



Research Paper

Genotype replacement of dengue virus type 3 and clade replacement of dengue virus type 2 genotype Cosmopolitan in Dhaka, Bangladesh in 2017

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ABSTRACT

Dengue is a mosquito-borne disease that has spread to > 100 countries and is caused by the dengue virus (DENV), which belongs to the *Flavivirus* genus of the family *Flaviviridae*. DENV comprises 4 serotypes (DENV-1 to -4), and each serotype is further divided into distinct genotypes. In India, it is reported that all 4 serotypes of DENV co-circulate. Although Bangladesh is a neighboring country of India, very few reports have published DENV sequence data for the country, especially after 2012. To understand the current distribution of DENV genotypes in Bangladesh, we determined the nucleotide sequences of envelope regions obtained from 58 DENV-positive patients diagnosed at Apollo Hospitals Dhaka during the period between September 2017 and February 2018. We found 5 DENV-1, 47 DENV-2, and 6 DENV-3 serotypes. A phylogenetic analysis of the obtained viral sequences revealed that DENV-3 genotype I was present instead of DENV-3 genotype II, which was predominant in Bangladesh between 2000 and 2009. Furthermore, we found two distinct lineages of the Cosmopolitan genotype of DENV-2, one of which was closely related to strains from Southeast Asia and has never been reported previously in Bangladesh. These results indicated that DENVs in Bangladesh have increased in genotypic diversity and suggest that the DENV genotypic shift observed in other Asian countries also might have been taking place in Bangladesh.

1. Introduction

Dengue virus (DENV) is a mosquito-borne virus that causes various clinical symptoms including dengue fever with and without warning signs and severe dengue classified by the WHO criteria of 2009 (Hadinegoro, 2012). DENV belongs to the *Flavivirus* genus of the family *Flaviviridae* and contains a single positive-stranded RNA of approximately 11 kb as a genome. The DENV genomic RNA has a single open reading frame (ORF) that encodes ten proteins consisting of three structural proteins (C, prM, and E) and seven non-structural proteins

(NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Henchal and Putnak, 1990). At both the 5' and 3' ends, the DENV ORF is flanked by untranslated regions (5'-UTR and 3'-UTR). There are four distinct serotypes of DENV (DENV-1, DENV-2, DENV-3, and DENV-4) (Holmes and Twiddy, 2003). Within each serotype of DENV, four to six geographically distinct genotypes have been reported. DENV-1 includes genotypes I, II, III (sylvatic), IV, V, and VI (Goncalvez et al., 2002; Pyke et al., 2016). DENV-2 includes Asian-I, Asian-II, Asian/American, American, Cosmopolitan, and sylvatic (Anez et al., 2011; Khan et al., 2013; Twiddy et al., 2002). DENV-3 includes genotypes I, II, III, IV, and

Abbreviations: DENV, dengue virus; ORF, open reading frame; CHIKV, chikungunya virus; tMRCA, time of the most recent common ancestor; MCMC, Markov chain Monte Carlo; UCLN, uncorrelated relaxed log-normal clock; MCC, Maximum Clade Credibility; FBS, fatal bovine serum; CPE, cytopathic effects; HPD, highest probability density

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V (Wittke et al., 2002). DENV-4 includes genotypes I, IIA, IIB, III, and sylvatic (AbuBakar et al., 2002; Klungthong et al., 2004; Lanciotti et al., 1997).

It has previously been reported that the replacement of a predominantly circulating dengue serotype and/or genotype is associated with increases in the incidence and severity of dengue (Nunes et al., 2016; Vu et al., 2010). In addition, viral evolution is one of the factors conferring higher DENV virulence (Rico-Hesse, 2010). Thus, the geographical movement as well as evolution of DENV need to be considered in order to control dengue infection (Kyle and Harris, 2008; Rico-Hesse, 2010).

Dhaka is the capital of Bangladesh with over 18 million people in an area of 1463.60 km². DENV infection is an emerging health problem in Bangladesh (Dhar-Chowdhury et al., 2017). From 2000 to 2002, there was a sudden surge of dengue fever cases with an average of 65 deaths (Rahman et al., 2002). Bangladesh is located next to India, which is a highly endemic area of dengue virus with all 4 serotypes of DENV having been reported (Racherla et al., 2018). DENV-3 was isolated for the first time from patients in Bangladesh in 1964 (Russell et al., 1966), and DENV-3 was found as the main circulating serotype during the 2000 to 2002 outbreaks (Aziz et al., 2002; Islam et al., 2006; Podder et al., 2006). Thereafter, serotype data were not available until 2012. High (80%) and widespread seroprevalence of DENV infections were reported in Dhaka in 2012 (Paul, 2014). A recent report showed that DENV-1 and DENV-2 were circulating serotypes in 3 major cities in Bangladesh, including Dhaka, during the years 2013–2016 (Muraduzzaman et al., 2018). Thus, the dominant dengue serotype in Dhaka changed to DENV-1 and DENV-2 from DENV-3 between 2002 and 2013. However, only a limited number of DENV sequences have been reported from Bangladesh, and no DENV sequences have been reported since 2012. We recently found that the genetic diversity of DENV-2 has increased and that the DENV-3 genotype has shifted from type II to III in Thailand (Phadungsombat et al., 2018). We speculated that a genotype shift might also be taking place in Bangladesh and started surveillance of DENV genotypes at Apollo Hospitals Dhaka in Bangladesh.

2. Materials and methods

2.1. Clinical specimens and ethical clearance

We conducted hospital-based surveillance at Apollo Hospitals Dhaka in Bangladesh. The study proposal was approved by the Research and Ethical Practice Committee of Apollo Hospitals Dhaka (approval number ERC 16/2018-3). As a routine assay for febrile patients who visited Apollo Hospitals Dhaka, 3 ml whole blood samples from adults and 0.5 ml to 1 ml from pediatric patients clinically suspected of having either chikungunya or dengue were collected. Viral RNA was extracted from 0.2 ml of serum according to the kit manufacturer's protocol (QIAamp MinElute Virus Spin Kit, Qiagen, Germany) and stored at –80 °C if not used immediately. Then, a CE-IVD approved commercial one-step reverse transcriptase real-time PCR kit (Fast Track Diagnostics, Luxembourg) was used for the detection of chikungunya virus (CHIKV) or DENV on a thermocycler, Rotor Gene Q (Qiagen, Germany), according to the kit manufacturer's instructions. Each run was performed with a negative control (no template) and positive controls for CHIKV and DENV. For DENV serotype identification, we used the commercial Genesig one-step reverse transcriptase real-time PCR kit from Primerdesign with the Rotor Gene Q thermocycler according to the kit manufacturer's instructions. Four DENV serotype-specific primers and probe mixes were provided in a single tube, and amplified products were detected through the four different channels as described in the kit contents (Rahman et al., 2019). All analyzed human samples were anonymized, and RNA stored at –80 °C was used for DENV envelope region analysis in this study. Two of DENV-1, seven of DENV-2, and two of DENV-3 sera, which contained high titers of viruses, were selected

and used for DENV isolation and whole genome analysis.

2.2. Sequence determination of envelope regions

Five microliters of stored DENV positive RNA were subjected to reverse transcription followed by PCR amplification of the entire envelope region of DENV by using One-step RT-PCR (Qiagen, Germany) in 50- μ l reactions. Five μ l of the amplified product were further subjected to nested PCR by using PrimeSTAR GXL polymerase (Takara, Japan) for amplification in 50- μ l reactions. The nested-PCR product was used for cycle sequencing with BigDye terminator ver 1.1 (Applied Biosystems, USA) and analyzed by an ABI 3130XL sequence analyzer. The details of the PCR and sequences of primer pairs are listed in S1 Table.

2.3. Phylogenetic analysis of the envelope region

The obtained DENV envelope sequences were aligned to the known genotype sequences retrieved from GenBank (<http://ncbi.nlm.nih.gov/genbank>) based on classification in previous studies (AbuBakar et al., 2002; Anez et al., 2011; Goncalvez et al., 2002; Khan et al., 2013; Klungthong et al., 2004; Lanciotti et al., 1994; Messer et al., 2003; Pyke et al., 2016; Twiddy et al., 2002; Wittke et al., 2002).

The DENV-1 genotyping dataset consisted of 27 envelope region sequences (1485 bp in length) including 6 distinct genotypes as follows: I ($n = 12$), II ($n = 1$), III ($n = 1$), IV ($n = 6$), V ($n = 6$), and VI ($n = 1$). We included 4 strains reported from Bangladesh and 5 sequences obtained in the present study. An additional 6 sequences that showed high levels of identity to the sequences obtained in the present study were also included. A total of 42 sequences were used for the phylogenetic analysis (S2 and S3 Tables).

The DENV-2 genotyping dataset (1485 bp in length) was composed of 42 sequences including 5 distinct genotypes as follows: Asian-I ($n = 9$), Asian-II ($n = 6$), Cosmopolitan ($n = 22$), American ($n = 2$), and Asian/American ($n = 3$). In addition, we included 12 sequences reported from Bangladesh and 47 sequences obtained in the present study. An additional 8 sequences that showed high levels of identity to the sequences obtained in the present study were also included. A total of 109 sequences were used for the phylogenetic analysis (S2 and S3 Tables).

The DENV-3 genotyping dataset (1475 bp in length) consisted of 30 sequences including 5 distinct genotypes as follows: I ($n = 7$), II ($n = 7$), III ($n = 7$), IV ($n = 3$), and V ($n = 6$). We included 35 previously reported sequences from Bangladesh and 6 sequences obtained in the present study. An additional 7 sequences that showed high levels of identity to the sequences obtained in the present study were also included. A total of 78 sequences were used for the phylogenetic analysis (S2 and S3 Tables).

Phylogenetic trees were inferred from the alignment using the maximum likelihood (ML) approach generated in W-IQ-TREE (Trifinopoulos et al., 2016), <http://iqtree.cibiv.univie.ac.at/>. The best-fit model was selected by ModelFinder (Kalyaanamoorthy et al., 2017), and an ultrafast bootstrap (Hoang et al., 2018) with 1000 replicates was calculated.

2.4. Divergence time estimation

Another 2 datasets of the envelope region were prepared for Bayesian phylogenetic analyses. The dataset of DENV-2 genotype Cosmopolitan was composed of 47 sequences obtained in the present study and 154 sequences retrieved from GenBank. The dataset of DENV-3 genotype I was composed of 6 sequences obtained in the present study and 146 sequences retrieved from GenBank. All of the DENV sequences were selected to represent samples obtained across a broad geographical area from multiple different countries in which the virus is known to have circulated during the interval from 1975 to 2017. The sequences of sylvatic strains, laboratory strains, and recombinants were

excluded from the dataset. All of the sequences were annotated in a format consisting of accession number-country-year of isolation. Before phylogenetic molecular clock analysis, the sequences within the 2 datasets of DENV-2 genotype Cosmopolitan and DENV-3 genotype I (S4 Table) were explored for temporal signals to identify the data quality, potential contaminants, and cryptic recombinants by investigating the regression of genetic divergence and sampling time by root-to-tip analysis using TempEst (Rambaut et al., 2016). The exhibition of a high degree of correlation indicated appropriate samplings for further molecular clock analysis. The time of the most recent common ancestor (tMRCA) and rate of evolution (substitution/site/year) were estimated using BEAST v1.8.4 (Drummond et al., 2012), in which Bayesian Markov chain Monte Carlo (MCMC) analysis was conducted using a SRD06 substitution model (Shapiro et al., 2006). The combinations of molecular clock models (strict and uncorrelated relaxed log-normal clock (UCLN) models) and the coalescent priors (Constant, Exponential, Bayesian Skyline, and GMRF Bayesian Skyride) were run with the MCMC length of chain for 50–200 million generations with sampling every 500–2000 and 10% burn-in. BEAGLE was used in parallel with BEAST to enhance the running speed (Ayes et al., 2012). The parameter traces were monitored using Tracer v1.7.1 with Effective Sample Size values over 200 ($ESS > 200$) (Rambaut et al., 2018). To select the best-fit model, the log marginal likelihoods of different models were estimated using path sampling and stepping-stone sampling methods for the Bayes factor (BF) test (Baele et al., 2012; Baele et al., 2013a; Baele et al., 2013b; Kass and Raftery, 1995). The strength of evidence against the null hypothesis (H_0) of the model showing lower marginal likelihood was evaluated as follows: $2\ln BF < 2$ indicates no evidence; 2–6 weak evidence; 6–10 strong evidence; and > 10 very strong evidence. The best-fit model was the combination of UCLN and Bayesian Skyline for either DENV-2 genotype Cosmopolitan or DENV-3 genotype I, respectively (S5 Table). The Maximum Clade Credibility (MCC) tree was generated and annotated with posterior probability by TreeAnnotator v1.8.4 (Drummond et al., 2012) and visualized in Figtree v 1.4.3.

2.5. Virus isolation, library construction, and next-generation sequencing

Among available serum specimens, we selected two of DENV-1, two of the minor lineage of DENV-2, five of the major lineage of DENV-2, and two of DENV-3 to isolate the viruses. The DENV positive sera were diluted with Leibovitz's L-15 medium (Hyclone, USA), filtered through sterile 0.22 μm membranes, and then inoculated to confluent *Ae. albopictus*-derived cells, C6/36. After overnight incubation at 28 °C, the inocula were removed and replaced with L-15 supplemented with 2% fetal bovine serum (FBS) and 0.3% tryptose phosphate broth. The cells were incubated at 28 °C until cytopathic effects (CPE) were observed. Viruses were further propagated in Vero cells in Minimum Essential Medium (Gibco, USA) supplemented with 2% FBS at 37 °C, 5% CO_2 . Viral RNA was extracted from culture supernatants of Vero cells using a QIAamp viral RNA mini kit (QIAGEN, Germany). To determine the whole genome sequence of DENV, two overlapping cDNA fragments covering the full-length viral genome including 3' and 5' non-coding regions were synthesized using a SuperScript III first-strand synthesis system (Invitrogen, USA) with primer pairs listed in S6 Table. The amplified double-stranded (Dhar-Chowdhury et al.) DNAs were quantified and normalized to 0.2 ng/ μl using a Qubit 2.0 fluorometer (Invitrogen, USA). Both the 3'- and 5'-amplicons of each virus isolate were pooled and processed for next-generation sequencing using an Illumina Nextera XT library preparation kit (Illumina, USA) to generate paired-end sequencing libraries. The paired-end sequencing of 2×250 bp was processed on the Illumina MiSeq platform using MiSeq v2 kit (500 cycles). FASTQ files were imported into CLC Genomics Workbench software (CLC Bio, QIAGEN). The forward and reverse reads were aligned to DENV1 Mochizuki (AB074760), DENV2 16,681 (NC 001474), DENV3 H87 (M93130), and DENV4 H241 (AY947539) using map read references, and the consensus sequences were

extracted.

2.6. Phylogenetic datasets for open reading frame (ORF) sequences

Eleven whole genome sequences obtained in the present study were trimmed to yield ORF regions of 10,179, 10,176, and 10,173 bp lengths for DENV-1, DENV-2, and DENV-3, respectively. For ORF datasets, available DENV coding sequences were retrieved from GenBank. The DENV-1 dataset consisted of 28 sequences including 6 distinct genotypes as follows: I ($n = 12$), II ($n = 1$), III ($n = 1$), IV ($n = 7$), V ($n = 6$), and VI ($n = 1$). The DENV-2 dataset was composed of 42 sequences including 5 distinct genotypes as follows: Asian-I ($n = 10$), Asian-II ($n = 6$), Cosmopolitan ($n = 21$), American ($n = 2$), and Asian/American ($n = 3$). Sylvatic DENV-2 strains were not included in this analysis. The DENV-3 dataset consisted of 26 sequences including 4 distinct genotypes as follows: I ($n = 7$), II ($n = 7$), III ($n = 6$), and V ($n = 6$). The DENV-4 dataset was composed of 27 sequences and included 4 distinct genotypes as follows: I ($n = 11$), II-A ($n = 5$), II-B ($n = 7$), and III ($n = 4$) (S7 Table). Zika virus (AY632535) was used as an outgroup.

3. Results

3.1. DENV-1 genotype V is circulating in Dhaka, Bangladesh

Among the 251 DENV-PCR positive specimens, stored RNA was available in 181 cases. These RNA specimens were analyzed by a Dengue Virus Serotyping Multiplex PCR Kit (Genesig) to determine the serotypes of DENV. There were 7 DENV-1, 147 DENV-2, and 7 DENV-3 cases (Rahman et al., 2019). We failed to determine the DENV serotypes of 20 samples, probably due to low levels of DENV RNA in those stored RNAs. Among the 7 DENV-1 specimens, we were able to determine the nucleotide sequences of envelope regions in 5 specimens. As shown in Fig. 1, the ML phylogenetic analysis revealed that the currently circulating DENV-1 in Bangladesh was genotype V. Only four DENV-1 envelope sequences from Bangladesh were deposited in the NCBI database before 2012 (Yang et al., 2018). All Bangladesh DENV-1 sequences were clustered together in the same cluster, which also contained sequences from India, Singapore (Hapuarachchi et al., 2016), and China (Wang et al., 2015; Zhou et al., 2016) (Fig. 1). These results indicated that DENV-1 genotype V is the predominant genotype within Bangladesh.

3.2. Clade replacement of DENV-2 cosmopolitan in Dhaka, Bangladesh

For DENV-2, we determined nucleotide sequences of the envelope regions in 47 specimens that were selected from 147 DENV-2 specimens because of their relatively high levels of DENV RNA. All of the 47 sequences obtained in the present study were genotyped as Cosmopolitan as shown in Fig. 2. The ML phylogenetic tree revealed that those Cosmopolitan sequences had separated into two statistically-supported distinct clusters (Bootstrap support = 100%). Almost all Bangladesh DENV-2 sequences (forty-four) grouped as a major cluster that was close to Singapore, Philippines, Malaysia, China, and Thailand DENV-2 sequences (Fig. 2). The other three minor sequences (14e-S1482-2017, 70e-S1285-2017, and 16e-S1431-2017) fell into another clade close to viruses from Ethiopia and India (Dash et al., 2013) within a monophyletic clade (Bootstrap support = 100%). On the other hand, the 12 Bangladesh DENV-2 Cosmopolitan envelope sequences deposited in the NCBI database collected between 2004 and 2011 (Moore et al., 2017; Shu et al., 2009) were located in a different branch from those of the 44 major sequences and three minor sequences obtained in the present study, and were more closely related to sequences from Sri Lanka, Pakistan (Akram et al., 2015), and India (Bootstrap support = 100%). These results indicated that two previously unrecognized clades of DENV-2 Cosmopolitan viruses had been introduced and might have

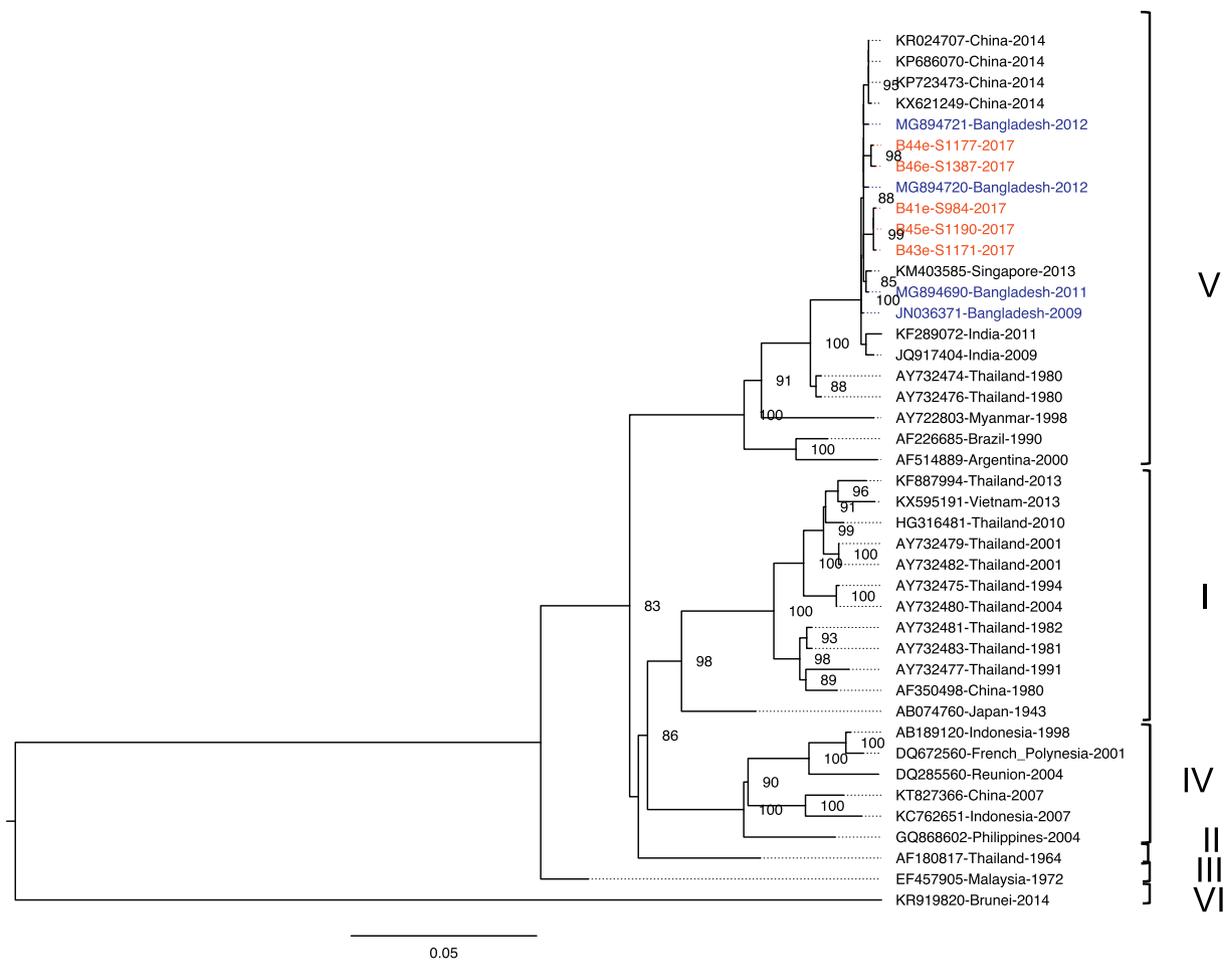


Fig. 1. Genotyping of the DENV-1 envelope region.

A maximum likelihood phylogenetic tree was constructed using the W-IQ-TREE program in ModelFinder, and an ultrafast bootstrap (UFBoot) of 1000 replicates was calculated. TIM2 + F + G4 was chosen as the best-fit model according to Bayesian information criteria. Data include DENV envelope-encoding sequences obtained in the present study (labeled in red) along with sequences with known genotypes obtained from GenBank. The sequences submitted from Bangladesh are shown in blue. Viral genotypes are indicated to the right. Virus names are shown as the accession number, country, and reported year of each sequence. Numbers to the right of the branches are UFBoot support values exceeding 75%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

replaced the previously predominant viruses in Dhaka, Bangladesh.

We then analyzed the molecular signatures of the newly emerged DENV-2 in Bangladesh. Variations in amino acid substitutions among DENV-2 were analyzed based on the envelope sequences in the corresponding phylogenetic tree (Fig. 2). In particular, our 44 major sequences shared amino acid residue 52H while 3 minor sequences (14e-S1482-2017, 16e-S1431-2017, and 70e-S1285-2017) shared 52Q. A substitution of T226I was uniquely observed in our 44 major sequences. Among these 44 sequences, 18 sequences possessed 164I, while the remaining 26 sequences shared 164V, and 3 sequences (62e-S1116-2017, 67e-S1218-2017, and 78e-S1570-2017) possessed the I322V substitution. Three other sequences (25e-S882-2017, 50e-S1051-2017, and 51e-S1056-2017) possessed the I378V substitution, which is rarely found in DENV-2. Furthermore, the amino acid residues 141I, 162V, and 322I were shared among the 3 minor sequences in the present study, LC121816 and JX475906, while 141V, 162I, and 322V were observed among previously reported Bangladeshi sequences. These results also support the hypothesis that there were 2 distinct novel clusters of DENV-2 Cosmopolitan circulating in Bangladesh in 2017.

3.3. Time of introduction of the new DENV-2 cosmopolitan lineage into Bangladesh

As shown in Fig. 3a, root-to-tip regression analysis of the DENV-2 Cosmopolitan envelope dataset (S4 Table) showed a high degree of correlation ($R^2 = 0.89$) between the collection year and divergence, suggesting appropriate samplings for molecular clock analysis. Bayesian phylogenetic analysis using this dataset, constructed using UCLN and the Bayesian Skyline coalescent prior as the best-fit model (S5 Table), revealed that the time of common ancestor of this dataset was in 1959.9 (95% highest probability density (HPD): 1955.1–1965.3). The mean substitution rate was 9.06×10^{-4} substitutions per site per year (95% HPD: 7.11×10^{-4} – 1.10×10^{-3} substitutions per site per year), which was similar to our previous report in Thailand, 8.81×10^{-4} substitutions per site per year (Phadungsombat et al., 2018). The Cosmopolitan Bayesian MCC phylogenetic tree comprised 3 lineages (posterior probabilities = 1) designated as lineages A, B, and C. The tMRCA of lineage A was estimated as 1967.4 (95% HPD: 1964.6–1970.3), and viral strains within this lineage were detected in Indonesia in 1975–1976, Burkina Faso in 1983, 1986, and 2016, Somalia in 2011, and Ivory Coast in 2017. Lineage B contained viruses isolated in South Asia (i.e., India, Pakistan, Bangladesh, and Sri Lanka) as well as China, Saudi Arabia, and recently found viruses in Kenya.

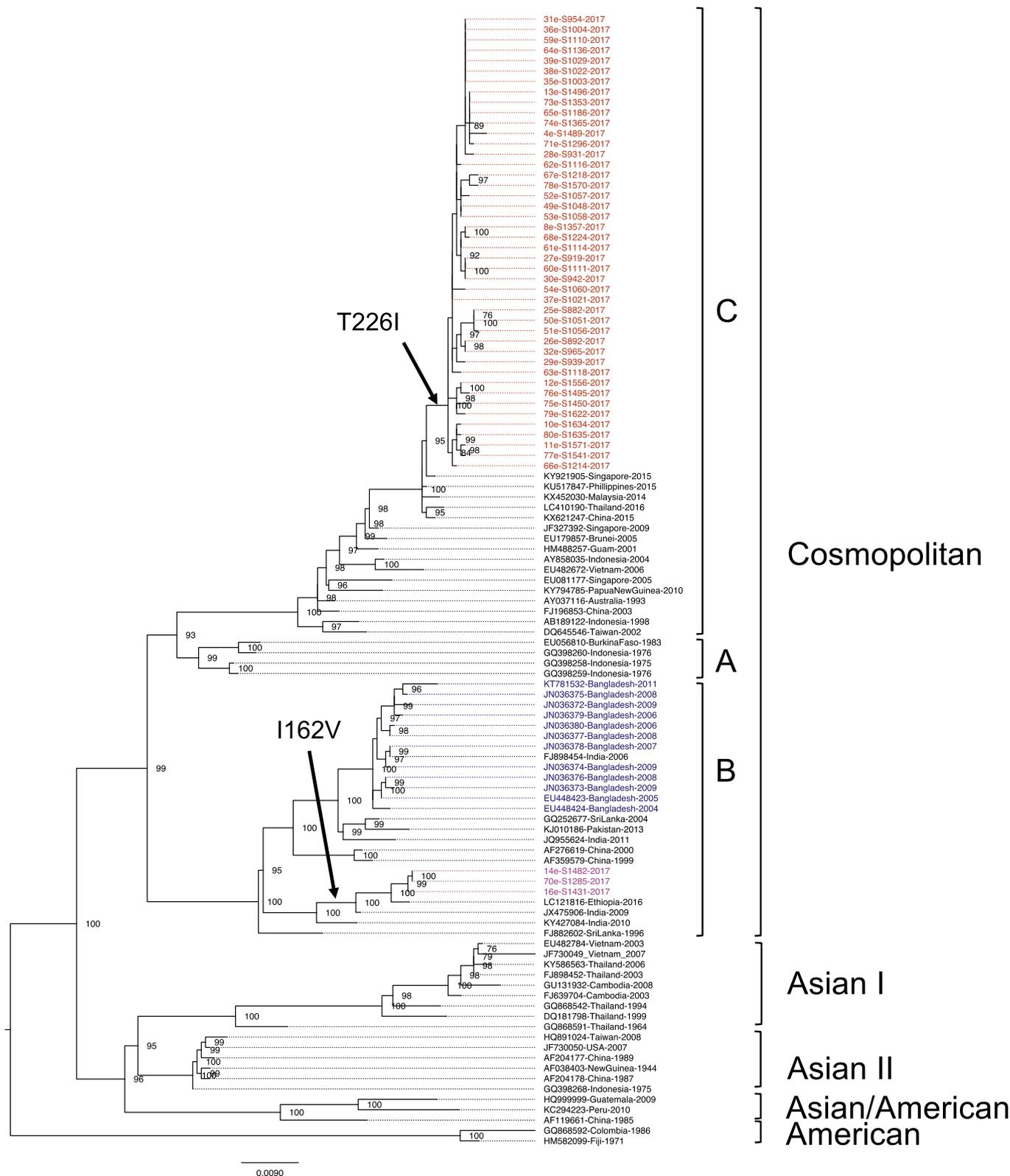
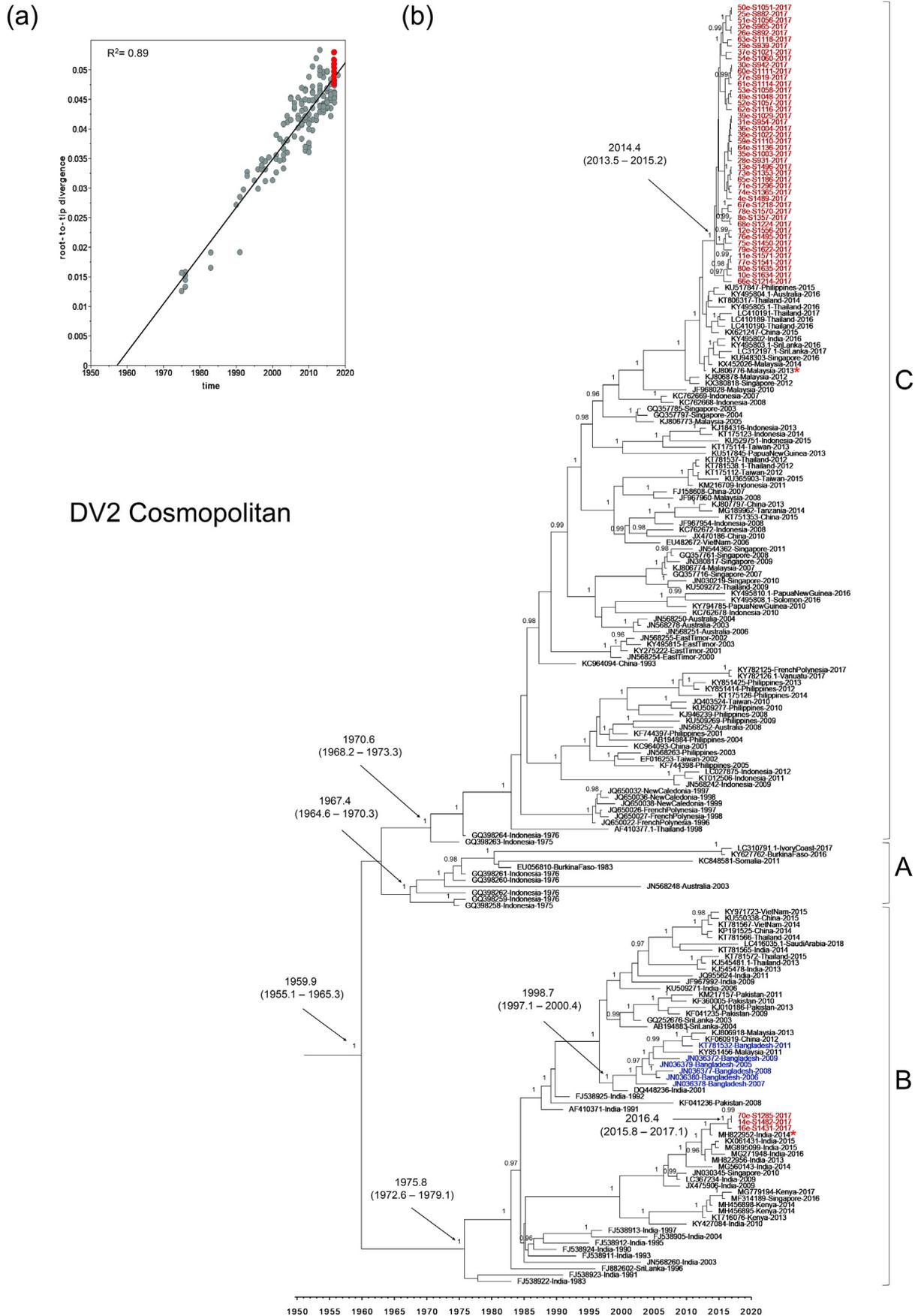


Fig. 2. Genotyping of DENV-2 in the envelope region.

A maximum likelihood phylogenetic tree was constructed using the W-IQ-TREE program in ModelFinder, and an ultrafast bootstrap (UFBoot) of 1000 replicates was calculated. TF + F + G4 was chosen as the best-fit model according to Bayesian information criteria. Data include DENV envelope-encoding sequences obtained in the present study (labeled in red and purple) along with sequences with known genotypes obtained from GenBank. The sequences submitted from Bangladesh are shown in blue. The viral genotypes are indicated to the right. Clusters corresponding to DENV-2 Cosmopolitan lineages A, B, and C, defined in Fig. 3b, are also indicated to the right. Virus names are shown as the accession number, country, and reported year of each sequence. Numbers to the right of the branches are UFBoot support values exceeding 75%. Unique amino acid substitutions shared in our sequences are shown with arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



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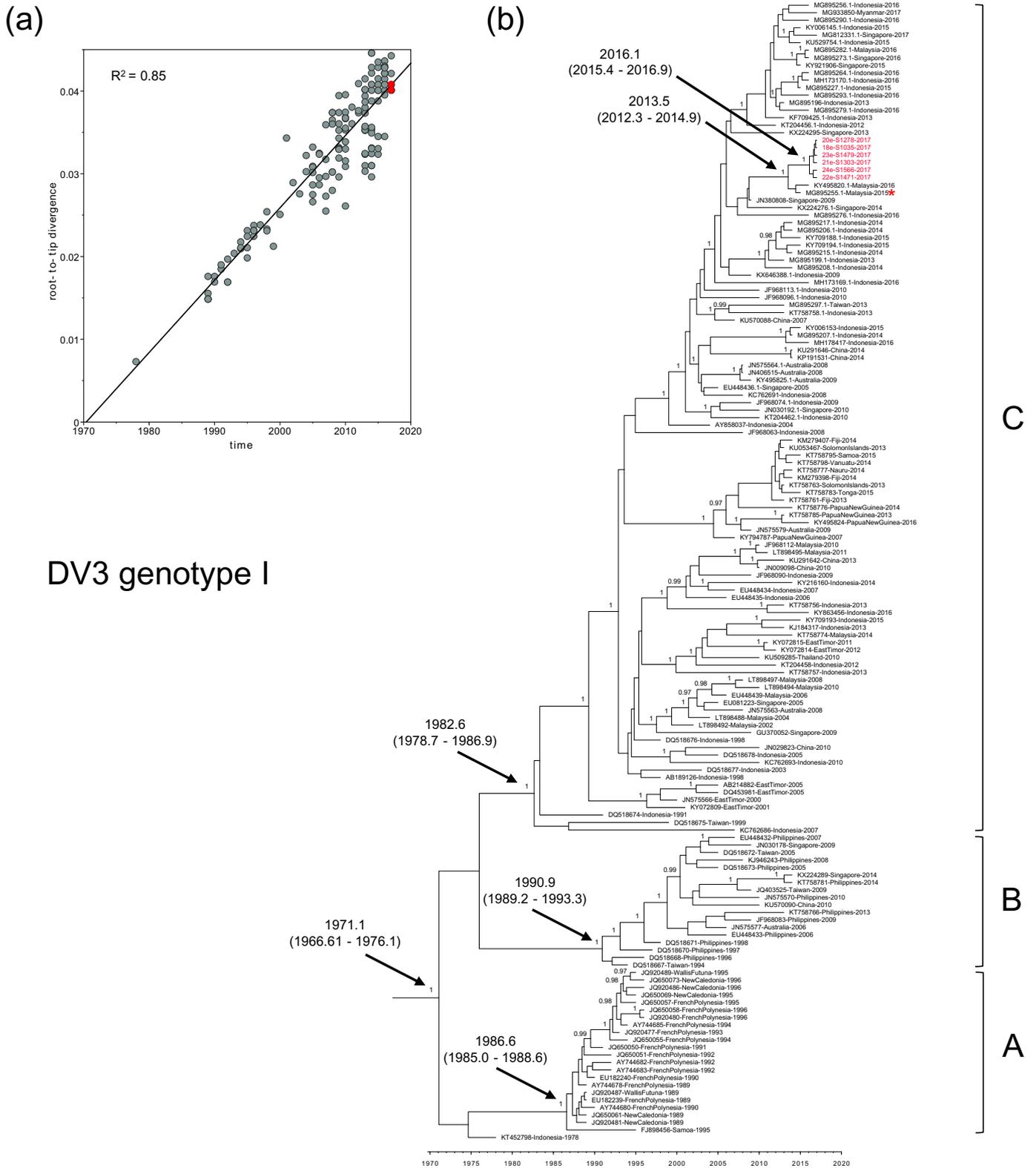


Fig. 5. Molecular clock analysis of DENV-3 genotype I envelope-encoding sequences. **(a)** Correlation of collection year and divergence from the maximum likelihood tree. The R^2 (coefficient of determination) of 0.85 was estimated using TempEst (shown at the top left). Red dots indicate sequences obtained in the present study. **(b)** Bayesian maximum clade credibility phylogenetic tree estimated using BEAST v1.8.4. The mean time of the most recent common ancestor (tMRC) and 95% highest probability density (HPD) (in calendar year and tenths of year) are indicated with black arrows, and posterior probability values are indicated adjacent to the node of interest. The name of each taxon is presented as the accession number, country, and year of collection. Sequences obtained in the present study are labeled in red. Lineages A, B, and C are shown to the right. The MG895255 virus is indicated by a red asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Dengue virus isolates with open reading frame sequences determined in the present study.

Isolate	Collection date	Serotype	Genotype	Accession no.
B17-1387DV1	October 2017	1	V	LC436667
B17-1547DV1	November 2017	1	V	LC436668
B17-1357DV2	October 2017	2	Cosmopolitan	LC436669
B17-1431DV2	October 2017	2	Cosmopolitan ^a	LC436670
B17-1482DV2	October 2017	2	Cosmopolitan ^a	LC436671
B17-1489DV2	October 2017	2	Cosmopolitan	LC436672
B17-1556DV2	November 2017	2	Cosmopolitan	LC436673
B17-1571DV2	November 2017	2	Cosmopolitan	LC436674
B17-1634DV2	December 2017	2	Cosmopolitan	LC436675
B17-1471DV3	October 2017	3	I	LC436676
B17-1479DV3	October 2017	3	I	LC436677

^a note minor lineage virus of DENV-2 Cosmopolitan genotype.

Viruses of lineage B shared a tMRCA of 1975.8 (95% HPD: 1972.6–1979.1), while those of lineage C showed a similar tMRCA of 1970.6 (95% HPD: 1968.2–1973.3). However, lineage C contained viral strains that predominated in Southeast Asia (i.e., Indonesia, Philippines, Singapore, and Malaysia), the Pacific islands (i.e., Papua New Guinea and New Caledonia), and Australia. The Cosmopolitan viruses obtained in the present study clustered within lineages B and C. The tMRCA of our 3 Bangladeshi viruses in lineage B was 2016.4 (95% HPD: 2015.8–2017.1) sharing the unique 162V together with viruses from India (JX475906, LC367234, MG271948, MG895099, MH822956, KX061431, and MH822952, but not MG560143) and Singapore (JN030345) in the same branch. The tMRCA of our 44 Bangladeshi viruses in lineage C was estimated as 2014.4 (95% HPD: 2013.5–2015.2). The 52H substitution was observed distinctly in this lineage.

The origin of DENV-2 Cosmopolitan lineage C viruses obtained in the present study (Figs. 2 and 3b) might be Malaysia, since a virus obtained in Malaysia in 2013 (KJ806776) is the common ancestor of our viruses (Fig. 3b). On the other hand, the origin of DENV-2 Cosmopolitan lineage B viruses obtained in the present study (Fig. 3b) was estimated to be India, since a virus obtained in India in 2014 (MH822952) was most closely related to these viruses. In addition, the origin of DENV-2 Cosmopolitan lineage B viruses reported previously from Bangladesh was also estimated to be India, since a virus obtained in India in 2001 (DQ448236) was most closely related to these viruses.

3.4. Genotype replacement of DENV-3 in Dhaka, Bangladesh

As shown in Fig. 4, the 35 envelope sequences deposited from Bangladesh to the NCBI database between 2000 and 2009 (Huang et al., 2007; Islam et al., 2006; Shu et al., 2009) were clustered in DENV-3 genotype II together with sequences from Vietnam, Taiwan (King et al., 2008), Thailand (Shihada et al., 2017), and China (Bai et al., 2013). Interestingly, however, 6 DENV-3 envelope sequences from Bangladesh in the present study belonged to DENV-3 genotype I and were closely related to sequences from Malaysia (Moore et al., 2017; Yang et al., 2018), Indonesia (Ong et al., 2008; Sasmono et al., 2015), Singapore, and Australia (Ritchie et al., 2013). These results suggested that the previously observed DENV-3 genotype II in Bangladesh might have been replaced by DENV-3 genotype I.

Variations in amino acid substitutions among DENV-3 were analyzed based on the envelope sequences. Thirty-six amino acid variations were detected among DENV-3 genotype I in the corresponding phylogenetic tree (Fig. 4). In particular, our viruses shared amino acid residues (i.e., 68V, 140I, 154E, 160A, 172I, 231K, 270T, 303A, 447S, and 489A) with all the DENV-3 genotype I viruses listed in Figs. 4, and 124L, 301S, and 377I were shared among several isolates of genotype I, while 223I was found only in the 6 viruses obtained in the present study and in sequences from Malaysia, MG895255 and KY495820. The amino

acid residue L at position 362 of six viruses obtained in the present study was shared with two Malaysian viruses and KY921906.

3.5. Time of introduction of the new DENV-3 genotype I into Bangladesh

Since DENV-3 sequence data from Bangladesh were not available for the years from 2010 to 2016, we estimated the time of the introduction of DENV-3 genotype I into Bangladesh by molecular clock analysis. For this purpose, we prepared another dataset of DENV-3 genotype I composed of those obtained from the present study along with other sequences from various countries from 1978 to 2016 (S4 Table). Root-to-tip regression analysis of these new datasets showed a correlation coefficient of 0.85, suggesting a sufficient temporal signal for proceeding with molecular clock analysis (Fig. 5a). Bayesian phylogenetic analysis (inferred using UCLN and the Bayesian Skyline coalescent prior, which was the best-fit model (S5 Table)) showed that the root of the DENV-3 genotype I tree was estimated as 1971.1 (95% HPD: 1966.6–1976.1). The mean substitution rate was 9.24×10^{-4} substitutions per site per year (95% HPD: 8.04×10^{-4} – 1.05×10^{-3} substitutions per site per year), which was slightly higher than 6.74×10^{-4} substitutions per site per year (95% HPD: 5.45×10^{-4} – 8.09×10^{-4} substitutions per site per year) obtained for DENV-3 genotype III in our previous report (Phadungsombat et al., 2018). The phylogenetic tree showed 3 lineages (posterior probability = 1). Lineage A contained viruses isolated from New Caledonia, French Polynesia, and Samoa between 1990 and 1996. The lineage A tMRCA was 1986.6 (95% HPD: 1985.0–1988.6). Lineage B consisted of viral strains circulating in the Philippines, Singapore, China, and Taiwan, and these viruses shared tMRCA around 1990.9 (95% HPD: 1989.2–1993.3). Lineage C comprised viruses isolated from Southeast Asia (Indonesia, Singapore, Malaysia, Thailand, and East Timor), Oceania (Australia, Fiji, Solomon, Nauru, Tonga, Vanuatu, and Papua New Guinea), and China, and showed a tMRCA of 1982.6 (95% HPD: 1978.7–1986.9). Our DENV-3 genotype I viruses (18e, 20e-24e) clustered within lineage C and appeared to be most closely related to viruses from Malaysia (KY495820 and MG895255). The tMRCA of this branch was estimated to be 2013.5 (95% HPD: 2012.3–2014.9), and the tMRCA of DENV-3 genotype I that we obtained in the present study was 2016.1 (95% HPD: 2015.4–2016.9) as shown in Fig. 5b.

The origin of DENV-3 genotype I viruses obtained in the present study (Figs. 4 and 5b) might be Malaysia, since a virus obtained in Malaysia in 2015 (MG895255) was most closely related to these viruses in the envelope region (Fig. 5b).

3.6. Full sequence analysis of encoded polyprotein

To confirm whether the conclusions obtained from the phylogenetic analysis based on envelope region sequences are the same as those obtained from the analysis based on the full-length sequences, we selected 2 DENV-1, 7 DENV-2, and 2 DENV-3 serum samples for virus isolation. We were able to isolate virus strains from these specimens and determined their nearly full genome sequences (Table 1). The phylogenetic analysis of the whole coding sequences confirmed the genotypes of the viruses described above for the envelope regions as genotype V of DENV-1, Cosmopolitan of DENV-2, and genotype I of DENV-3 (Fig. 6). The whole coding sequences of our DENV-2 Cosmopolitan viruses separated into two clusters as observed in the envelope sequences (Fig. 6). Except for 1547, we were also able to amplify the envelope region from sera as described above. Sequence comparisons revealed no difference between the PCR-amplified envelope region of stored RNA from serum and isolated virus strains.

4. Discussion

To understand the current DENV diversity in Bangladesh, we determined the nucleotide sequences of DENV envelope regions from

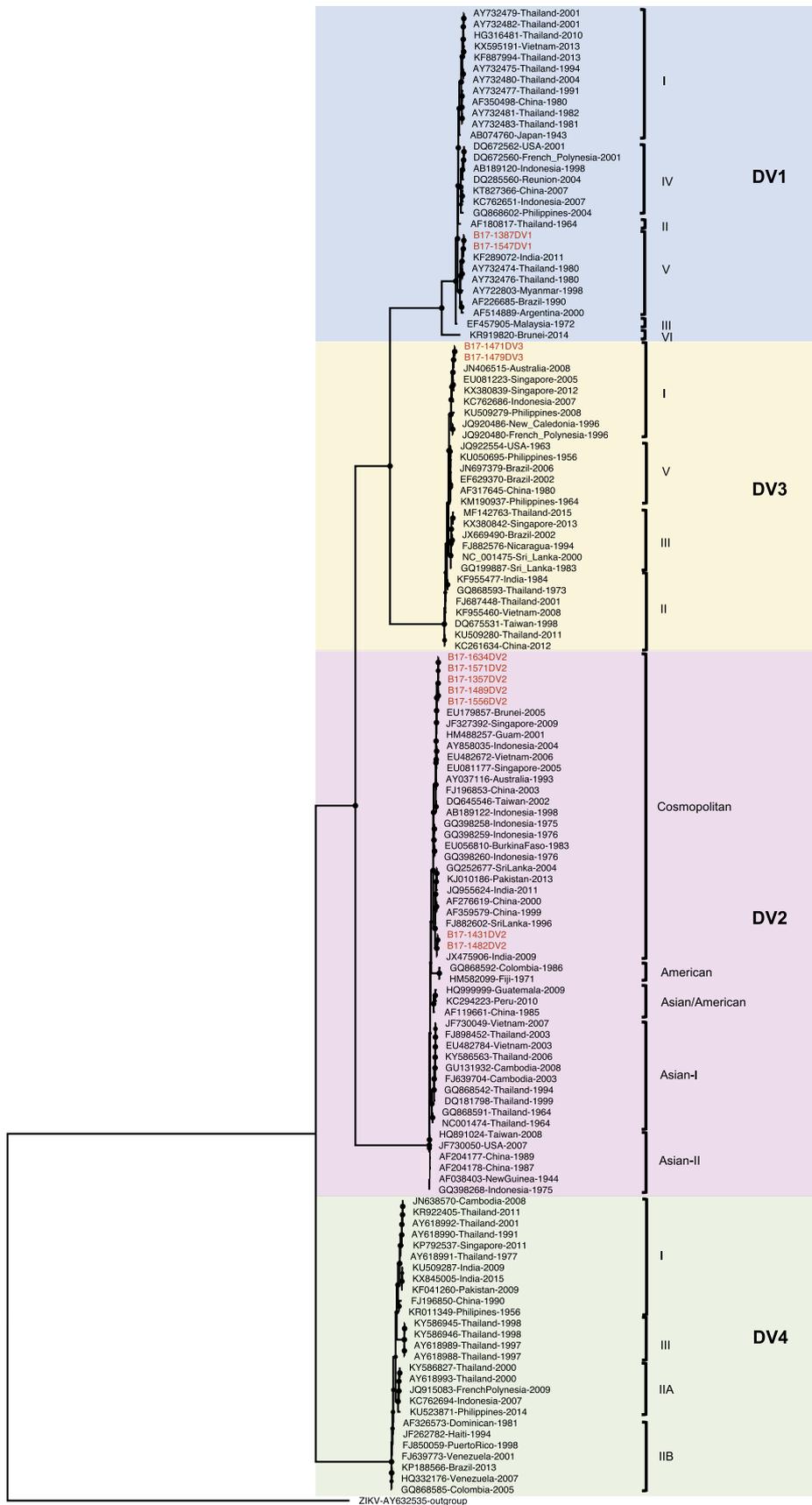


Fig. 6. Isolated virus genotypes based on the coding region.

A maximum likelihood phylogenetic tree was constructed in the W-IQ-TREE program using ModelFinder, and an ultrafast bootstrap (UFBoot) of 1000 replicates was calculated. GTR + F + I + G4 was chosen as the best-fit model according to Bayesian information criteria. Data included sequences obtained from isolated viruses in the present study (labeled in red) along with sequences obtained from GenBank. The viral serotypes and genotypes are indicated to the right. Virus names are shown as the accession number, country, and reported year of each sequence. Black circles on the branches denote bootstrap support values with 100%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DENV-positive serum from patients diagnosed in Apollo Hospitals Dhaka between September 2017 and February 2018. We found that DENV-2 was dominant in this period. Phylogenetic analysis of the envelope region revealed that all the DENV-2 sequences were genotyped as Cosmopolitan. There were two distinct lineages of the DENV-2 Cosmopolitan genotype, one of which has never been reported from Bangladesh. Furthermore, DENV-3 genotype I was found for the first time in this country instead of DENV-3 genotype II. The majority of DENV-2 Cosmopolitan and DENV-3 genotype I found in the present study were closely related to respective strains in Malaysia.

In the present study, we found that the circulating DENV-2 genotype Cosmopolitan lineage C viruses in Dhaka in 2017 were distinct from DENV-2 genotype Cosmopolitan lineage B viruses reported before 2011 (Moore et al., 2017; Shu et al., 2009). The tMRCA of our DENV-2 genotype Cosmopolitan lineage C viruses was 2014, suggesting recent introduction in Dhaka. DENV-2 Cosmopolitan was reported as a predominant DENV in Singapore during 2003–2012 before DENV-1 became predominant (Lee et al., 2012; Ng et al., 2015; Rajarethinam et al., 2018). DENV-2 Cosmopolitan also replaced DENV-3 and DENV-4 in the southern part of Peninsular (West) Malaysia in 2013 (Ng et al., 2015). Similar results were also found in our previous report, which showed that DENV2 Cosmopolitan was introduced to Thailand in late 2014 (Phadungsombat et al., 2018).

On the other hand, we found that 3 out of 47 sequences of DENV-2 were clustered in Cosmopolitan lineage B and were closely related to sequences from India. India is a major endemic country of DENV, and all 4 serotypes are present there. The circulating DENV-2 in Delhi in 2012–2015 was reported as Cosmopolitan (Afreen et al., 2014; Choudhary et al., 2017; Kar et al., 2018). It is reasonable to infer that the DENV-2 genotype Cosmopolitan lineage B reached Bangladesh from India more than once, since three sequences (14e-S1482, 16e-S1431, and 70e-S1285) in the present study clustered in a different branch from the sequences previously reported from Bangladesh. The tMRCA of these three sequences was 2016, also suggesting recent introduction in Dhaka. It will be interesting to see whether this minor virus of DENV-2 Cosmopolitan lineage B will become common or disappear in the future.

In the NCBI database, 35 envelope sequences of Bangladesh DENV-3 were available. From our phylogenetic analysis, the previously circulating DENV-3 was genotype II, while the viruses in our study belonged to genotype I, which might have come to Bangladesh through Malaysia. This is the first report of DENV-3 genotype I in South Asia, since the DENV-3 genotype I was exclusively found before either in Indonesia, Oceania, Malaysia, Singapore, or China (Haryanto et al., 2016; Lanciotti et al., 1994; Roche et al., 2007; Suppiah et al., 2018; Tan et al., 2017). In Malaysia, the genotype shift of DENV-3 recurrently occurred, and genotypes II, I, and III were dominant genotypes in 1987–2002, 2004–2007, and 2012–2013, respectively. Moreover, co-circulation of DENV-3 genotypes I and III was observed in 2014–2017 (Suppiah et al., 2018; Tan et al., 2017).

Bangladesh and India are neighboring countries, and people in business frequently travel between them. However, a different DENV-3 genotype, genotype III, was dominant in India in 2016 (Parveen et al., 2018). In the case of Thailand, a genotype shift from DENV-3 genotype II to III, which might have come to Thailand through India, was recently found (Phadungsombat et al., 2018). Due to frequent population exchanges between East and Southeast Asian countries where different DENV-3 genotypes have circulated, it would be very difficult to predict future endemic genotypes of DENV. It is thus necessary to continue a DENV surveillance program in order to provide early warning of possible genotype variations.

In conclusion, we observed clade shifts in the DENV-2 Cosmopolitan genotype and a genotype shift in DENV-3 in 2017 in Dhaka, Bangladesh. Shirin et al. recently reported that the number of DENV cases increased in 2018 in Dhaka (Shirin et al., 2019). They also reported increased frequency of DENV-3, since 31% of dengue cases were

infected with DENV-3. It is thus important to sample serotypes and genotypes of DENV each year to understand its epidemiology. Further, detailed clinical analyses of dengue-confirmed patients would provide a deeper understanding of the impact of the serotype and genotype changes in the community.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2019.103977>.

Declaration of Competing Interests

Keita Suzuki is a paid employee of Tanaka Kikinzoku Kogyo. Tanaka Kikinzoku Kogyo had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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