MOLECULAR EPIDEMIOLOGY OF DENGUE VIRUSES ISOLATED FROM PATIENTS WITH SUSPECTED DENGUE FEVER IN BANGKOK, THAILAND DURING 2006-2015

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Abstract. Dengue virus serotypes 1, 2, 3, and 4 (DENV1-DENV4), Family Flaviviridae, genus *Flavivirus* cause mosquito-borne diseases, such as dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in sub-tropical and tropical regions. In addition, different genotypes of each DENV serotype are involved in severity among dengue patients. This study evaluated the molecular epidemiology of patients' sera DENV isolates grown in C6/36 cells using quantitative (q)RT-PCR, DNA sequencing and construction of phylogenetic tree. QRT-PCR revealed 75 isolates consisting of DENV1 (n = 15), DENV2 (n = 20), DENV3 (n = 28), and DENV4 (n = 12). DNA sequencing and phylogenetic tree analysis demonstrated genotype I of DENV1, genotype Asian I of DENV3 consisting of genotype II (n = 5) and genotype III (n = 23), and genotype I of DENV4. Survey of dengue in Thailand showed presence of DENV3 genotype II since 1973, and genotype III since 2008. Our study reveals genetic information, which complements current knowledge on dengue epidemiology, evolution and transmission dynamics. Understanding of dengue epidemiology at the molecular level will be of particular importance in dengue disease control and prevention.

Keywords: dengue virus, DNA sequencing, epidemiology, phylogenetic tree analysis

INTRODUCTION

Dengue virus (DENV) belongs to the genus *Flavivirus*, family Flaviviridae. There are four antigenically distinct DENV serotypes, namely, DENV1, DENV2, DENV3, and DENV4 (Guzman *et al*, 2016). DENV genome consists of single-stranded positive-sense RNA approximately 11 kb in length that is capped at the 5′ end and lacks a 3′ polyadenylated sequence. DENV genome is translated as a single polypeptide and post-translationally cleaved into three structural proteins [capsid (C), premembrane (prM) and envelope (E)] and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Guzman *et al*, 2016).

DENV is transmitted to humans through the bite of infected *Aedes* mosqui-

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toes, particularly *Ae. aegypti* and *Ae. albopictus* (Hugo *et al*, 2014). Infection with any of the four DENV causes a wide spectrum of clinical features ranging from nearly asymptomatic disease, an undifferentiated febrile illness, dengue fever (DF), to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). DENV affects 50-200 million people and leads to approximately 20,000 deaths annually in tropical and subtropical regions of the world. The mortality rate of patients with severe dengue diseases is about 1-2.5% (Martina *et al*, 2009; Guzman *et al*, 2016). In Thailand, a total of 142,925 dengue cases (morbidity rate of 222.56/100,000 population) with 147 deaths (mortality rate of 0.23/100,000 population) were reported in 2015 (BOE, 2016). In endemic areas, co-circulation of multiple DENV serotypes has been shown (Holmes *et al*, 2009). In addition, each DENV serotype shows phylogenetically distinct genotypes (Klungthong *et al*, 2008; Teoh *et al*, 2013). Genotype and clade replacements in DENV serotypes are associated with prevalence of dengue disease (Zhang *et al*, 2005).

In this study, we evaluated the molecular epidemiology of DENV isolated from patients' sera, obtained in Bangkok, Thailand during 2006 to 2015.

MATERIALS AND METHODS

Viruses and cell culture

DENV1 (16007-strain), DENV2 (16681-strain), DENV3 (16562-strain) and DENV4 (1036-strain) passaged 50 times in C6/36 cells at a viral titer of 1×10^5 plaque-forming units (pfus)/ml were used as sources of viral nucleic acid/positive standards.

C6/36 cells were cultured in modified Eagle medium (MEM; GIBCO, Grands Island, NY) containing 10% fetal bovine serum (FBS; GIBCO), 2 mM L-glutamine (GIBCO), 1% sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 1% non-essential amino acid (GIBCO) at 32°C.

Patients' sera

Sera from 326 patients with suspected dengue and subsequently clinical diagnosed by Professor Dr Ampaiwan Juansamrit, Ramathibodi Hospital and Professor Dr Kulkanya Chokephaibulkit, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok during 2006-2015 were screened by SD Bioline Dengue Duo Rapid Test (Standard Diagnostics, Gyeonggi-do, Korea) and stored at -80°C until used.

Virus infection of C6/36 cells

A 100 µl aliquot of patient's serum was added to complete medium (MEM+10% FBS) containing C6/36 cells, which were serially passaged three times over a period of 7 days at 32°C. DENV in infected C6/36 cells and culture supernatant was detected by indirect immunofluorescent assay (IFA) and quantitative (q)RT-PCR), respectively.

IFA

Mouse monoclonal antibodies (mAbs) to DENV1 (15F3-1, ATCC HB-47), DENV2 (3H5-1, ATCC HB-46), DENV3 (5D4-11, ATCC HB-49) and DENV4 (1H10-6, ATCC HB-48) in tissue culture fluid were used as the primary Abs and goat FITC-conjugated anti-mouse IgG (Thermo Scientific, Amarillo, TX) was used as secondary Ab.

Uninfected and DENV-infected cells were spotted on slides and fixed with cold acetone. Each anti-DENV mouse mAb [diluted 1:100 in phosphate-buffered saline pH 7.4 (PBS)] was applied onto slides for 45 minutes at 37°C. Goat Ab conjugate (diluted 1:50 in PBS) then was applied and incubated for 45 minutes at 37°C. Slides were mounted with 50% buffered glycerol

Table 1
Primers and probes used in the study.

Name	Nucleotide sequence (5′→3′)	Genome position
QRT-PCR		
DENV1 F	CAAAAGGAAGTCGTGCAATA	8936-8955
DENV1 C	CTGAGTGAATTCTCTCTACTGAACC	9023-9047
DENV1 probe	FAM-CATGTGGTTGGGAGCACGC-BHQ1	8961-8979
DENV2 F	CAGGTTATGGCACTGTCACGAT	1426-1447
DENV2 C	CCATCTGCAGCAACACCATCTC	1482-1504
DENV2 probe	HEX-CTCTCCGAGAACAGGCCTCGACTTCAA-BHQ1	1454-1480
DENV3 F	GGACTGGACACACGCACTCA	701-720
DENV3 C	CATGTCTCTACCTTCTCGACTTGTCT	749-775
DENV3 probe	TXR-ACCTGGATGTCGGCTGAAGGAGCTTG-BHQ2	722-747
DENV4 F	TTGTCCTAATGATGCTGGTCG	884-904
DENV4 C	TCCACCTGAGACTCCTTCCA	953-992
DENV4 probe	Cy5-TTCCTACTCCTACGCATCGCATTCCG-BHQ3	939-960
RT-PCR and DNA sequencing		
DG1 (E1)-F	AGTAGAGACTTGGGCTCTGA	802-821
DG1 (E2)-R	CCAGTTGATTACACATCCCG	2424-2443
DG2 (E1)-F	CAGCTGTCGCTCCTTCA	914-930
DG2 (E2)-R	GCTCTAGATCGGCCTGCACCAT	2410-2432
DG3 (E1)-F	GCCCATTACATAGGCACTTCC	857-877
DG3 (E2)-R	ACACAYCCCATGTCAGCTTG	2408-2427
DG4 (E1)-F	CTCTTGGCAGGATTYATGGC	843-862
DG4 (E2)-R	CACTCCATGACACCACACAACC	2430-2451

Y = C or T.

and inspected under an UV microscope (BX60 model, Olympus, Tokyo, Japan).

QRT-PCR

RNA was extracted from culture supernatant using E.Z.N.A viral RNA mini kit (Omega biotek, Norcross, GA) and a small aliquot was used for qRT-PCR with primers specific for each of the DENV (Table 1). Reaction solutions were prepared using KAPA PROBE FAST universal one-step qRT-PCR master mix kit (KAPA biosystems, Wilmington, MA) and were thermocycled in a Chromo4 (Bio-Rad, Hercules, CA) as previously described (Johnson *et al*, 2005). Positive controls were viruses extracted from cell culture, and negative control was nuclease-free water.

DENV E gene sequencing and phylogenetic tree construction

DENV genotyping was performed using E gene sequence (1,485 nt). Viral RNA was reverse-transcribed into cDNA using Maxima H minus-first strand cDNA synthesis kit (Thermo Scientific), which then was used as template for PCR amplification using Phusion Flash high-fidelity PCR master mix (Thermo Scientific) and DENV E-specific primers (Table 1). Amplicons were sequenced by First BASE Laboratories (Selangor, Malaysia), and sequences were aligned using BioEdit

sequence alignment editor (www.mbio.ncsu.edu/BioEdit/bioedit.html) and analyzed using basic local alignment search tool (BLAST; https://www.ncbi.nlm.nih.gov/BLAST/). DENV E gene sequence data from GenBank were included. Sequence multiple alignments were performed using ClustalW (Thompson *et al*, 1994). The phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software (Tamura *et al*, 2013). Neighbor-joining tree was constructed with 1,000 bootstrap replicates. Genotypes of DENV were classified as previously described (Klungthong *et al*, 2008). The E gene sequences obtained in the study were deposited in GenBank with accession numbers MG564068-MG564138, KT026308-KT026310 and KR922405 (Table 2).

RESULTS

In order to evaluate the molecular epidemiology of DENV isolated from patients' sera obtained from two hospitals in Bangkok during 2006 to 2015, 326 dengue patients' sera were screened using SD Bioline dengue duo rapid test, of which 143 (44%) were positive for dengue NS1 Ag, 221 (68%) positive for dengue IgM and 173 (53%) positive for IgG. When the 326 patients' sera were directly inoculated to C6/36 cells for 3 passages, IFA revealed 75 (23.0%) DENV-positive isolates (bright green apple fluorescence in cytoplasm) and 251 (77%) DENV-negative virus isolates (dull green fluorescence in cytoplasm) (data not shown). The 75 DENV isolates were categorized as DENV1 (n = 15, 20%), DENV2 (n = 20, 27%), DENV3 (n = 28, 37%), and DENV4 (n = 12, 16%). QRT-PCR quantified DENV titers as ranging from 7.75×10^2 to 2.26×10^6 copies/µl cell culture supernatant (data not shown).

In order to evaluate the genotypes within each serotype, genotyping based on the E gene was conducted. The amplicon sequences were employed to construct phylogenetic tree for each DENV type revealing that all DENV1 isolates (n = 15) belonged to genotype I (Fig 1A), all DENV2 isolates (n = 20) to Asian I genotype (Fig 1B), DENV3 isolates to genotype II (n = 5) and genotype III (n = 23) (Fig 1C), and all DENV4 isolates (n = 12) to genotype I (Fig 1D).

DISCUSSION

Thailand is one of DENV hyper endemic area where all four DENV serotypes have spread to all provinces of the country (BOE, 2016). Currently, only limited DENV genetic information is available in Thailand. Molecular epidemiology of each DENV serotype can aid in a better management of dengue (Lestari *et al*, 2017). DENV3 was the predominant serotype in 2015 as determined from patients' sera in Bangkok during 2006-2015 (Table 2). This result is consistent with the reported by the Bureau of Epidemiology (BOE, 2016). Previous reports demonstrated co-circulation of the four DENV serotypes caused DF, DHF and DSS (Klungthong *et al*, 2008). To date, we found that the co-circulation of multiple genotypes and genotype replacement increased with the rise dengue cases in Thailand (Klungthong *et al*, 2004; Zhang *et al*, 2005; *ibid*, 2006).

Of the five DENV1 genotypes (I, II, III, IV and V), in Thailand, genotype I is a major genotype while genotype II (Halstead and Simasthien, 1970) and genotype V are minor genotypes (Zhang *et al*, 2005; Klungthong *et al*, 2008). In this study, only DENV1 genotype I was found in agreement with previous reports (Zhang *et al*, 2005; Klungthong *et al*, 2008) indicating

Table 2
Serotype, genotype and year of DENV isolates.

No.	Isolate ID	Serotype	Genotype	Isolation year	GenBank Accession No.
1	06/177	DENV1	I	2006	MG564069
2	11/1193	DENV1	I	2011	MG564070
3	11/1590	DENV1	I	2011	MG564072
4	11/1660	DENV1	I	2011	MG564073
5	11/172	DENV1	I	2011	MG564074
6	11/265	DENV1	I	2011	MG564075
7	11/606	DENV1	I	2011	MG564076
8	11/69	DENV1	I	2011	MG564068
9	15/1310	DENV1	I	2011	MG564080
10	15/1321	DENV1	I	2011	MG564081
11	15/1323	DENV1	I	2015	MG564082
12	15/1075	DENV1	I	2015	MG564079
13	15/1048	DENV1	I	2015	MG564078
14	11/1212	DENV1	I	2015	MG564071
15	11/976	DENV1	I	2015	MG564077
16	11/1688	DENV2	Asian I	2011	MG564091
17	11/1694	DENV2	Asian I	2011	MG564092
18	11/1657	DENV2	Asian I	2011	MG564090
19	11/151	DENV2	Asian I	2011	MG564088
20	11/941	DENV2	Asian I	2011	MG564095
21	11/1236	DENV2	Asian I	2011	MG564083
22	11/1253	DENV2	Asian I	2011	MG564084
23	11/1380	DENV2	Asian I	2011	MG564085
24	11/1387	DENV2	Asian I	2011	MG564086
25	11/1414	DENV2	Asian I	2011	MG564087
26	11/1569	DENV2	Asian I	2011	MG564089
27	11/1695	DENV2	Asian I	2011	MG564093
28	11/1707	DENV2	Asian I	2011	MG564094
29	12/436	DENV2	Asian I	2012	MG564096
30	13/37	DENV2	Asian I	2013	MG564097
31	13/45	DENV2	Asian I	2013	MG564098
32	15/1057	DENV2	Asian I	2015	MG564100
33	15/1082	DENV2	Asian I	2015	MG564101
34	15/1252	DENV2	Asian I	2015	MG564102
35	15/1046	DENV2	Asian I	2015	MG564099
36	06/429	DENV3	II	2006	MG564130
37	11/586	DENV3	II	2011	MG564106
38	11/372	DENV3	II	2011	MG564105
39	15/1072	DENV3	II	2014	MG564117
40	15/1328	DENV3	II	2015	MG564107
41	15/1329	DENV3	III	2006	MG564129
42	06/129	DENV3	III	2011	MG564103
43	11/1230	DENV3	III	2014	MG564104
44	14/141	DENV3	III	2014	MG564108

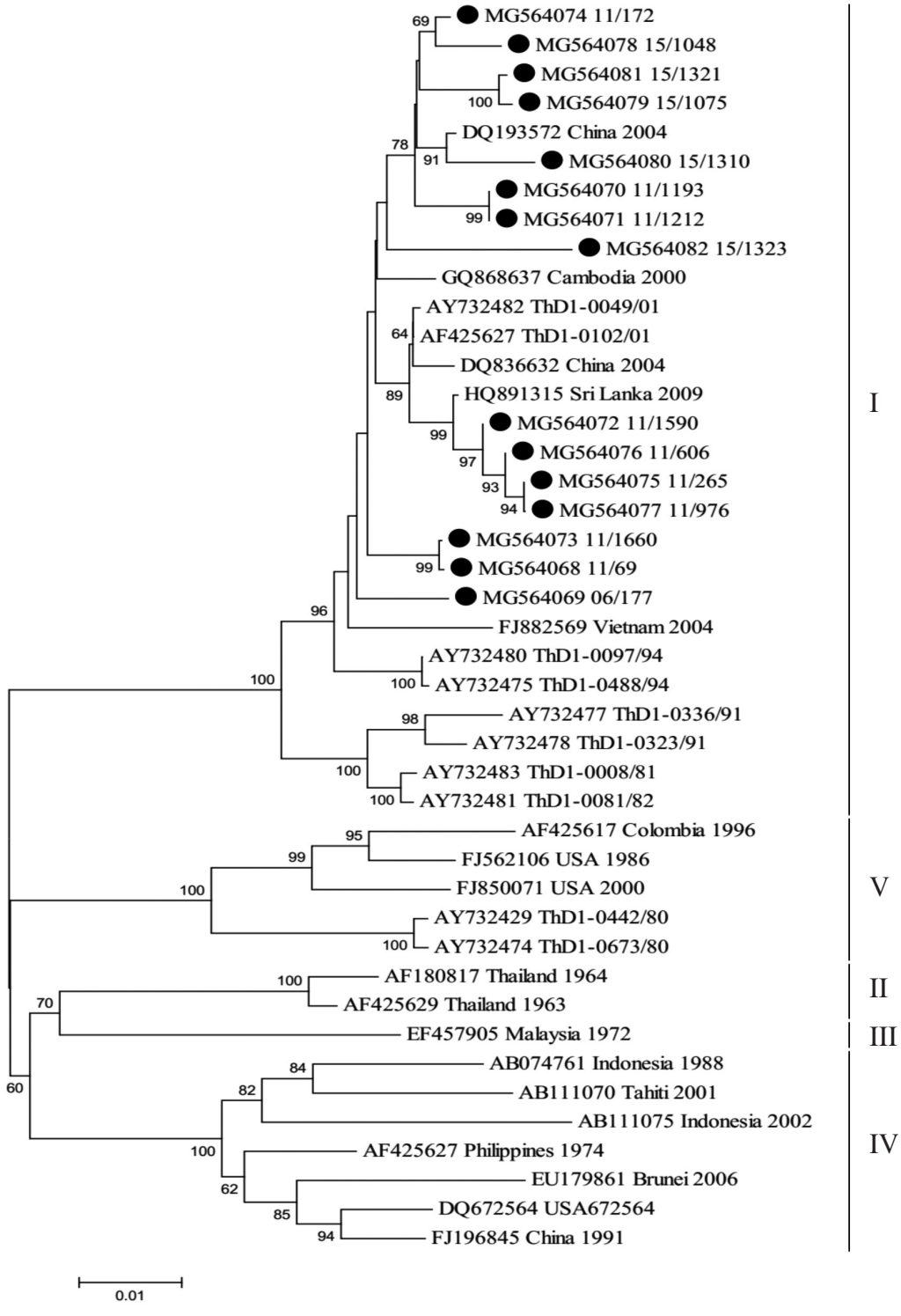
Table 2 (Continued).

No.	Isolate ID	Serotype	Genotype	Isolation year	GenBank Accession No.
45	15/1052	DENV3	III	2014	MG564113
46	15/1053	DENV3	III	2014	MG564114
47	15/1066	DENV3	III	2015	MG564115
48	15/1068	DENV3	III	2015	MG564116
49	15/1084	DENV3	III	2015	MG564118
50	15/1090	DENV3	III	2015	MG564119
51	15/1091	DENV3	III	2015	MG564120
52	15/1092	DENV3	III	2015	MG564121
53	15/1093	DENV3	III	2015	MG564122
54	15/1303	DENV3	III	2015	MG564123
55	15/1312	DENV3	III	2015	MG564124
56	15/1313	DENV3	III	2015	MG564125
57	15/1316	DENV3	III	2015	MG564126
58	15/1317	DENV3	III	2015	MG564127
59	15/1319	DENV3	III	2015	MG564128
60	14/164	DENV3	III	2015	MG564109
61	14/170	DENV3	III	2015	MG564110
62	14/177	DENV3	III	2015	MG564111
63	14/182	DENV3	III	2015	MG564112
64	11/1373	DENV4	I	2011	KT026309
65	11/1404	DENV4	I	2011	KT026310
66	11/1194	DENV4	I	2011	KT026308
67	11/1666	DENV4	I	2011	KR922405
68	15/1074	DENV4	I	2015	MG564131
69	15/1105	DENV4	I	2015	MG564132
70	15/1305	DENV4	I	2015	MG564133
71	15/1306	DENV4	I	2015	MG564134
72	15/1307	DENV4	I	2015	MG564135
73	15/1315	DENV4	I	2015	MG564136
74	15/1322	DENV4	I	2015	MG564137
75	15/1324	DENV4	I	2015	MG564138

this genotype circulated in Bangkok during the period of the study. DENV1 genotype I has been predominated in Southeast Asia, such as Cambodia (Shu *et al*, 2009), Indonesia (Yamanaka *et al*, 2011; Sasmono *et al*, 2015), Lao PDR (Dubot-Peres *et al*, 2013), Malaysia (Ng *et al*, 2015), Myanmar (Ngwe Tun *et al*, 2016; Kyaw *et al*, 2017), Singapore (Schreiber *et al*, 2009; Lee *et al*, 2012) and Vietnam (Shu *et al*, 2009).

Similarly, of the five DENV2 genotypes (Asian I, Asian II, Asian/American, Cosmopolitan, and American) (Klungthong *et al*, 2008) and of the three DENV4 genotypes (I, II and III) (Klungthong *et al*, 2004) this study found only DENV2 Asian I genotype, and DENV4 genotype I in agreement with previous reports (Wang *et al*, 2000; Klungthong *et al*, 2004; Zhang *et al*, 2006; Klungthong *et al*, 2008). Circulating DENV2 Asian I genotype has been predominated in Southeast Asia,

(A)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MG564078 15/1048
MG564081 15/1321
MG564079 15/1075
DQ193572 China 2004
MG564080 15/1310
MG564070 11/1193
MG564071 11/1212
MG564082 15/1323
GQ868637 Cambodia 2000
AY732482 ThD1-0049/01
AF425627 ThD1-0102/01
DQ836632 China 2004
HQ891315 Sri Lanka 2009
MG564072 11/1590
MG564076 11/606
MG564075 11/265
MG564077 11/976
MG564073 11/1660
MG564068 11/69
MG564069 06/177
FJ882569 Vietnam 2004
AY732480 ThD1-0097/94
AY732475 ThD1-0488/94
AY732477 ThD1-0336/91
AY732478 ThD1-0323/91
AY732483 ThD1-0008/81
AY732481 ThD1-0081/82
AF425617 Colombia 1996
FJ562106 USA 1986
FJ850071 USA 2000
AY732429 ThD1-0442/80
AY732474 ThD1-0673/80
AF180817 Thailand 1964
AF425629 Thailand 1963
EF457905 Malaysia 1972
AB074761 Indonesia 1988
AB111070 Tahiti 2001
AB111075 Indonesia 2002
AF425627 Philippines 1974
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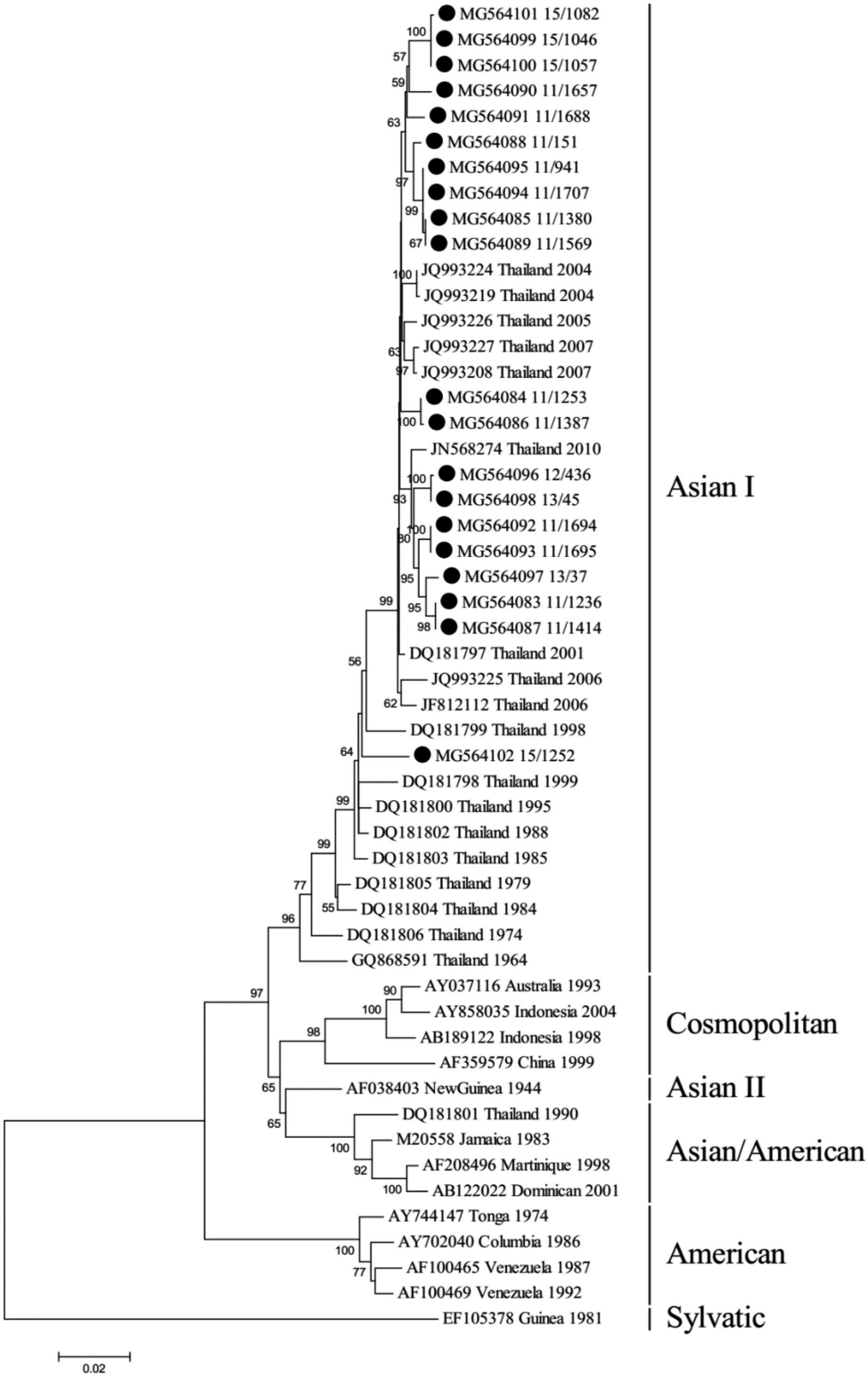
DQ672564 USA672564
FJ196845 China 1991
I
V
II
III
IV
0.01

(**B**)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MG564094 11/1707
MG564085 11/1380
MG564089 11/1569
JQ993224 Thailand 2004
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JQ993227 Thailand 2007
JQ993208 Thailand 2007
MG564084 11/1253
MG564086 11/1387
JN568274 Thailand 2010
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MG564098 13/45
MG564092 11/1694
MG564093 11/1695
MG564097 13/37
MG564083 11/1236
MG564087 11/1414
DQ181797 Thailand 2001
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MG564102 15/1252
DQ181798 Thailand 1999
DQ181800 Thailand 1995
DQ181802 Thailand 1988
DQ181803 Thailand 1985
DQ181805 Thailand 1979
DQ181804 Thailand 1984
DQ181806 Thailand 1974
GQ868591 Thailand 1964
AY037116 Australia 1993
AY858035 Indonesia 2004
AB189122 Indonesia 1998
AF359579 China 1999
AF038403 NewGuinea 1944
DQ181801 Thailand 1990
M20558 Jamaica 1983
AF208496 Martinique 1998
AB122022 Dominican 2001
AY744147 Tonga 1974
AY702040 Columbia 1986
AF100465 Venezuela 1987
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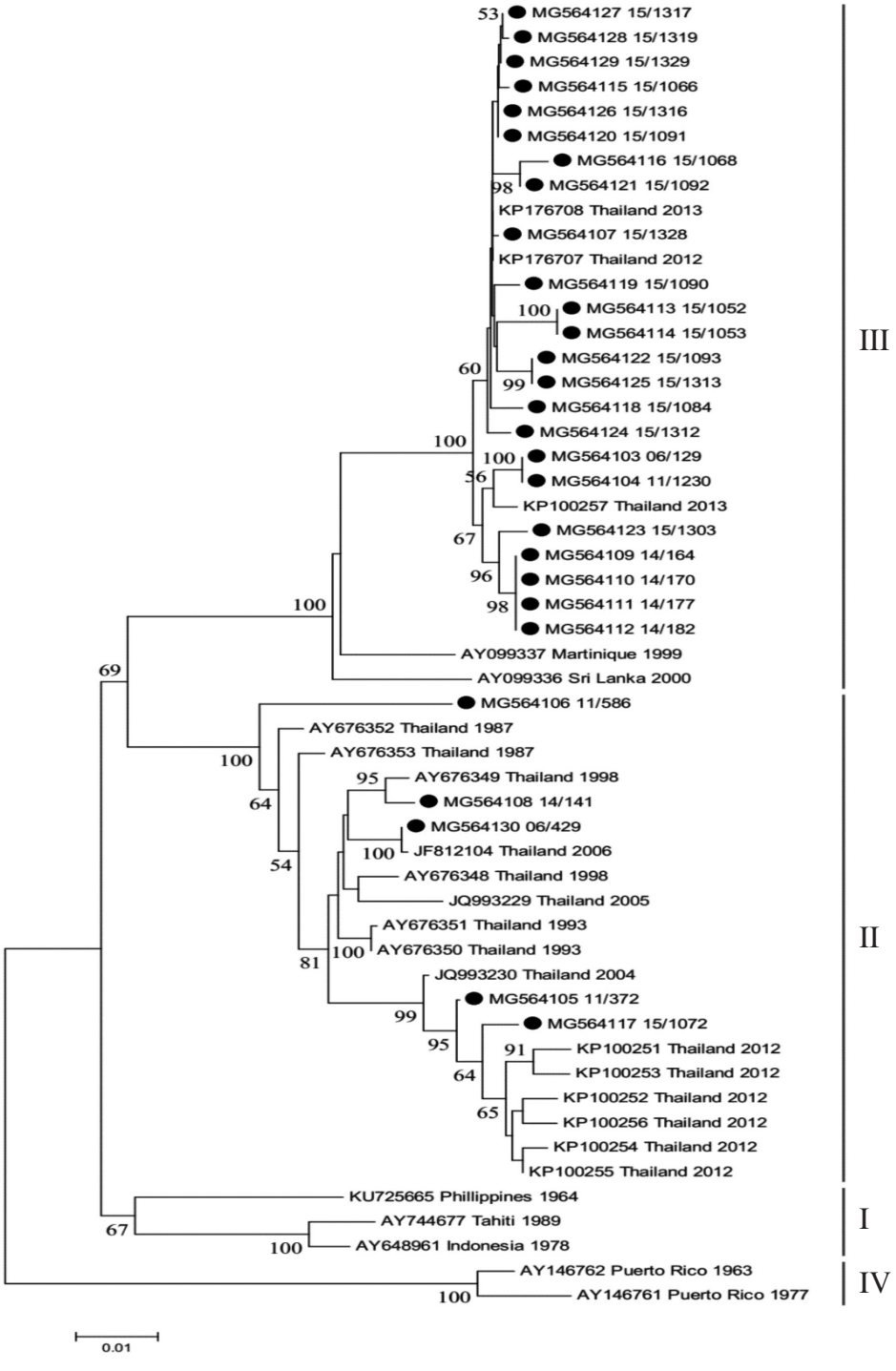
EF105378 Guinea 1981
Asian I
Cosmopolitan
Asian II
Asian/American
American
Sylvatic
0.02

(**C**)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MG564124 15/1312
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MG564110 14/170
MG564111 14/177
MG564112 14/182
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AY676349 Thailand 1998
MG564108 14/141
MG564130 06/429
JF812104 Thailand 2006
AY676348 Thailand 1998
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JQ993230 Thailand 2004
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MG564117 15/1072
KP100251 Thailand 2012
KP100253 Thailand 2012
KP100252 Thailand 2012
KP100256 Thailand 2012
KP100254 Thailand 2012
KP100255 Thailand 2012
KU725665 Phillippines 1964
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III
II
I
IV
0.01

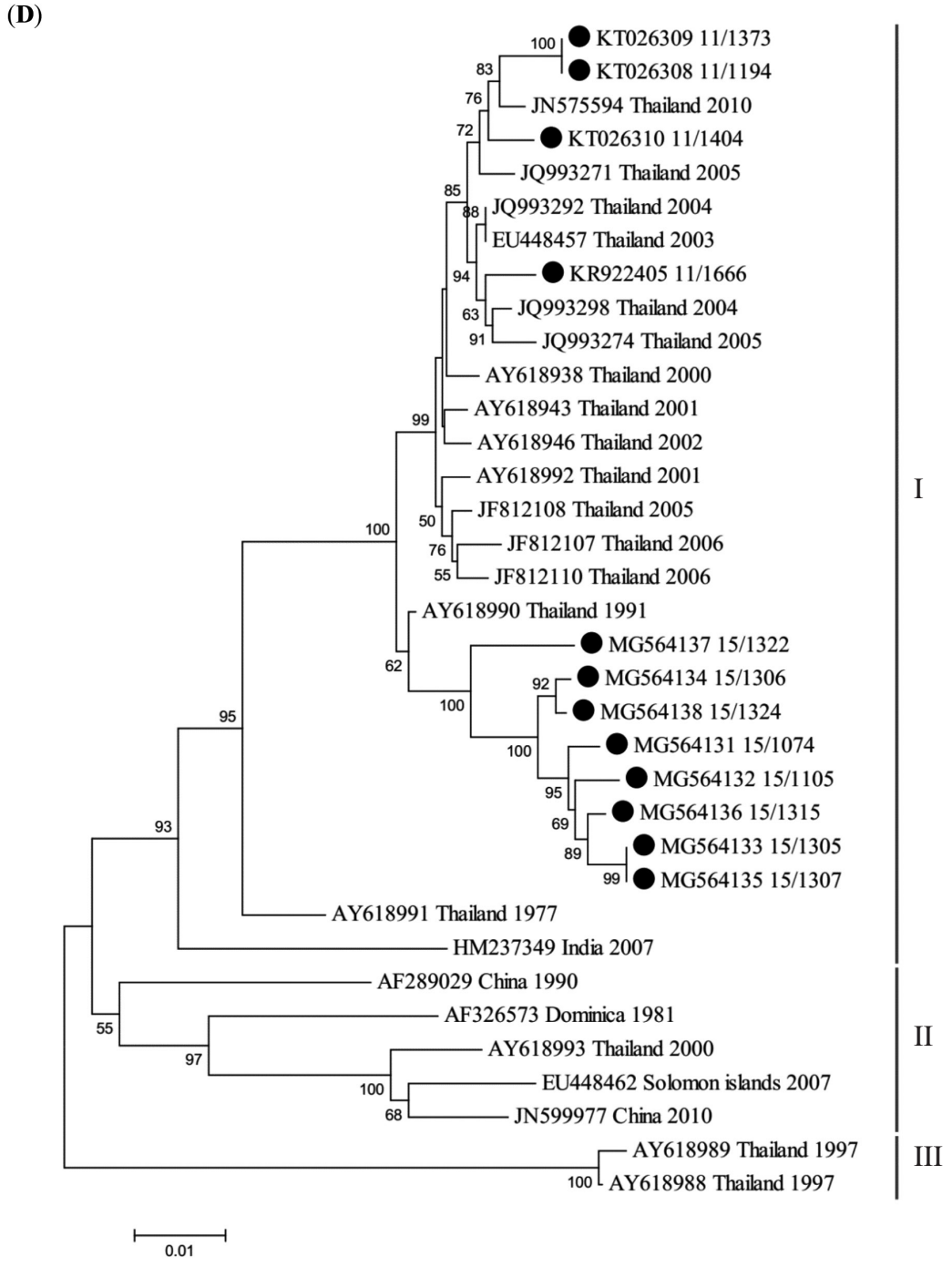

Fig 1–Neighbor joining tree of (A) DENV1, (B) DENV2, (C) DENV3, and (D) DENV4. Black dot denotes DENV used in the study. Scale bar indicates evolutionary distance. Bootstrap values ≥ 50% are shown.

such as Cambodia (Huang *et al*, 2012). Lao PDR (Huang *et al*, 2012; Ernst *et al*, 2015), Myanmar (Thant *et al*, 2015; Kyaw *et al*, 2017) and Vietnam (Vu *et al*, 2010). However, in 1969 and 1998, DENV2 Cosmopolitan genotype (Twiddy *et al*, 2002; Zhang *et al*, 2006) and in 1980-1991 Asian/American genotype (Zhang *et al*, 2006; Klungthong *et al*, 2008) were reported in Bangkok. Likewise, DENV4 genotype I has been reported to be predominant in Cambodia (Tuiskunen *et al*, 2011), Myanmar (Thant *et al*, 2015; Kyaw *et al*, 2017) and Vietnam (Takamatsu *et al*, 2015). But DENV4 genotypes II and III were reported during 1997-2001 in Bangkok (Klungthong *et al*, 2004). Genotype II was first reported in Thailand in 2012 (Kittichai *et al*, 2015) and is predominant in Indonesia (Haryanto *et al*, 2016), Malaysia (Holmes *et al*, 2009), Singapore (Lee *et al*, 2012).

DENV3 consists of four genotypes (I, II, III and IV) (Klungthong *et al*, 2008) and genotypes II and III were found in this survey. DENV3 genotype III was shown to be predominant in Latin America region (Aquino *et al*, 2008**;** Kochel *et al*, 2008) and South Asia (Patil *et al*, 2008; Koo *et al*, 2013) while genotype II has been predominant in Thailand since the 1970s (Lanciotti *et al*, 1994; Zhang *et al*, 2005; Klungthong *et al*, 2008; Chen, 2013). Genotype III has been circulating predominantly in Thailand and Lao PDR since 2008 (Huang *et al*, 2012; Lao *et al*, 2014) and also in Lao PDR (Lao *et al*, 2014), Myanmar (Thant *et al*, 2015: Kyaw *et al*, 2017), Singapore (Lee *et al*, 2012) and Vietnam (Phu Ly *et al*, 2015). In addition, genotype II has been circulated in Lao PDR (Lao *et al*, 2014), Malaysia (Fong *et al*, 2004), Myanmar (Shu *et al*, 2009; Thant *et al*, 2015), Singapore (Lee *et al*, 2012) and Vietnam (Huang *et al*, 2007).

In summary, this study describes the molecular epidemiology of dengue infection in Bangkok during 2006-2015. All four DENV serotypes circulated in Bangkok during this period. DENV1, DENV2 or DENV4 each have only one genotype while DENV3 has two genotypes (II and III), with DENV3 genotype III becoming predominant in recent years. The study provides genetic information that complements current knowledge on dengue epidemiology, evolution and transmission dynamics. The data should prove useful in future studies on molecular epidemiology in the prevention and control of dengue in Thailand.

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