	0.91% (1.03%)	1.14%	1.15%	0.97% (1.13%)	1.01%	1.02% (1.20)	1.12%	

[a] (YNSW2: +1).
[b] (YNSW1: +2).

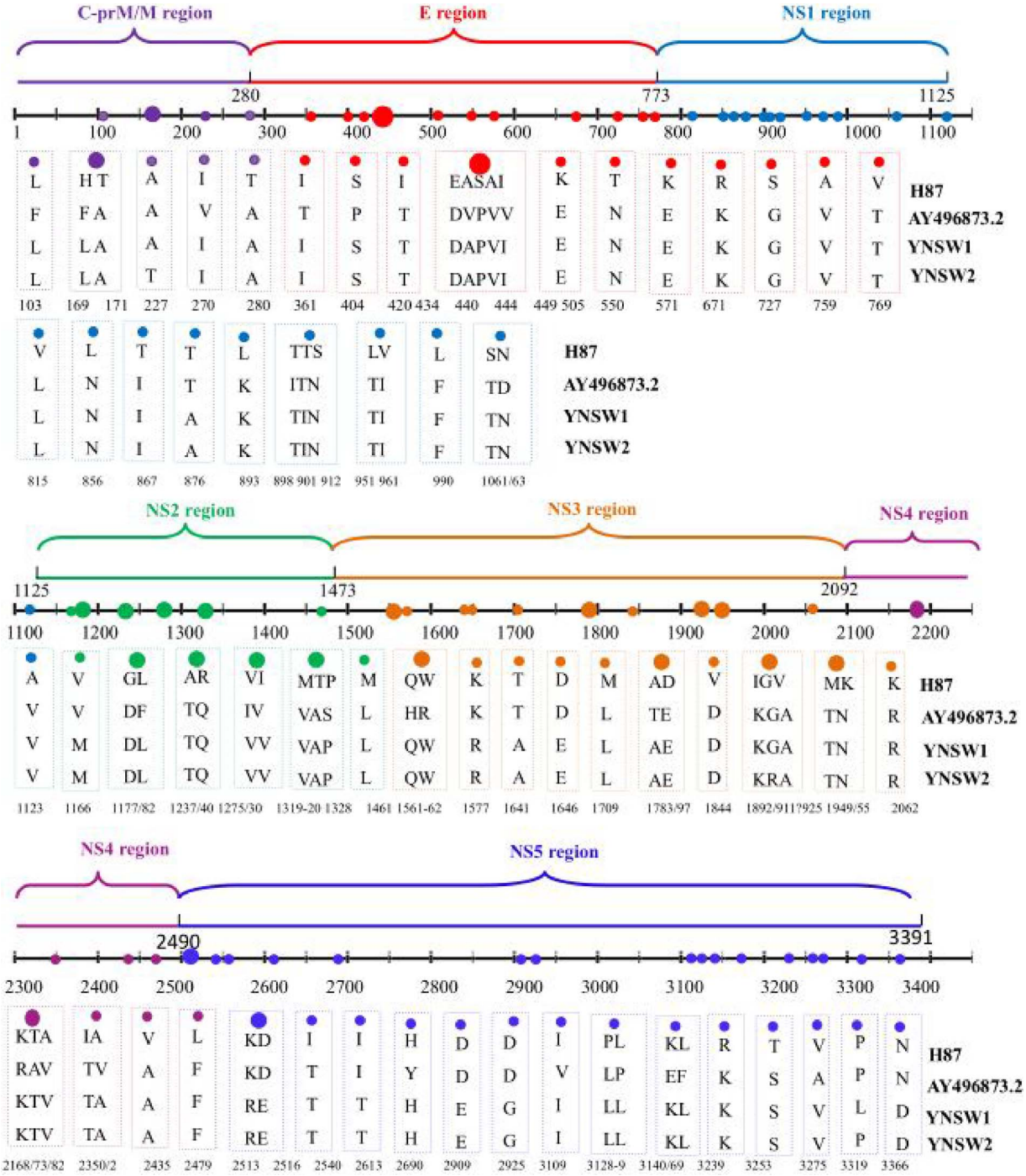

Fig. 2. Amino acid substitutions in the YNSW1 and YNSW2, compared to the DENV-3 standard strain H87 and DENV-3 genotype II strain AY496873.2.

from nonpolar to polar (Fig. 2 and S5 Table). The A3955G (V194M) modification cause polarity reversal from polar to nonpolar. In the NS2B region, there was only one sense amino acid mutation M (Methionine) to L (Leucine), which was caused by the A4381C base substitution. Compared with AY496873.2, there was no amino acid mutation in the NS2B region, but only 4 non-synonymous substitutions in NS2A (41:V → M; 57: F → L; 150:I → V; 203:S → P).

The total number of base substitution mutations in the NS3 region was 103, and the number of non-synonymous substitutions was 12 when compared with H87. Compared with AY496873.2, the number of substitution mutations in the NS3 region was 34, and 6 (YNSW2: 7) non-synonymous substitutions in the NS3 region. (Fig. 2 and S6 Table). Compared with H87 or AY496873.2, there were only four amino acid substitutions in NS4A-4B. The total number of base substitution mutations in the NS4A–4B region was 60 compared with H87, and 12 base substitution mutations in the NS4A–4B region compared with AY496873.2. The T7049C and I235T substitutions resulted in a polarity reversal from nonpolar to polar (Fig. 2 and S7 Table). Compared with H87, the total number of base substitutions in the NS5 region was 138 (YNSW1:140); there were 11 non-synonymous substitutions found in this region, with a substitution rate of 1.22%. Compared with AY496873.2, the total number of base substitutions in the NS5 region was 42 (YNSW2: 44); there were 12 (YNSW2:13) non-synonymous substitutions found in this region. (Fig. 2 and S8 Table) However, the physicochemical properties of the amino acids in those substitutions did not change.

3.4. Selection pressure analysis

The site specific selection pressure in DENV-3 was analyzed by CodeML. There was no positive selection in the sequence alignment of DENV structuraland nonstructural genes. The selective pressure analysis results are summarized in Table 2.

4. Discussion

Yunnan Province, which is located in Southwest China, has tropical and subtropical regions and shares a long border with its neighboring countries Laos, Myanmar and Vietnam, all of which are dengue-endemic areas in 2013. During the 2013 epidemic, a total of 1331 cases of dengue were reported in Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China. Of these cases, 1269 cases were reported in Jinghong City (including 1253 indigenous and 16 imported cases), 45 cases were reported in Mengla County (including 34 indigenous and 11 imported cases), and 17 imported cases were reported in Menghai County. (Hu et al., 2017).

Xishuangbanna Dai Autonomous Prefecture is located in Southeast Asia. Because of the relatively close geographical relations and outbreak of dengue fever in 2013, we selected isolated strains of Laos, Myanmar, Vietnam, Indonesia, Thailand and Singapore to carry out phylogenetic tree analysis. According to phylogenetic analysis, dengue virus 3 genotype I was distributed in 2013 in Indonesia; genotype II was distributed in Laos, and Viet Nam; Thailand and Singapore were genotype III. The newly isolated autochthonous strains YNSW1 and YNSW2 belonged to genotype II, and both of them were very closely to Laos (KF816161.1, KF816158.1, LC147061.1, LC147059.1, KF816162.1). In addition, both these Laos isolated strains and YNSW1/2 were close to Bangladesh-2002 (AY496873.2). Geographically, Bangladesh is next to Myanmar, the neighboring country of Laos. Although those two patients were never going abroad, but DENV could be transmitted by mosquito-borne. Both those Laos isolated strains and YNSW1/2 were strong possibility originated in the Bangladesh.

Table 2
The results of selective pressure analysis.

Regions	2ΔlnL		Positive selection
	M1 vs M2	M7 vs M8	
C-prM-M	0.03	0.25	NA
E	0	0	NA
NS1	0	0	NA
NS2	0	0	NA
NS3	0	0	NA
NS4	0	0	NA
NS5	0	0	NA

The degrees of freedom in M1 vs M2 or M7 vs M8 is 2.

In order to investigate amino acid substitution in the YNSW1 and YNSW2, both DENV-3 genotype II strain AY496873.2 (Bangladesh-2002) and genotype I strain H87 (Philippine-1990) for analysis, the result showed that amino acid substitution occur in both structure and non-structure proteins of YNSW1 and YNSW2, which may result in structural and functional changes of the protein.

As indicated in previous studies, the capsid protein (C) is essential for RNA genome encapsidation, which has an unusually high net charge (Chang et al., 2001). The membrane protein (prM), which is cleaved in the trans-Golgi network (TGN) by a cell-encoded furin-like protease during the late stages of virus assembly, is necessary for the release of mature virions (Kuhn et al., 2002). There were 3 non-synonymous substitutions in C-prM region compared with AY496873.2.

The E protein is responsible for cell receptor binding, which is the main target of neutralizing antibodies and vaccine development (Lindenbach et al., 2007; Webster et al., 2009; Whitehead et al., 2007). There were some nucleotide changes identified in the sequences coding for the E protein, and this led to amino acid substitutions. The transitions of amino acids in the E region may led to structural changes in protein cell entry and virus immunogenicity. Previous reports indicated that there are 10 highly conserved regions in E protein, namely, N8-G14, V24-D42, R73-E79, V97-S102, D192-M196, V208-W220, V252-H261, G281-C285, E314-T319, E370-G374, K394-G399, and R411-S424 (Mazumder et al., 2007). In this study, no amino acid mutations were found in those highly conserved regions. Each of the monomer subunits in the E protein comprises three distinct domains: (1) Domain I: α β-barrel structure oriented parallel to the viral membrane, and the α β-barrel is assembled by α-helixes and β-sheets; the amino acid substitution of S (Serine) to P (Proline) (T1330C) may influence the β-barrel structure and its bonding function. (2) Domain II, a finger-like structure composed of a pair of discontinuous loops, one of which is highly conserved among flavivirus and contains the hydrophobic 'fusion' peptide (residues 98–110) essential for virus-cell fusion; three amino acid substitutions were identified in this domain; however, no relevant amino acid transitions led to possible secondary structural changes. Compare with H87, the amino acid substitution of V (Valine) to T (Threonine), A(Alanine) to T (Threonine), A(Alanine) to V (Valine) in the transmembrane domain of the C-terminal (TM1 and TM2) (452–496) (Modis et al., 2004; Zhang et al., 2003) changed this area to hydrophilic and receded the bonding between the virus and cell member, none amino acid substitution, compared with AY496873.2.

Nonstructural proteins are essential for viral replication. NS1 can be secreted as a soluble hexamer in the sera of patients, which forms a lipoprotein particle with an open-barrel protein shell and a prominent central channel rich in lipids. NS1 is used as a diagnostic marker to detect acute DENV infections (Chuang et al., 2013; Gutsche et al., 2011). This study identified 49 single nucleotide changes in the NS1 region, non-synonymous substitutions, including 12 non-synonymous substitutions compared with H87, 4 non-synonymous substitutions compared with AY496873.2. Mutations leading to a polarity reversal may modify protein folding and skew the diagnostic marker results.

The NS2A and NS2B proteins could regulate viral protease activity and are essential for viral replication (Li et al., 2015; Nemesio and Villalain, 2014). Compared with H87, there were 7 non-synonymous substitutions found in NS2A; however, only 1 was identified in NS2B.

Compared with AY496873.2, there were 4 non-synonymous substitutions found in NS2A; none was identified in NS2B. DENV RNA replication was reported to occur within endoplasmic reticulum (ER)-derived structures called vesicle packets (VPs) (Welsch et al., 2009). NS3 is responsible for processing the polyprotein at all junctions on the cytoplasmic side of the ER and also assists the RNA-dependent RNA polymerase residing in NS5, which mediates the capping of the neo synthesized viral genomes (Chatel-Chaix et al., 2015). There were 12 non-synonymous substitutions identified in the NS3 protein compared with H87, 7 non-synonymous substitutions compared with AY496873.2.

Previous studies have reported that among nonstructural proteins, NS4 and NS5 are the largest enzymes and the most conserved protein components of the multi-protein replication complex (Zhao et al., 2015). However, compared with AY496873.2, 2 non-synonymous substitutions were still found in the NS4 and NS5 regions, respectively.

Nucleotide substitutions in viral genomes may affect virus assembly and host immunologic responses. Our study may help us understand the intrinsic geographical relatedness and biological differences of Dengue viruse and contributes further to research on their infectivity, pathogenicity and vaccine development.

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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.2017.06.015.

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