Molecular epidemiology of mosquito-borne viruses from imported

Name of researcher: Pei-Yun Shu, Cheng-Fen Yang, Chien-Ling Su, Tung-Chien Hsu, Shu-Fen Chang, Chien-Chou Lin
Affiliation: Centers for Disease Control, Taiwan

Summary:

Dengue and chikungunya are the most common imported mosquito-borne diseases to Taiwan. Dengue is not considered endemic in Taiwan and the constant importation of dengue viruses (DENVs) from the neighboring countries through close commercial links and air travel is responsible for the local outbreaks each year. To reduce the imported mosquito-borne viruses and their local spread, laboratory-based arboviral surveillance system has been established for early identification of imported cases. In 2014, a total of 240 imported dengue cases were identified, among them, 117 (48.8%) were detected through airport and seaport fever screening surveillance systems. Imported dengue cases were arriving from 16 countries. Malaysia (71 cases), Indonesia (58 cases), the Philippines (33 cases) and China (23 cases) were the most frequent importing countries. From the acute phase serum samples of all imported dengue cases, 80 DENV-1, 53 DENV-2, 25 DENV-3, and 12 DENV-4 strains were identified. Sequence analysis showed that genotype I of DENV-1, cosmopolitan genotype of DENV-2 and genotype I of DENV-3 strains were the predominant DENV strains circulating in Southeast Asia. In addition, 8 imported chikungunya cases were identified in 2014. Travelers were arriving from the Philippines (1 case), Indonesia (6 cases) and Guatemala (1 case). All of the imported chikungunya virus (CHIKV) strains from the Philippines and Indonesia belonged to Asian genotype.

Purpose:

The geographic spread of mosquito-borne viruses is increasing throughout the world. Understanding the epidemiological situations of the diseases and the phenotypic and genotypic characteristics of viruses contributes to the development of new strategies for prevention and control. In order to promote communication and exchange of the information of dengue and other mosquito-borne viruses, laboratory network between Asia and Pacific Rim should be developed and strengthened.

Methods:

1 Clinical samples and laboratory diagnosis

Human serum samples from clinically suspected DENV and other arbovirus infections were submitted to the Vector-Borne Viral and Rickettsial Diseases Laboratory, Center for Research, Diagnostics and Vaccine Development, Centers for Disease Control, Taiwan (Taiwan CDC) for laboratory diagnosis. A

confirmed dengue or chikungunya case was defined as febrile illness associated with a positive real-time reverse transcription (RT)-PCR test, isolation of DENV or CHIKV, or the detection of DENV- or CHIKV-specific IgM and IgG antibodies (1-3). A multiplex one-step real-time RT-PCR was developed to simultaneously detect and differentiate various flaviviruses and alphaviruses in the acute-phase serum samples using group-specific and virus-specific primers. In addition, a flavivirus/alphavirus-specific capture ELISA was developed to detect and differentiate various flavivirus/alphavirus infections.

2 Virus isolation and identification

DENVs and CHIKVs were isolated from the acute phase serum samples of confirmed cases. The virus isolation was performed using mosquito cell line (clone C6/36 of *Aedes albopictus* cells). For each acute-phase serum sample, 50 μl of diluted serum samples at ratios of 1:20, 1:40, 1:80, and 1:160 (diluted with RPMI , Gibco/BRL, Life Technologies, containing 1% FCS), were added to a 96-well microtiter plate, and 10^5 cells/100 μl/well of C6/36 were then added to the microtiter plate, followed by incubation for 7 days at 28℃. Cells were harvested, and infection was confirmed via immunofluorescence assay using dengue or chikunkunya serotype-specific monoclonal antibodies. The viruses were subcultured in C6/36 cells and harvested for nucleotide sequencing after the first or second passage. Isolated viruses were identified using the nomenclature of serotype/country/strain/year of isolation.

3. Primers used for RT-PCR of arboviruses

To screen viremic fever patients with flavivirus and alphavirus infections, a multiplex one-step SYBR Green I-based real-time RT-PCR was developed (1,3,4). A cocktail consisted of two sets of primers (Flavivirus consensus primer set 1370F: 5’-TGY GTB TAC AAC ATG ATG GG-3’, 1442F: 5’-ATA TGG TAC ATG TGG CTA GGA GC-3’, 1620R: 5’-GTG TCC CAN CCH GCT GTG TCA-3’; JEV specific primer set JE3F1: 5’-CCC TCA GAA CCG TCT CGG AA-3’, JE3R1: 5’-CTA TTC CCA GGT GTC AAT ATG CTG T-3’), a cocktail consisted of two sets of primers (DENV group specific primer set R36: 5’-CAA TAT GCT GAA ACG CGA GAG AAA -3’, R169: 5’-CCC CAT CTA ACC AAT ATT CCT GCT-3’, R170: 5’-CCC CAT CTG TTC AGT ATC CCT GCT-3’; SFTSV specific primer set SFTS-1F: 5’-GGA AAC TGG RAG AGA GAA CT -3’, SFTS-1R: 5’-GAA GTG AAC AAG TGG TGG TT -3’), and a cocktail consisted of three sets of primers (alphavirus-specific primer set AL-2: 5’-AAG CTY CGC GTC CTT TAC CAA AG-3’ and AL-3: 5’-GTG GTG TCA AAC CCT ATC CA-3’ targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all alphaviruses, CHIKV-specific primer set CHIK-F: 5’-AAG CTY CGC GTC CTT TAC CAA AG-3’ and CHIK-R: 5’-CCA AAT TGT CCY GGT CTT CCT-3’ targeted a region of the envelope protein 1 (E1) gene of CHIKVs (7), Ross River virus-specific primer set RRV-1: 5’-GGG TAG AGA GAA GTT

YGT GGT YAG-3' and RRV-2: 5'-CGG TAT ATC TGG YGG TGT RTG C-3' targeted a region of the envelope protein 2 (E2) gene of Ross River virus) were used for RT-PCR screening. Positive results were then confirmed by sequence analysis.

4. Preparation of viral RNA, RT-PCR amplification and nucleotide sequencing

Viral RNAs were extracted from either acute phase serum samples or culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For full-length structural protein gene sequencing, extracted viral RNA from culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains was used as the template for cDNA synthesis, which subsequently was used for PCR amplification. Primers used for amplification and sequencing of complete structural protein gene sequences of DENVs and CHIKV were described previously (5-7). The RT-PCR reaction was carried out with the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen). PCR products were purified using the Qiagen QIA quick Gel Extraction kit (QIAGEN). Nucleotide sequences were determined by the ABI Prism automated DNA sequencing kit and the ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocols. Overlapping nucleotide sequences were combined for analysis and edited with the Lasergene software package (DNASTAR Inc, Madison, WI).

5. Phylogenetic analysis

The nucleotide sequences of complete E genes of DENV strains were aligned, edited and analyzed using Clustal W software. The phylogenetic analysis was performed using MEGA version 6 (http://www.megasoftware.net/). Phylogenetic trees were constructed using the maximum likelihood method based on the General Time Reversible (GTR) model. The reliability of the analysis was evaluated by a bootstrap test with 1,000 replications.

Results:

1. Imported dengue cases in Taiwan, 2014

A total of 240 laboratory confirmed imported dengue cases were identified in 2014. Among these patients, 117 (48.8%) were detected through the airport and seaport fever screening surveillance systems. Table 1 showed the summary of countries of origin and DENV serotypes of imported cases. Most cases arrived from Southeast Asian countries, with Malaysia, Indonesia, the Philippines and China were the most frequent importing countries. In addition, cases were also imported from India subcontinent (India, Bangladesh), the South Pacific region

(Nauru, Tuvalu and French Polynesia), and Middle East (Saudi Arabia). The results reflected the frequency of air travel between Taiwan and these nations, as well as the intensity of massive dengue outbreaks in the country origin during the same period.

2. Nucleotide sequencing and phylogenetic analysis

From the 240 imported dengue cases, 80, 53, 25, and 12 cases were determined to be infected with the DENV-1, DENV-2, DENV-3, and DENV-4, respectively (Table 1). Among them, 139 DENV strains were isolated from acute-phase serum samples of patients. A summary of the serotype and genotype distributions of these isolates by country is summarized in Table 2. The DENV-1 genotype I and DENV-2 cosmopolitan genotype were the most dominant genotypes of DENV strains isolated from imported cases arriving from Southeast Asia.

Figure 1 showed the phylogenetic tree of E gene sequences of DENV-1 strains isolated from indigenous and imported dengue cases. The result showed that epidemic DENV-1 strains (D1/Taiwan/806KH/2014) in the genotype I causing large outbreak in Southern Taiwan is most closely related to virus from Indonesia. All of the DENV-1 isolated from imported cases from Indonesia belonged to genotype I. DENV-1 strains isolated from imported cases from China fell into genotype I and III, indicating multiple DENV-1 strains were circulating in China in 2014. Genotype III of DENV-1 was newly emerging in several Southeast Asian countries.

Figure 2 showed the phylogenetic tree of E gene sequences of DENV-2 strains. Most of DENV-2 strains isolated from imported cases from Southeast Asia belonged to cosmopolitan genotype. A few DENV-2 isolates from Malaysia, Myanmar and Thailand belonged to Asian 1 genotype. No Asian 2 genotype and Asian/American genotype strains were found in imported cases in 2014.

Figure 3 showed the phylogenetic tree of E gene sequences of DENV-3 strains. DENV-3 genotype I contains viruses from Indonesia and the Philippines, genotype II contains virus from Cambodia, and genotype III contains viruses from Malaysia and Thailand.

Figure 4 showed the phylogenetic tree of E gene sequences of DENV-4 strains. DENV-4 genotype I contains viruses from Myanmar, Thailand, and Vietnam, and genotype II contains viruses from Malaysia, Indonesia, the Philippines and Singapore.

3. CHIKV identification and characterization

For other arboviruses, we identified 8 imported CHIK cases in 2014. Among these travelers, 1, 6, 1 arrived from the Philippines, Indonesia, and Guatemala, respectively. Phylogenetic analysis showed that all CHIKV strains from Indonesia and the Philippines belonged to Asian genotype (Figure 5).

Discussion:

Studies on imported cases with arboviral infections have provided useful information of geographic distribution and global movement of arboviruses. A total of 240 imported dengue cases and 8 chikungunya cases were identified in 2014. Phylogenetic analysis of virus sequences demonstrated that DENV-1 genotype I and DENV-2 cosmopolitan genotype comprised the predominant genotypes circulating in Southeast Asia. DENV-1 genotype III strains were newly emerging in Malaysia, China and Singapore. DENV-4 genotype II strains were re-emerging in the Philippines. The genetic database generated from these isolated imported strains provides useful information for the understanding of global distributions and movements of various DENV and CHIKV serotypes and genotypes. Understanding the genetic changes and the mode of transmission of these viruses is important toward the development of effective control measures.

Reference list:

1. Shu PY, Chen LK, Chang SF, Su CL, Chien LJ, Chin C, Lin TH, Huang JH. Dengue virus serotyping based on envelope and membrane and nonstructural protein NS1 serotype-specific capture immunoglobulin M enzyme-linked immunosorbent assays. J Clin Microbiol. 2004;42:2489-94.
2. Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, Chien LJ, Chin C, Lin TH, Huang JH. Comparison of capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary dengue virus infections. Clin Diagn Lab Immunol. 2003;10:622-30.
3. Shu PY, Yang CF, Su CL, Chen CY, Chang SF, Tsai KH, Cheng CH, Huang JH. Two imported chikungunya cases, Taiwan. Emerg Infect Dis. 2008;14:1326-7.
4. Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. J Virol Methods. 2005;124:65-71.
5. Huang JH, Liao TL, Chang SF, Su CL, Chien LJ, Kuo YC, Yang CF, Lin CC, Shu PY. Laboratory-based dengue surveillance in Taiwan, 2005: a molecular epidemiologic study. Am J Trop Med Hyg. 2007;77:903-9.
6. Shu PY, Su CL, Liao TL, Yang CF, Chang SF, Lin CC, Chang MC, Hu HC, Huang JH. Molecular characterization of dengue viruses imported into Taiwan during 2003-2007: geographic distribution and genotype shift. Am J Trop Med Hyg. 2009;80:1039-46.
7. Huang JH, Yang CF, Su CL, Chang SF, Cheng CH, Yu SK, Lin CC, Shu PY. Imported chikungunya virus strains, Taiwan, 2006-2009. Emerg Infect Dis. 2009;15:1854-6.

Table 1. Countries of origin and DENV serotypes of imported cases in Taiwan, 2014.

Country	Case	Fever screening	DENV-1	DENV-2	DENV-3	DENV-4	Unknown
Malaysia	71	41	30	24	2	1	14
Indonesia	58	35	12	10	20	2	14
Philippines	33	12	7	6	1	5	14
China	23	7	14	2	-	-	7
Singapore	12	9	7	2	-	1	2
Myanmar	10	3	2	3	-	1	4
Thailand	10	3	2	3	1	1	3
Vietnam	9	3	3	1	-	1	4
Cambodia	3	1	1	-	1	-	1
India	3	2	1	-	-	-	2
Bangladesh	2	-	1	-	-	-	1
Nauru	2	-	-	-	-	-	2
French Polynesia	1	-	-	-	-	-	1
Tuvalu	1	1	-	1	-	-	0
Saudi Arabia	1	-	-	1	-	-	0
Japan	1	-	-	-	-	-	1
Total	240	117	80	53	25	12	70

Table 2. Summary of serotype and genotype distributions of DENV strains isolated from imported cases in Taiwan, 2014

Serotype	DENV-1			DENV-2		DENV-3			DENV-4		
Genotype	I	II	III	Cosmopolitan	Asian 1	I	II	III	I	II	Total
Malaysia	22	1	5	16	1	0	0	1	0	1	47
Indonesia	12	0	0	7	0	16	0	0	0	2	37
Philippines	0	7	0	6	0	1	0	0	0	5	19
China	5	0	3	2	0	0	0	0	0	0	10
Singapore	0	0	5	2	0	0	0	0	0	1	8
Myanmar	2	0	0	0	3	0	0	0	1	0	6
Thailand	0	0	0	1	1	0	0	1	1	0	4
Vietnam	2	0	0	1	0	0	0	0	1	0	4
Cambodia	0	0	0	0	0	0	1	0	0	0	1
India	0	0	1	0	0	0	0	0	0	0	1
Tuvalu	0	0	0	1	0	0	0	0	0	0	1
Saudi Arabia	0	0	0	1	0	0	0	0	0	0	1
Total	43	8	14	37	5	17	1	2	3	9	139

Table 3. Imported chikungunya cases in Taiwan, 2014.

No.	Year	Seq name	Location	E1-226	Genotype
CK10300001	2014	1401aTW	Philippines	A	Asian
CK10300010	2014	1403aTW	Indonesia	A	Asian
CK10300011	2014	1403bTW	Indonesia	A	Asian
CK10300013	2014	1404aTW	Indonesia	A	Asian
CK10300024	2014	1406aTW	Indonesia	A	Asian
CK10300032	2014	1408aTw	Indonesia	A	Asian
CK10300055	2014	1410aTw	Indonesia	A	Asian

Figure 1A

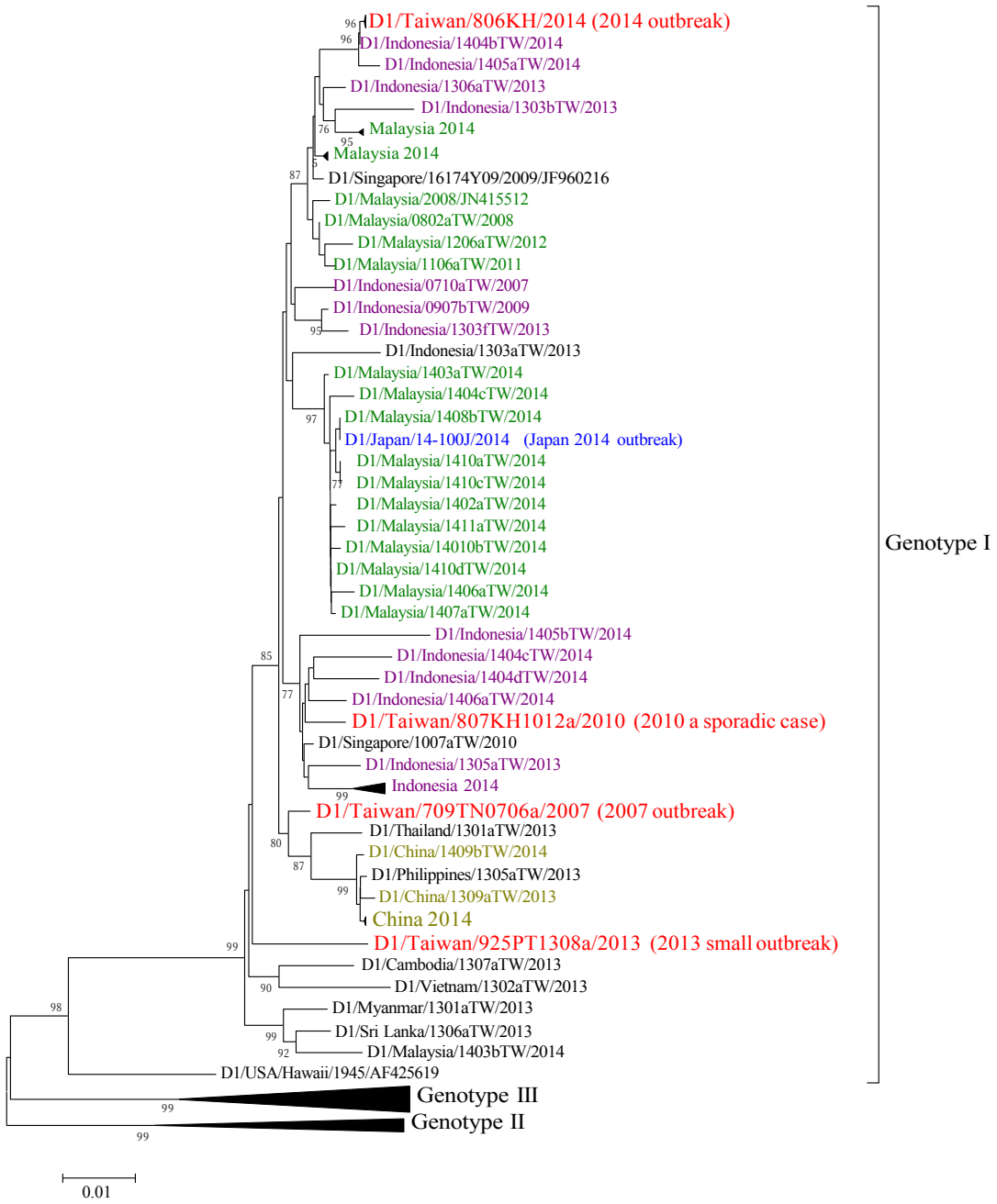
D1/Taiwan/806KH/2014 (2014 outbreak)
D1/Indonesia/1404bTW/2014
D1/Indonesia/1405aTW/2014
D1/Indonesia/1306aTW/2013
D1/Indonesia/1303bTW/2013
Malaysia 2014
Malaysia 2014
D1/Singapore/16174Y09/2009/JF960216
D1/Malaysia/2008/JN415512
D1/Malaysia/0802aTW/2008
D1/Malaysia/1206aTW/2012
D1/Malaysia/1106aTW/2011
D1/Indonesia/0710aTW/2007
D1/Indonesia/0907bTW/2009
D1/Indonesia/1303fTW/2013
D1/Indonesia/1303aTW/2013
D1/Malaysia/1403aTW/2014
D1/Malaysia/1404cTW/2014
D1/Malaysia/1408bTW/2014
D1/Japan/14-100J/2014 (Japan 2014 outbreak)
D1/Malaysia/1410aTW/2014
D1/Malaysia/1410cTW/2014
D1/Malaysia/1402aTW/2014
D1/Malaysia/1411aTW/2014
D1/Malaysia/14010bTW/2014
D1/Malaysia/1410dTW/2014
D1/Malaysia/1406aTW/2014
D1/Malaysia/1407aTW/2014
D1/Indonesia/1405bTW/2014
D1/Indonesia/1404cTW/2014
D1/Indonesia/1404dTW/2014
D1/Indonesia/1406aTW/2014
D1/Taiwan/807KH1012a/2010 (2010 a sporadic case)
D1/Singapore/1007aTW/2010
D1/Indonesia/1305aTW/2013
Indonesia 2014
D1/Taiwan/709TN0706a/2007 (2007 outbreak)
D1/Thailand/1301aTW/2013
D1/China/1409bTW/2014
D1/Philippines/1305aTW/2013
D1/China/1309aTW/2013
China 2014
D1/Taiwan/925PT1308a/2013 (2013 small outbreak)
D1/Cambodia/1307aTW/2013
D1/Vietnam/1302aTW/2013
D1/Myanmar/1301aTW/2013
D1/Sri Lanka/1306aTW/2013
D1/Malaysia/1403bTW/2014
D1/USA/Hawaii/1945/AF425619
Genotype III
Genotype II
Genotype I
0.01

Figure 1B

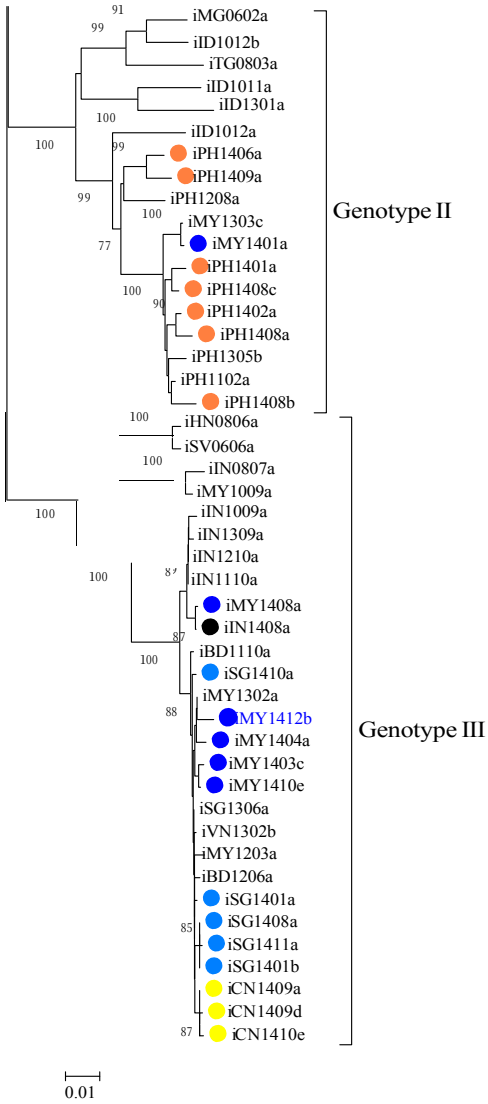

Figure 1. Phylogenetic trees derived from full-length E gene sequences of DENV-1 strains isolated from confirmed dengue cases. The DENV-1 Genotype I (1A) and genotype II and III (1B) are showed. The tree was constructed by the maximal likelihood method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation (1A), i (imported)/MG (Madagascar), ID (Indonesia), PH (Philippines), MY (Malaysia), IN (India), BD (Bangladesh), SG (Singapore), VN (Vietnam), CN (China)/year and month of isolation.

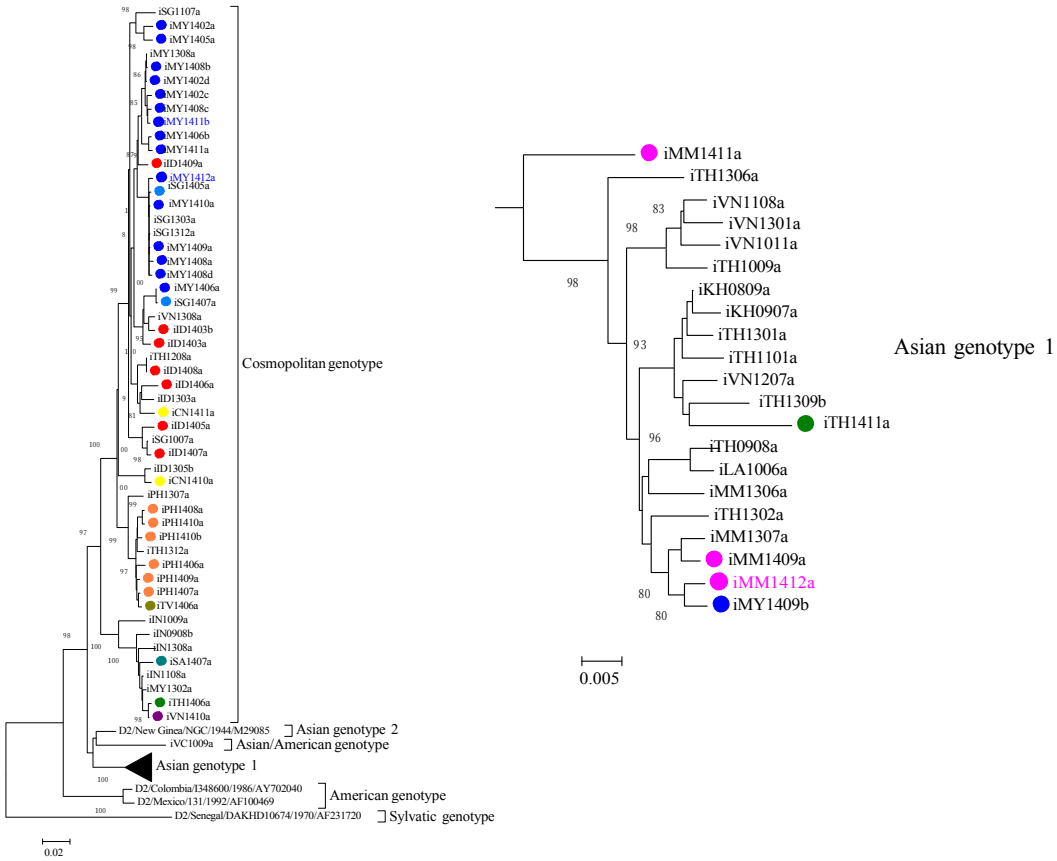

Figure 2. Phylogenetic trees derived from full-length E gene sequences of DENV-2 strains isolated from confirmed dengue cases. Viruses were identified using the nomenclature: i (imported)/ID (Indonesia), PH (Philippines), MY (Malaysia), IN (India), SG (Singapore), VN (Vietnam), CN (China), TH (Thailand), TV (Tuvalu), MM (Myanmar), KH (Cambodia)/year and month of isolation.

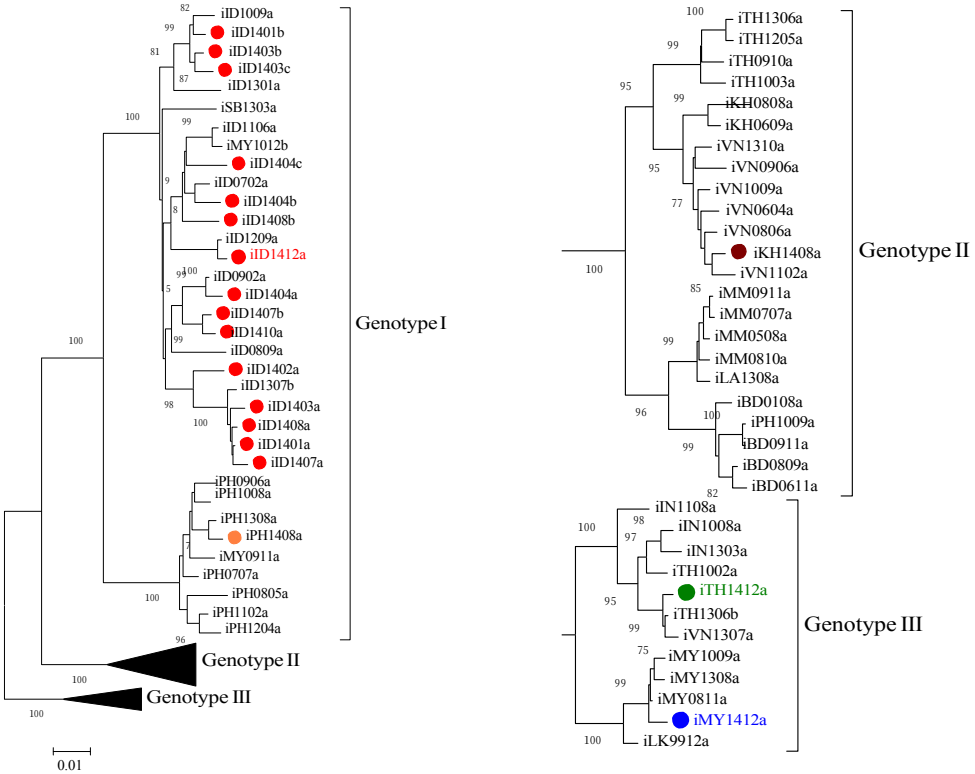

Figure 3. Phylogenetic trees derived from full-length E gene sequences of DENV-3 strains isolated from confirmed dengue cases. Viruses were identified using the nomenclature: i (imported)/ID (Indonesia), PH (Philippines), MY (Malaysia), VN (Vietnam), BD (Bangladesh), TH (Thailand), MM (Myanmar), KH (Cambodia)/year and month of isolation.

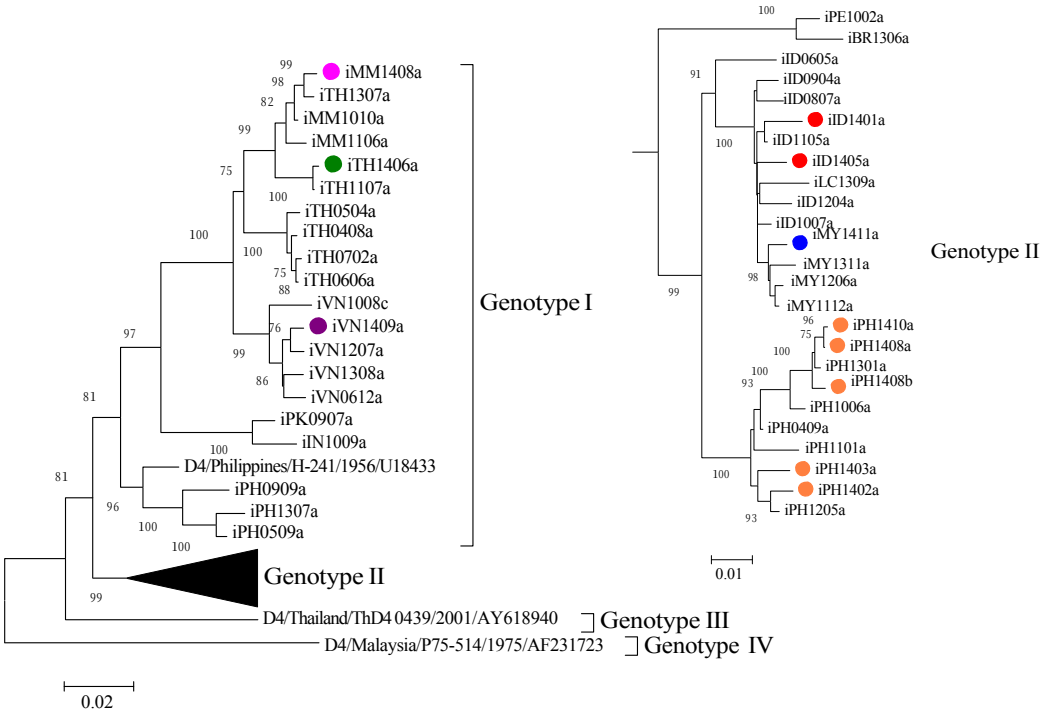

Figure 4. Phylogenetic trees derived from full-length E gene sequences of DENV-4 strains isolated from confirmed dengue cases. Viruses were identified using the nomenclature: i (imported)/ID (Indonesia), PH (Philippines), MY (Malaysia), VN (Vietnam), BD (Bangladesh), TH (Thailand), MM (Myanmar), KH (Cambodia)/year and month of isolation.

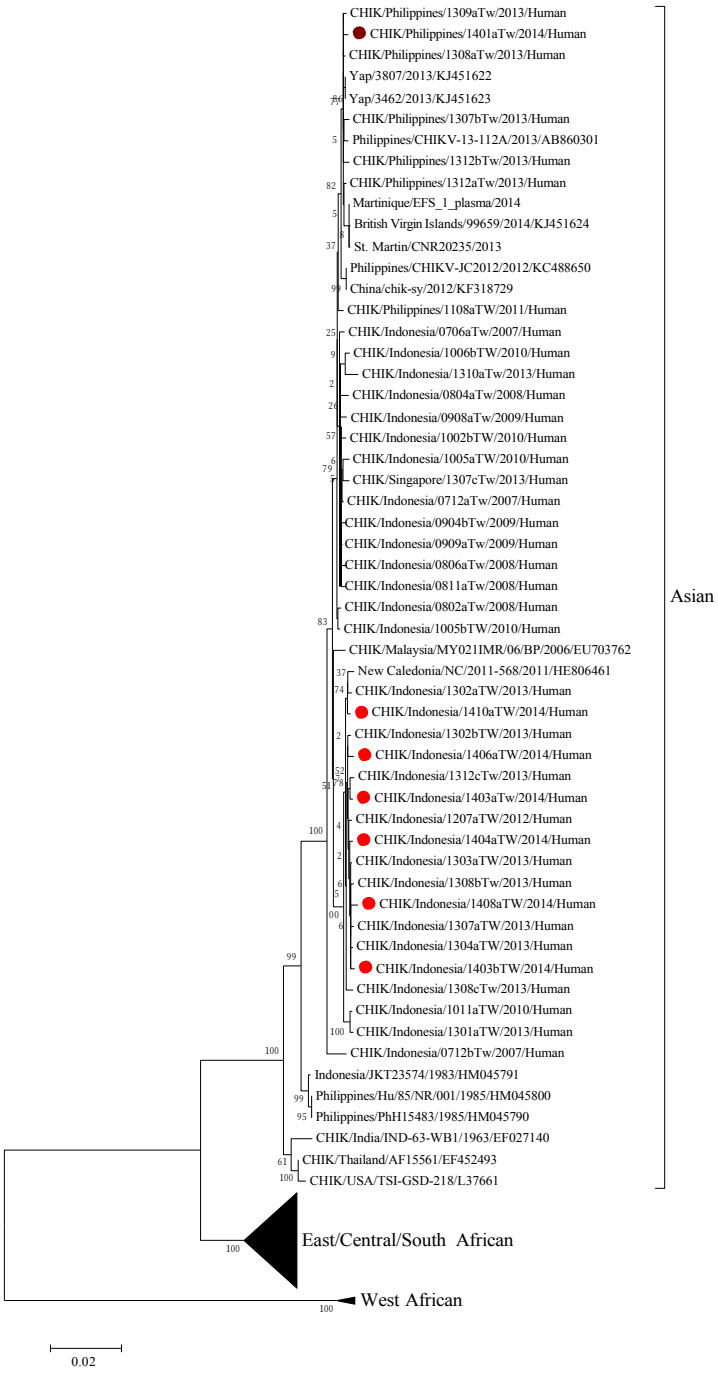

Figure 5. Phylogenetic relationships of chikungunya virus (CHIKV) isolates from 7 imported cases in Taiwan. The tree was constructed by the maximal likelihood method using complete structural protein gene sequences (3747 bp). The 7 imported CHIKV strains in 2014 are designated by close circles. Viruses were identified using the nomenclature of virus/country/strain/year of isolation. The scale bar on the left indicates substitutions per site.