

## Original Article

# An Epidemic of Dengue Virus Serotype-4 during the 2015 - 2017: the Emergence of a Novel Genotype IIa of DENV-4 in the Philippines

Mark Anthony de Vera Luz<sup>1,2†</sup>, Takeshi Nabeshima<sup>1†</sup>, Meng Ling Moi<sup>1\*</sup>, Maria Terrese Alonzo Dimamay<sup>3</sup>, Lady-Anne Suarez Pangilinan<sup>3</sup>, Mark Pierre Sijo Dimamay<sup>3</sup>, Ronald Roll Matias<sup>3</sup>, Cynthia Abad Mapua<sup>3</sup>, Corazon Cerilla Buerano<sup>3</sup>, Ferdinand de Guzman<sup>4</sup>, Edith Sangalang Tria<sup>4</sup>, Filipinas Florendo Natividad<sup>3</sup>, Maria Luisa de Guzman Daroy<sup>3</sup>, Taichiro Takemura<sup>1</sup>, Futoshi Hasebe<sup>1</sup>, and Kouichi Morita<sup>1</sup>

<sup>1</sup>Department of Virology, Institute of Tropical Medicine (NEKKEN), and <sup>2</sup>Leading Program, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; <sup>3</sup>Research and Biotechnology, St. Luke's Medical Center, Manila, Philippines; and <sup>4</sup>San Lazaro Hospital, Manila, Philippines.

**SUMMARY:** Dengue remains a major public health problem in the Philippines. In this study, we determined the circulating dengue serotypes in the Philippines during the 2015–2017 outbreaks using a total of 678 serum samples from 537 individual dengue patients. Following an increase in the number of DENV-4 patients in recent years, we conducted a comprehensive molecular and epidemiology analysis on the DENV-4 strains isolated recently in the Philippines. Two genotypes of DENV-4 have been isolated in the Philippines since 1956: GI and GIIa. The GIIa DENV strains that were isolated in the present study were closely related to a distinct group of GIIa strains that were isolated from the Philippines in 2004. A majority of the isolates of this sub-group have been identified in the Philippines, suggesting that this lineage may have been introduced in the Philippines, and evolved to form the distinct sub-group within GIIa strains. The increase in DENV-4 activity also coincided with the appearance of the GIIa sub-group and the phasing-out of the GI lineage in the Philippines. Overall, our study demonstrates a shift in DENV-4 genotype and epidemic dynamics in a hyperendemic region, suggesting the importance of DENV genetic evolution in establishing and sustaining transmission.

## INTRODUCTION

Dengue fever is the most prevalent arthropod-borne viral disease in humans. There are approximately 390 million infections per year, which account for approximately 96 million of the clinically apparent cases (1). The virus causes various clinical symptoms ranging from mild fever with or without warning signs to severe dengue as defined by the WHO (2). The dengue virus (DENV) is a member of the genus *Flavivirus* of the family *Flaviviridae*, which is antigenically divided into four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). Any of these serotypes can cause mild febrile self-limiting illness that can progress to bleeding manifestations leading to severe dengue (2).

Dengue is endemic in the Philippines where all the causative agents—the four dengue virus serotypes—are circulating. It has been a notifiable disease in the Philippines since 1958 (3) and is one of the country's eight pervasive infectious diseases (4). In 1954, an epidemic characterized by symptoms of fever and hemorrhages among infants and children, which led to

the deaths of some infected individuals, was reported in the Philippines (5). In 1956, in an outbreak that occurred in Manila—where there were over 750 dengue cases and a mortality of approximately 10%—the dengue viruses were first associated with hemorrhagic fever (6). Although it is not the most prevalent isolated serotype, DENV-4 was reported to be associated with an outbreak for the first time. In 1964, another outbreak of mosquito-borne hemorrhagic fever occurred in Manila and nearby areas (7). This was the only time when DENV-4 was reported to be the most prevalent serotype during an outbreak in the Philippines. Circulation of the dominant serotypes has demonstrated distinct cyclic patterns in the Philippines, with a cycle of 2–4 years for each major circulating serotype. Towards the end of the 20th century and at the beginning of the 21st century, DENV-1 and DENV-2 were reported to be the major circulating serotypes, with DENV-3 predominating towards the end of the first decade of the 21st century (8). While DENV serotypes have played an important role in DENV outbreaks in the Philippines, DENV-4 has been consistently the least detected serotype (9), and may amount for only 7% of reported cases (8). Globally, DENV-4 has been re-emerging in various parts of the world. DENV-4 is stratified into eight lineages with unique spatiotemporal characteristics and limited genetic recombination (10).

Previous studies have suggested the important role of the introduction of new genotypes and serotypes in changing the dynamics of DENV outbreaks. Notably, between 1995–2002 in the Philippines, the predominant serotype shift to DENV-2 coincides with

Received June 7, 2019. Accepted July 16, 2019.  
J-STAGE Advance Publication August 30, 2019.  
DOI: 10.7883/yoken.JJID.2019.208

\*Corresponding Author: Mailing Address: Department of Virology, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki, Nagasaki 852-8523, Japan. Tel: +81- 95-819-7829, Fax: +81-95-819-7830, E-mail: sherry@nagasaki-u.ac.jp

†These authors contributed equally to this work.

the DENV-2 genotype replacement from the Asian-2 genotype to the Cosmopolitan genotype (11). In the present study, phylogenetic analyses of the full-length virus genome using next-generation sequencing approach were performed to better understand the molecular epidemiology of the recent DENV-4 emergence. Here, we show the emergence and circulation of DENV-4 genotype IIa and demonstrate the recent changes in DENV-4 circulation dynamics in a hyperendemic region.

## MATERIALS AND METHODS

**Clinical samples, patient data, and ethics statement:** A total of 678 serum or plasma samples were obtained from 537 febrile patients (145 in-patients and 392 out-patients) who sought medical treatments at St. Luke’s Medical Center (SLMC) and San Lazaro Hospital from 2015–2017. Blood specimens were collected from all patients within 5 days of the onset of fever after written consents from patients or guardians. Subsequent blood specimens that originated mostly from in-patients were collected after 6 days or more after the onset of fever. Patients were confirmed to have dengue infection using IgM capture ELISA at SLMC (12) and detection of dengue virus gene was performed using a real-time RT-PCR assay (13,14). A primary and secondary type of infections was determined using DENV IgG ELISA kit according to the manufacturer’s instructions (Panbio, Queensland, Australia) (15). Virus isolation was carried out by inoculating 10 µl serum or plasma sample into a confluent monolayer of *Aedes albopictus* C6/36 cells following a protocol as previously described (16). The ethical clearance for this

study was approved by the SLMC Institutional Ethics Review Committee (EC Reference number: 14018) and Institute of Tropical Medicine, Nagasaki University (no. 20144004).

**Virus serotyping and sequence analyses:** Virus serotyping was performed either by a conventional RT-PCR assay (17) or by using fluorogenic Taqman primers that are specific to each serotype (14,18). For conventional sequencing methods, the E region of the virus was amplified by the Qiagen One-step RT-PCR kit (Qiagen, Hilden, Germany) using primers flanking the whole envelope gene. The resulting 2014 bp product was then sequenced using sequencing primers according to the methods previously described (19).

To sequence the whole dengue virus genome using a next-generation sequencing approach, cDNA libraries were synthesized with the SuperScript III first-strand synthesis system (Invitrogen, Waltham, MA, USA) using random primers according to the manufacturer’s instructions. The second strand was synthesized using Ultra II Non-directional RNA second strand synthesis module (NEB, Ipswich, MA, USA). The amplified double-stranded (ds) DNA was quantified and normalized using Qubit 2.0 fluorometer (Invitrogen). The amplicons were fragmented with transposome according to manufacturer’s instructions (Illumina, San Diego, CA, USA). The library was then generated with Illumina Nextera XT library preparation kit (Illumina), purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, South Kraemer Blvd Brea, CA, USA), and then quantified by Qubit 2.0 fluorometer. Pooled libraries were denatured with 0.2 M NaOH, and the sample was loaded onto Miseq v2 kit (500 cycles) for

Table 1. Dengue virus (DENV) serotypes in Metro Manila, 2003-2017<sup>2)</sup>

Year	DENV serotype				Total by year
	DENV-1	DENV-2	DENV-3	DENV-4	
2003	16 (80) <sup>1)</sup>	1 ( 5)	1 ( 5)	2 (10)	<b>20</b>
2004	3 (20)	5 (33)	2 (13)	5 (33)	<b>15</b>
2005	7 (16)	9 (20)	17 (39)	11 (25)	<b>44</b>
2006	4 ( 3)	6 ( 5)	120 (90)	3 ( 2)	<b>133</b>
2007	0 ( 0)	1 ( 7)	12 (86)	1 ( 7)	<b>14</b>
2008	3 ( 5)	24 (36)	38 (58)	1 ( 2)	<b>66</b>
2009	3 ( 1)	198 (92)	11 ( 5)	3 ( 1)	<b>215</b>
2010	15 (17)	35 (41)	30 (35)	6 ( 7)	<b>86</b>
2011	8 (73)	1 ( 9)	2 (18)	0 ( 0)	<b>11</b>
2012	12 (52)	1 ( 4)	2 ( 9)	8 (35)	<b>23</b>
2013	4 (16)	11 (44)	2 ( 8)	8 (32)	<b>25</b>
2014	ND <sup>3)</sup>	ND	ND	ND	<b>ND</b>
2015	54 (32)	44 (26)	32 (19)	38 (23)	<b>168</b>
2016	17 (19)	21 (23)	36 (40)	16 (18)	<b>90</b>
2017	28 (23)	10 ( 8)	67 (56)	15 (13)	<b>120</b>
<b>Total by serotype</b>	<b>174 (17)</b>	<b>367 (36)</b>	<b>372 (36)</b>	<b>117 (11)</b>	<b>1030</b>

<sup>1)</sup>: Number in brackets indicate percentage of total serotype by year, and underline indicates the serotype with the highest percentage in the specific year.

<sup>2)</sup>: Data source from SLMC database.

<sup>3)</sup>: ND indicates no data.

paired-end sequencing on the Illumina Miseq platform. Image processing and base calling were generated as FASTQ files. Quality assessment was performed using the Miseq control software, in which samples with a Q score of >30 were used for further analyses (20).

**Phylogenetic analyses and molecular dating:** Low-quality sequences were removed by FASTX-Toolkit Ver 0.0.14 (21) from the input data file. Before and after the quality filtering, sequence quality was assessed using FastQC Ver 0.11.7 (22). Trinity Ver 2.8.4 (21) was used for de novo assembly. Using Entrez-edirect (23) and BLASTN Ver 2.7.1 (24), the consensus sequence was chosen from INSDC. Reads from trimmed fastq data set were mapped by BWA Ver 0.7.17 (25) to the reference sequence. Using Varscan Ver 2.4.3 (26) and Samtools Ver 1.9 (27), a consensus sequence was then constructed. Due to limited full-length genome analyses of DENV-4, sequences from the E-gene were used for the phylogenetic analyses (Table 2). The sequences of the E protein-coding region were aligned using MAFFT Ver 7.407 (28). The substitution model were selected by JModeltest Ver 2.1.10 (29) and bModelTest 1.1.2 (30). Bayesian Markov Chain Monte Carlo (MCMC) analysis was conducted using the GTR substitution model by BEAST Ver 1.10.4 (31) and BEAGLE Ver 3.1.2 (32). The MCMC length of chain

was for 100,000,000 generations. The Maximum Clade Credibility (MCC) tree was generated and annotated with posterior probability by TreeAnnotator Ver 1.10.4 (31). The phylogenetic tree was drawn by Figtree Ver 1.4.4. Gaussian Markov random field (GMRF) Skyride plot was constructed using Tracer Ver 1.7.1 (33).

**Statistical analyses:** Data were described using descriptive statistics such as mean and standard deviation. Student's *t*-test was used to compare the means, and the difference between means was considered to be significant for *P*-value less than 0.05.

## RESULTS

In this study, a total of 537 DENV cases (678 serum and plasma samples) were confirmed by real-time RT-PCR and anti-DENV IgM ELISA. A total of 261 patients were confirmed to have secondary DENV infection ( $N_{\text{out-patient}} = 204/347$  [58.8%] and  $N_{\text{in-patient}} = 57/131$  [43.5%]). DENV was isolated from 141 out of 167 samples. Among the DENV cases, patients with signs of severe dengue were admitted as "in-patient" and other patients who did not require hospitalization were grouped as "out-patient". Most of the in-patient cases were infected with DENV-3 ( $N=5$ ), while one case was that of DENV-4 infection. There were no DENV-1

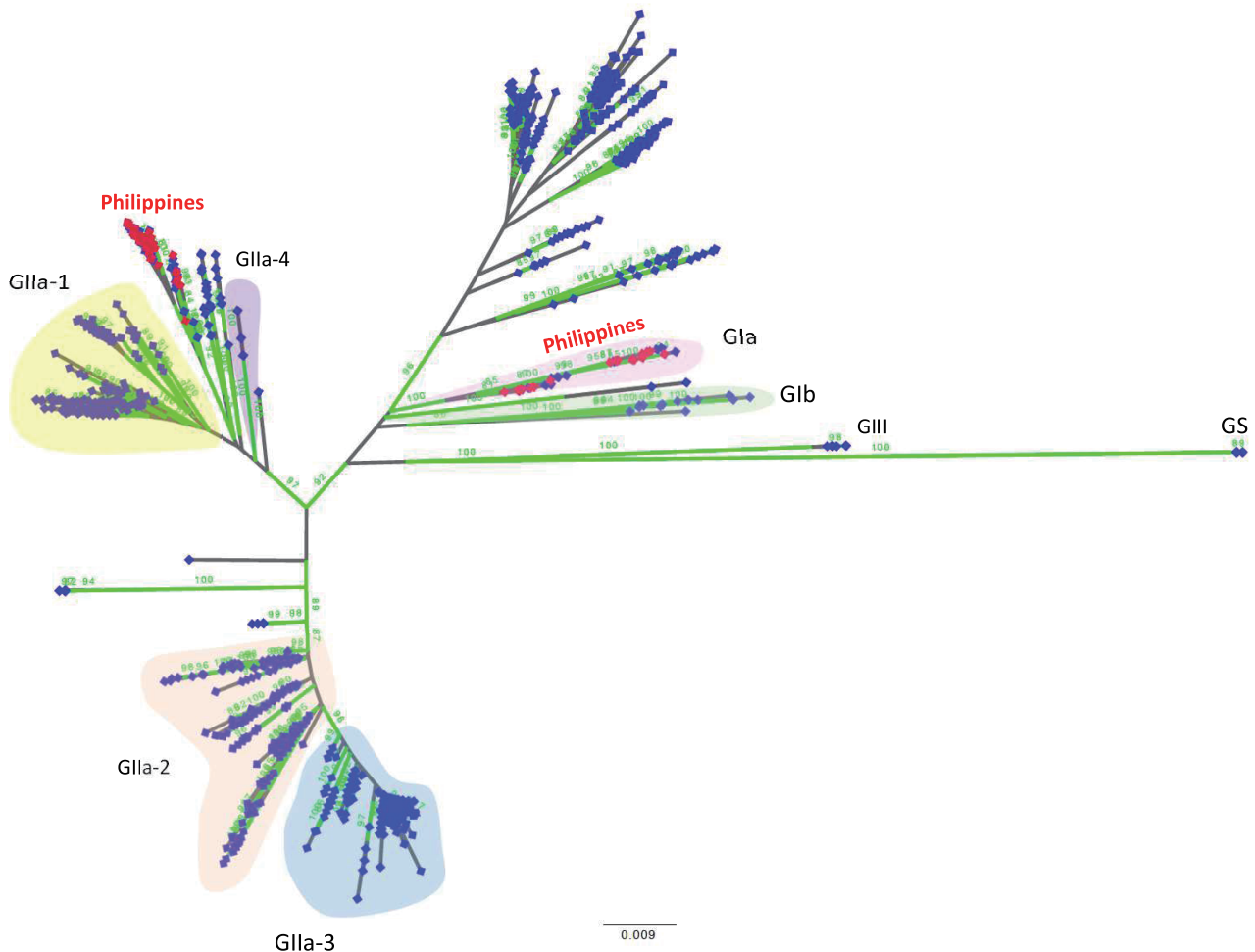


Fig. 1. Phylogenetic tree of global DENV-4 strains. The tree was constructed by the neighbor-joining (NJ) method (Seaview version 4.7) with 100 bootstrap replicates, by using 1683 global DENV-4 sequences. Branches of the tree that are color-coded green indicate bootstrap values of >80. DENV-4 strains from the Philippines are color-coded as red.

Table 2. DENV isolates characterized in the present study

Isolate	Sex	Age	Days of Fever	Date of sample collection	DENV-4 real-time PCR (ct values)	GenBank accession number
173	F	13	4	2015/7/ 9	29.8	MN027545
310	F	12	3	2015/8/10	32.4	MN027546
322	M	18	3	2015/8/14	29.2	MN027547
334	M	35	4	2015/8/18	28.3	MN027548
336	F	7	4	2015/8/18	32.0	MN027549
337	M	30	3	2015/8/17	31.8	MN027550
363	M	27	4	2015/8/22	31.4	MN027551
379	M	27	4	2015/8/25	35.1	MN027552
380	F	7	4	2015/8/25	31.0	MN027553
382	M	32	3	2015/8/23	28.7	MN027554
402	M	35	4	2015/8/29	14.48	MN027555
405	M	9	3	2015/8/29	32.4	MN027556
444	M	16	6	2015/9/15	34.0	MN027557
573	F	61	4	2015/11/15	33.0	MN027558
991	F	20	5	2016/10/21	ND <sup>1)</sup>	MN027559

<sup>1)</sup>: ND indicates no data for the sample.

and DENV-2 cases in the in-patient group. In contrast, 28.0%, 28.0%, 21.0%, and 23.0% of out-patient samples were positive for DENV1, DENV2, DENV3, and DENV4, respectively. While there was an increase in the percentage of patients with DENV-4, the clinical signs including body temperature and blood chemistry were not significantly different from those of other serotypes (data not shown).

Following an increase in the percentage of DENV-4 carrying patients in recent years, we conducted a comprehensive molecular epidemiology analysis on the DENV-4 strain isolated from the Philippines over several decades. In addition to the 15 DENV-4 isolates obtained in this study, 97 published strains isolated from the Philippines between 1956 to 2016 (available from public databases), were used to perform a phylogenetic analysis. A neighbor-joining (NJ) phylogeny revealed two DENV-4 genotype lineages (GI and GII) in the Philippines (Fig. 1). DENV-4 GI was associated with two different major lineages, one which consists of strains isolated in 1956, and the second group of GI lineages consists of strains that are distinct from the strains isolated in the 1950s. The GI group consists of more recent DENV-4 isolates, and these strains were isolated co-incidentally in a subsequent wave of increased DENV-4 activity, involving a 10-year span between 2003–2013.

Following a period of relatively low levels of DENV-4 activity between 2006–2011 (Table 1), a new genotype (GIIa) was detected during the early 2000s. Subsequently, a majority of the isolates from the lineage (L3, Fig. 2a) were recently identified between 2012–2016. The GIIa lineage from the Philippines forms a distinct and well-supported monolignage that originated from strains isolated in East Timor in 2001 (KY275251, KY275252). The MCC analyses revealed a distinct lineage (L3) for the recent isolates in the GIIa group, which consists of strains from the early 2000s to the

present. The L2 lineage sub-groups in the L3-1-1a and the L3-1-1b lineage, however, have not been detected in recent years, thus, demonstrating a pattern of active sub-lineage replacements between 2010–2015.

Interestingly, while there was an increase of GIIa isolates, there were no DENV-4 GI isolates after 2013. The turnover from GI to GII also coincides with a period of relatively high DENV-4 activity between 2012–2016. Additionally, the mechanisms between the genotype shift and DENV-4 activity in the Philippines were further examined by the GMRF Skyride plot (Fig. 2b). The GMRF Skyride plot suggests that the DENV-4 in the Philippines appears to have a phase of the exponential increase of  $N_e$  during 2000–2010. This inference is consistent with the appearance of GIIa lineage and the phasing-out of GI lineage in the Philippines. Out of a total 84 strains that clustered within the distinct GIIa lineage of the Philippines (Fig. 1), 73 were isolated from the Philippines (2004–2015), while the remaining strains were isolated from Indonesia (2016), East Timor (2001), Singapore (2005, 2013–2014), China (2012) and Thailand (2014). Because most of the isolates of the GIIa lineage have been identified in the Philippines, the lineage may have been introduced and evolved in the country to form a distinct group within GIIa strains.

## DISCUSSION

Two genotypes of DENV-4 have been isolated in the Philippines since 1956: GI and GIIa. The DENV-4 strains isolated in this study from 2015–2017 were closely related to previous GIIa strain isolated from the Philippines since 2004. Although all four serotypes co-circulate in the Philippines, there are limited data on outbreaks associated with DENV-4 and evolutionary trajectory. Here, we present a comprehensive analysis of an important DENV serotype, DENV-4, in the Philippines over recent decades.

## Emergence of DENV-4 Genotype IIa, the Philippines

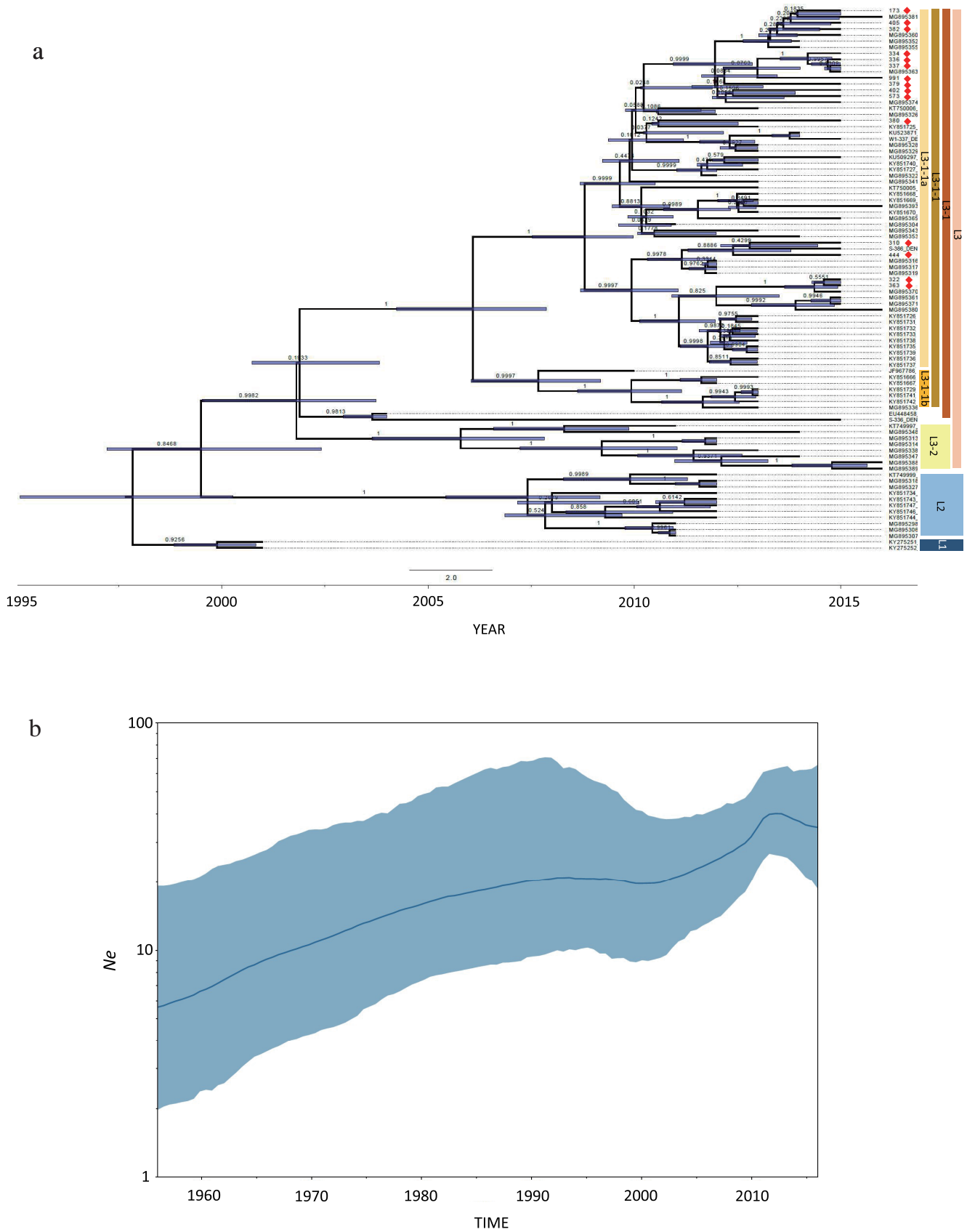


Fig. 2. Molecular clock analysis of DENV-4 genotype IIa sequences in the Philippines. (a) Bayesian maximum clade credibility (MCC) phylogenetic tree estimated by using BEAST Ver 1.10.4 and BEAGLE Ver 3.1.2. The MCMC length of chain was for 100,000,000 generations, in which the generated MCC tree was annotated with percentage of highest posterior density (95% HPD) by TreeAnnotator Ver 1.10.4. The 95% HPD is indicated adjacent to nodes. Strains isolated from this study are indicated as solid red diamonds. (b) Gaussian Markov random field (GMRF) Skyride plot showing the demographic history of DENV-4 GI and GII as constructed by using Tracer Ver 1.7.1. Blue area indicates variations in effective population size (Y axis,  $N_e$ ) whereas blue line indicates estimated mean of  $N_e$  variations.

Phylogenetic analyses on the DENV-4 serotype isolated since 1956 demonstrated several lineage turnovers, associated with changes in DENV serotype dynamics. By using analyses of all strains isolated in the Philippines, we found that the introduction of distinct lineages was followed by large DENV-4 outbreaks, and an incident of higher DENV-4 activity occurred between 2004–2006 and 2012–2016. Although the number of proportion of DENV-4 patients increased, there was no significant increase in the number of DENV-4 patients with severe dengue. During our study period, clinical signs, blood chemistry and viremia levels of DENV-4 patients did not significantly differ from those of hospitalized patients with other serotypes. While our results suggest that higher DENV-4 activity may not be consistent with an increase in the number of severe dengue cases, further studies are needed to define the association between the virus pathogenicity and enhanced transmission efficiency.

The GI lineage had been identified in the Philippines since 1956 and persisted until at least 2013; the GI group was predominantly replaced by GIIa since 2011. The emergence of GIIa in the Philippines was estimated to be between 2004 to 2007, and the genotype turnover, as defined in a switch in dominance, took almost seven years. Introduction of GIIa in the Philippines also coincided with the period in which there was an increase in DENV-4 GIIa outbreaks in neighboring regions including Malaysia (34). Further studies are needed to determine the patterns of genotype shift in the region. Interestingly, the GIIa strain, identified in the Philippines, forms a distinct group among the global GIIa strains (Fig. 1), suggesting that the strain may have evolved independently in the Philippines and is unique to the region.

Several shifts in the predominant DENV-4 genotype lineages occurred in the Philippines between 2004–2016. In concurrence with other studies in which genotype and lineage turnover coincide with changes in epidemic activity (19, 35–38), our study demonstrated an increase in the number of DENV-4 patients during the turnover period. While the GIIa lineage in the Philippines forms a distinct lineage, processes of genotype replacement may have been a result of introductory strains and after that on-going local evolution, with the dominance of lineage L3 from the replacement of lineage L2 and sub-lineages within L3 (L3-1-1b). One possible factor that is associated with driving the turnover may be an immune escape, in which positive selection could confer a selective advantage over other strains (39,40). Based on the high number of DENV-4 cases that coincided with the genotype turnover, the GIIa virus may also possess enhanced ability to replicate or transmit at the population level in the Philippines or possess higher fitness as compared to prior strains. While this study did not include any participants from the recent national dengue vaccination program, further studies on viral dynamics and herd immunity would clarify the factors associated with DENV evolutionary trajectory. Further studies on the viral dynamics in the region would also be of importance, particularly the introduction of strains between hyperendemic regions, to better understand the factors that drive epidemic dynamics.

DENV remains a major public health problem in the

Philippines. Based on the DENV-4 sequence evolution study spanning over 6 decades, we found that DENV-4 has been evolving rapidly in recent years in this DENV hyperendemic region, with a major genotype turnover to GIIa and the subsequent disappearance of GI. GIIa strains associated with the 2004 outbreaks and beyond were marked with local evolution in the Philippines, rapid and frequent inter-genotype lineage turnover and increased DENV-4 epidemic activity. Overall, our study demonstrated a shift in DENV-4 genotype and epidemic dynamics in a hyperendemic region, suggesting the importance of DENV genetic evolution in establishing and sustaining transmission.

**Acknowledgments** We would like to thank all staff and members of the Department of Virology, NEKKEN, Nagasaki University, Japan for providing technical support and advice. Our special thanks to the staff of the Pavilion II and the Central Laboratory of San Lazaro Hospital for their kind assistance during patient recruitment and data collection. We are also very grateful for the support of the Senior Vice President and Head of Research and Biotechnology (R&B) Group of St. Luke's Medical Center, Dr. Isaac David E. Ampil II. Finally, our sincere thanks to the members of R&B's dengue research group for kindly preparing the samples to be transported to NEKKEN.

**Conflict of interest** None to declare.

## REFERENCES

1. Bhatt S, Gething PW, Brady OJ, et al. The global distribution and burden of dengue. *Nature*. 2013;496:504-7.
2. World Health Organization. Dengue: guidelines for diagnosis, treatment, prevention and control. Available at <<https://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf>>. Accessed July 16, 2019.
3. Dominguez NN. Current DF/DHF prevention and control program in the Philippines. *Dengue Bull*. 1997;21: 41–6.
4. Edillo FE, Halasa YA, Largo FM, et al. Economic cost and burden of dengue in the Philippines. *Am J Trop Med Hyg*. 2015;92:360-6.
5. Quintos FN, Lim LE, Juliano L, et al. Hemorrhagic fever observed among children in the Philippines. *Philippines J Pediat*. 1954;3:1-19.
6. Hammon WM, Rudnick A, Sather GE. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. *Science*. 1960;131:1102-3.
7. Basaca-Sevilla V, Halstead SB. Recent virological studies on haemorrhagic fever and other arthropod-borne virus infections in the Philippines. *J Trop Med Hyg*. 1966;69:203-8.
8. Bravo L, Roque VG, Brett J, et al. Epidemiology of dengue disease in the Philippines (2000-2011): a systematic literature review. *PLoS Negl Trop Dis*. 2014;8:e3027.
9. Matias R. A historical review of dengue virus research in the Philippines. *St. Luke's J Med*. 2003;1:3-8.
10. Waman VP, Kasibhatla SM, Kale MM, et al. Population genomics of dengue virus serotype 4: insights into genetic structure and evolution. *Arch Virol*. 2016;161:2133-48.
11. Salda L, Parquet M, Matias RR, et al. Molecular Epidemiology of dengue 2 Viruses in the Philippines. *Am J Trop Med Hyg*. 2005;73:796-802.
12. Buerano CC, Ibrahim IN, Contreras RC, et al. Dengue Network Philippines. IgM-capture ELISA of serum samples collected from Filipino dengue patients. *Southeast Asian J Trop Med Pub Health*. 2000;31:524-9.
13. Alm E, Lesko B, Lindegren G, et al. Universal single-probe RT-PCR assay for diagnosis of dengue virus infections. *PLoS Negl Trop Dis*. 2014;8:e3416.
14. Ito M, Takasaki T, Yamada K, et al. Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. *J Clin Microbiol*. 2004;42:5935-7.
15. Moi ML, Omatsu T, Tajima S, et al. Detection of dengue virus nonstructural protein 1 (NS1) by using ELISA as a useful laboratory diagnostic method for dengue virus infection of international

## Emergence of DENV-4 Genotype IIa, the Philippines

- travelers. *J Travel Med.* 2013;20:185-93.
16. Buerano CC, Natividad FF, Contreras RC, et al. Antigen sandwich ELISA predicts RT-PCR detection of dengue virus genome in infected culture fluids of *Aedes albopictus* C6/36 cells. *Southeast Asian J Trop Med Pub Health.* 2008;817-21.
  17. Morita K, Tanaka M, Igarashi A. Rapid identification of dengue virus serotypes by using polymerase chain reaction. *J Clin Microbiol.* 1991;29:2107-10.
  18. Alm E, Lindegren G, Falk KI, et al. One-step real-time RT-PCR assays for serotyping dengue virus in clinical samples. *BMC Infect Dis.* 2015;15:493.
  19. Takamatsu Y, Nabeshima T, Nguyen TT, et al. A Dengue virus serotype-4-dominated outbreak in central Vietnam. *J Clin Virol.* 2015;66:24-6.
  20. Bui TT, Moi ML, Nabeshima T, et al. A single amino acid substitution in the NS4B protein of dengue virus confers enhanced virus growth and fitness in human cells in vitro through IFN-dependent host response. *J Gen Virol.* 2018;99:1044-57.
  21. Grabherr MG, Haas BJ, Yassour M, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol.* 2011;29(7):644-52.
  22. Shen W, Le S, Li Y, et al. SeqKit: A Cross-Platform and ultrafast toolkit for FASTA/Q file manipulation. *PLoS One.* 2016;11:e0163962.
  23. Kans J. Entrez Direct: E-utilities on the UNIX Command Line. Available at <<https://www.ncbi.nlm.nih.gov/books/NBK179288/>>. Accessed July 16, 2019.
  24. Camacho C, Coulouris G, Avagyan V, et al. BLAST+: architecture and applications. *BMC Bioinformatics.* 2009;10:421.
  25. Li H, Durbin, R. Fast and accurate long-read alignment with burrows-wheeler transform. *Bioinformatics.* 2010;26:589-95.
  26. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* 2012;22:568-76.
  27. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* 2009;25:2078-9.
  28. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol.* 2013;30:772-80.
  29. Darriba D, Taboada GL, Doallo R, et al. jModelTest 2: more models, new heuristics and parallel computing. *Nat Meth.* 2012;9:772.
  30. Bouckaert R & Drummond AJ. bModelTest: Bayesian phylogenetic site model averaging and model comparison. *BMC Evol Biol.* 2017;17:42.
  31. Drummond AJ, Suchard MA, Xie D, et al. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol.* 2012; 29:1969-73.
  32. Ayres DL, Darling A, Zwickl DJ, et al. BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. *Syst Biol.* 2012; 61:170-3.
  33. Rambaut A, Drummond AJ, Xie D, et al. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Syst Biol.* 2018;67:901-4.
  34. Abu Bakar, S Wong PF, Chan YF. Emergence of dengue virus type 4 genotype IIA in Malaysia. *J Gen Virol.* 2002;83:2437-42.
  35. Kotaki T, Yamanaka A, Mulyatno KC, et al. Divergence of the dengue virus type 2 cosmopolitan genotype associated with two predominant serotype shifts between 1 and 2 in Surabaya, Indonesia, 2008-2014. *Infect Genet Evol.* 2016;37:88-93.
  36. Martin E, Chirivella M, Co JKG, et al. Insights into the molecular evolution of dengue virus type 4 in Puerto Rico over two decades of emergence. *Virus Res.* 2016;213:23-31.
  37. Phadungsombat J, Lin MYC, Srimark N, et al. Emergence of genotype cosmopolitan of dengue virus type 2 and genotype III of dengue virus type 3 in Thailand. *PLoS One.* 2018;3:e 0207220.
  38. Shu PY, Su CL, Liao TL, et al. Molecular characterization of dengue viruses imported into Taiwan during 2003-2007: distribution and genotype shift. *Am J Trop Med Hyg.* 2009;80:1039-46.
  39. Azami NAM, Moi ML, Ami Y, et al. Genotype-specific and cross-reactive neutralizing antibodies induced by dengue virus infection: detection of antibodies with different levels of neutralizing activities against homologous and heterologous genotypes of dengue virus type 2 in common marmosets (*Callithrix jacchus*). *Virol J.* 2018;15:51.
  40. Zellweger RM, Tang WW, Eddy WE, et al. CD8<sup>+</sup> T cells can mediate short-term protection against heterotypic dengue virus reinfection in mice. *J Virol.* 2015;89:6494-505.