Molecular Characterization and Phylogenetic Analysis of Dengue Viruses Imported into Taiwan during 2008–2010

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Abstract. We present our surveillance results on imported dengue cases in Taiwan during 2008–2010. A total of 734 imported dengue patients were identified. The travelers were arriving from 18 countries, including Southeast Asia, the Indian subcontinent, South Pacific islands, and Latin America. Gene sequences from 358 dengue virus (DENV) isolates were used to perform phylogenetic analyses, thus, providing an updated view of the geographic distribution and dynamic transmission of DENV strains circulating in these countries. Our results suggest that the DENV-1 genotype I and DENV-2 Cosmopolitan genotype comprise the predominant DENV strains circulating in Southeast Asian countries. The DENV-3 Genotype III strain was found to be newly emerging in several Southeast Asian countries, however, the Asian genotype 2 and the Asian/American genotype of DENV-2 strains appeared to be less prevalent in Southeast Asia. Furthermore, imported dengue viruses are representative of the overall patterns of serotype/genotype frequencies of dengue outbreaks that occurred in Taiwan.

INTRODUCTION

Dengue is the most prevalent arthropod-borne viral infection of humans in tropical and subtropical countries around the world. The incidence of dengue has grown dramatically in recent decades. Approximately 2.5 billion people are now at risk for dengue, and there are an estimated 50 million dengue infections worldwide every year.¹ The disease symptoms can vary and include asymptomatic cases, febrile disease, classic dengue fever (DF), and complications such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Dengue virus (DENV) belongs to the genus *Flavivirus* in the family *Flaviviridae*. The DENV genome consists of a single-stranded, positive-sense RNA, which is ~11 kb and contains a long open reading frame encoding three structural proteins (capsid [C], premembrane/membrane [M], and envelope [E] proteins), and seven nonstructural proteins.^{2,3} There are four genetically and antigenically distinct DENV serotypes (DENV-1 through DENV-4). The DENVs are transmitted to humans through the bite of an infective female *Aedes* mosquitoes.⁴ In recent decades, the number of reported dengue cases worldwide and the number of countries with endemic dengue has increased dramatically. At fault for this increase is the growing habitat of the mosquito vectors *Aedes* sp., the growing number of susceptible human hosts, and a higher DENV exposure caused by rapid and frequent global travel.^{5,6}

Dengue is endemic to most Southeast Asian countries. These countries endure nearly 75% of the current global dengue disease burden.⁷ High numbers of cases are reported each year, and all four DENV strains currently circulate. With the increase in worldwide travel, the rapid expansion of DENV strains to different parts of the world has been well documented.^{8–14} Studies on imported dengue cases can provide useful information for the geographic distribution and global movement of DENV strains.

Taiwan is an East Asian island off the southeastern coast of mainland China in the western Pacific Ocean. The island

straddles the Tropic of Cancer, giving it a warm tropical-subtropical climate. *Aedes albopictus* is found throughout Taiwan, whereas *Aedes aegypti* is distributed in the south.¹⁵ Dengue is not considered endemic in Taiwan, thus, the close commercial ties and air travel from neighboring Southeast Asian countries are responsible for the constant importation of multiple DENVs and the outbreaks that occur each year.¹⁶ We previously reported the molecular characterization of DENV strains imported into Taiwan during 2003–2007.¹⁴ These results provided valuable information on the geographic distributions and dynamic transmission of DENV strains in Southeast Asian countries, notably, that the genotype distribution of DENV strains isolated from imported dengue cases from these countries can be divided into two geographic regions. A northern region includes Vietnam, Thailand, Cambodia, and Myanmar, and a southern region includes Indonesia, the Philippines, Malaysia, and Singapore. In each of these two regions, the DENV strains are from closely related clades in the same genotypes, suggesting a close genetic relationship and frequent flow of viruses in these countries. However, inconsistent genotype distributions were observed, indicating multiple introductions and expansions of viral strains in some of these countries. Therefore, although the geographic distributions of genotypes of DENV-3 isolated from Southeast Asian countries remain unchanged, the introduction and local expansion of the DENV-1, DENV-2, and DENV-4 strains into new areas in Asia were observed between 2003 and 2007. In this study, we present the current dynamics of DENV strains isolated from imported dengue cases in Taiwan during 2008–2010.

MATERIALS AND METHODS

Human serum samples. Dengue (DF and DHF) are reportable infectious diseases in Taiwan and suspected cases must be reported within 24 hours of clinical diagnosis. To provide effective surveillance, both passive (the hospital-based reporting system) and active (such as fever screening at airports, self-reporting, expanded screening for contacts of confirmed cases, patients with fever of unknown origin, and school-based reporting) surveillance systems were implemented by the central

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and local health departments in Taiwan. Human serum samples of suspected dengue cases were submitted to the Research and Diagnostic Center, Taiwan Centers for Disease Control and Prevention (CDC), for confirmation of DENV infection. Human serum samples used in this study were derived from confirmed dengue cases submitted to Taiwan CDC during 2008–2010. An imported dengue case was defined as an infected patient who had traveled abroad more than 2 weeks before the onset of illness, as it is likely the patient was infected abroad. An indigenous case was recorded when no overseas travel was indicated.

Laboratory diagnosis. The DENV infection was defined as a febrile illness associated with at least a 4-fold increase in DENV-specific immunoglobulin M (IgM) or IgG antibodies, the isolation of DENV, or the detection of DENV RNA by reverse transcription-polymerase chain reaction (RT-PCR). To detect and differentiate DENV serotypes in the acute phase samples, we performed one-step, SYBR Green I, real-time RT-PCR (QuantiTect SYBR Green RT-PCR kit, Qiagen, Hilden, Germany) in the Mx3000P quantitative PCR system (Stratagene, La Jolla, CA).¹⁷ Real-time RT-PCR was performed using two sets of consensus primers, one primer set targeting a region of the nonstructural protein 5 (NS5) genes to detect all of the flaviviruses and the other primer set targeting a region of the C gene to detect all of the DENV serotypes. Positive samples were confirmed by DENV serotyping using four sets of serotype-specific primers targeting the C gene. E/M-specific capture IgM and IgG enzyme-linked immunosorbent assay were used to detect DENV-specific IgM and IgG antibodies as previously described.¹⁸ Isolation of DENV was performed using a mosquito cell line (clone C6/36 of *Ae. albopictus* cells). For each acute-phase serum sample, 50 μ L of diluted serum samples at ratios of 1:20, 1:40, 1:80, and 1:160 (diluted with RPMI 1640 medium, Gibco/BRL, Life Technologies, Auckland, New Zealand, containing 1% fetal calf serum [FCS]), were added to a 96-well microtiter plate, and 10^5 cells/100 μ L/well of C6/36 were then added to the microtiter plate, followed by incubation for 7 days at 28°C. Cells were harvested, and infection was confirmed by immunofluorescence assay using dengue 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.

Preparation of viral RNA, RT-PCR amplification, and nucleotide sequencing. Viral RNA was extracted from either acute-phase serum samples or the culture supernatant of C6/36 cell line infected with each of the isolated DENV strains using the QIAamp Viral RNA Mini kit (Qiagen). Primers used for amplification and sequencing of C, premembrane (prM), and E-gene sequences of DENVs were described previously.^{14,16} The RT-PCR reaction was carried out with the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen). The cDNA synthesis step was performed at 55°C for 30 min, PCR at 94°C for 2 min, 40 cycles of 94°C for 15 sec, 50°C for 30 sec, and 68°C for 1 min; and a prolonged elongation at 68°C for 5 min. The 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, Foster City, CA) according to the manufacturer's protocols. Overlapping nucleotide sequences were combined for analysis and edited with the Lasergene software

package (DNASTAR Inc., Madison, WI). Nucleotide sequences of the complete E-gene of the DENV strains described in this study were submitted to GenBank and their accession nos.: 167 DENV-1 strains (JF967793-JF967953 and JQ403516-JQ403521), 105 DENV-2 strains (JF967954-JF968052, HM067745-HM067747, and JQ403522-JQ403524), 62 DENV-3 strains (JF968053-JF968113 and JQ403525), and 34 DENV-4 strains (JF967759-JF967792 and JQ403526).

Phylogenetic analysis. A total of 358 DENV isolates imported into Taiwan and 11 DENV isolates from local dengue outbreaks were analyzed in combination with various global reference sequences of different genotypes available from GenBank. The nucleotide sequences of complete E-genes of DENV strains were aligned, edited, and analyzed using ClustalW software. The phylogenetic analysis was performed using MEGA version 5 (<http://www.megasoftware.net/>).¹⁹ Phylogenetic trees were constructed using the maximum likelihood method based on the general time-reversible model. The reliability of the analysis was evaluated by a bootstrap test with 1,000 replications. Sequences of D3/Philippines/H-87/1956 strain (M93130), D2/Senegal/DAKHD10674/1970/AF231720, D1/USA/Hawaii/1945 strain (AF425619), and D2/NewGuinea/NGC/1944 strain (M29095), were used as out-group to root the tree of the DENV-1, DENV-2, DENV-3, and DENV-4 strains, respectively.

RESULTS

Imported dengue cases in Taiwan during 2008–2010. A total of 734 imported dengue cases (226, 204, and 304 cases for 2008, 2009, and 2010, respectively) were identified in Taiwan during 2008–2010. Among them, 362 cases (49.3%) were identified by fever screening at airports (Table 1). Most cases arrived from Southeast Asian countries, with Indonesia, Vietnam, Thailand, and the Philippines the most frequent importing countries. In addition, cases were also imported from the Indian subcontinent (India, Bangladesh, Pakistan, Sri Lanka, and Maldives), the South Pacific region (Tonga), and Latin America (Honduras, Peru, and Saint Vincent and the Grenadines).

Serotype and genotype distributions of imported DENV strains during 2008–2010. From the 734 imported dengue cases, 186, 129, 92, and 45 cases were determined to be infected with the DENV-1, DENV-2, DENV-3, and DENV-4 strains, respectively (Table 1). Among them, 358 DENV strains were isolated from acute-phase serum samples of patients arriving from 16 countries. A summary of the serotype and genotype distributions of these isolates by country is summarized in Table 2. Phylogenetic analyses of the E-gene sequences of DENV strains isolated from imported cases were conducted to determine the genetic relationship of these viral strains. The designations of DENV genotypes are based on the classification of A-Nuegoonpipat and others,²⁰ Twiddy and others,²¹ Lanciotti and others,²² and Klunthong and others²³ for the DENV-1, DENV-2, DENV-3, and DENV-4 strains, respectively. The phylogenetic trees of the representative E-gene sequences, which cover the full range of genetic diversity of 358 DENV strains observed in the phylogenetic trees (data not shown), are shown in Figures 1–4. From 2008 to 2010, the combined imported and indigenous dengue case data contain 57 DENV-1 sequences, 54 DENV-2 sequences, 38 DENV-3 sequences, and 23 DENV-4 sequences, and an additional 28 DENV-1 reference sequences, 21 DENV-2

TABLE 1
Serotype distributions of DENV strains from imported dengue cases in Taiwan during 2008–2010

Country origin	Case	Fever screening	DENV-1	DENV-2	DENV-3	DENV-4	Unknown
Indonesia	217	126	40	46	39	30	62
Vietnam	195	90	80	13	13	6	83
Thailand	88	42	28	17	8	0	35
Philippines	78	37	5	19	17	4	33
Cambodia	39	10	7	11	2	0	19
Malaysia	30	22	11	8	4	1	6
Myanmar	26	8	4	1	4	1	16
Singapore	14	8	4	6	0	0	4
Laos	3	1	0	2	0	0	1
India	21	7	3	3	2	1	12
Bangladesh	5	3	0	0	3	0	2
Pakistan	2	2	0	0	0	1	1
Sri Lanka	1	0	0	0	0	0	1
Maldives	1	0	0	0	0	0	1
Tonga	4	1	1	0	0	0	3
Saint Vincent and the Grenadines	1	0	0	1	0	0	0
Peru	1	1	0	0	0	1	0
Honduras	1	1	1	0	0	0	0
Unknown	7	3	2	2	0	0	3
Total	734	362	186	129	92	45	282

reference sequences, 25 DENV-3 reference sequences, and 21 DENV-4 global reference sequences of different genotypes available from GenBank.

Phylogenetic analysis of DENV-1 isolates. The DENV-1 genotype I was the most dominant genotype of DENV strains isolated from dengue cases arriving from Southeast Asian countries. All of the DENV-1 strains isolated from imported dengue cases from Vietnam, Thailand, Cambodia, Myanmar, Malaysia, and Singapore fell into this genotype (Table 2; Figure 1). Viral strains from Vietnam and Thailand showed a high degree of genetic diversity and strains in different lineages that were co-circulating in these countries. The DENV-1 strains from Cambodia are closely related to viruses from Vietnam, whereas viral strains from Myanmar are clustered with strains from Thailand. Genotype I was the predominant genotype of DENV-1 in Southeast Asian countries except in the Philippines. DENV-1 strains isolated from imported cases from the Philippines belong to genotype II and form a distinct clade, which is consistent with its geographical locality. A

DENV-1 strain (D1/Tonga/0803aTw/2008) isolated from an imported case from Tonga belongs to genotype II and is closely related to the viral strain from Tahiti (French Polynesia). Genotype III contained viruses from diverse geographical localities including Asia, the Pacific Islands, the Americas, and parts of Africa. Viruses isolated from imported cases from India belong to this genotype. A 2010 strain from Malaysia also belongs to this genotype and is closely related to viruses from India and Singapore. In addition, a 2008 strain from Honduras belongs to genotype III and is clustered with strains from its neighboring countries, including El Salvador and Nicaragua.

Phylogenetic analysis of DENV-2 isolates. The DENV-2 strains isolated from imported cases during 2008–2010 fell into three genotypes (Table 2; Figure 2). The Asian genotype 1 contains viruses from Southeast Asia, including viruses imported from Vietnam, Thailand, Cambodia, Myanmar, and Laos. The Cosmopolitan genotype has a wide geographic distribution, which includes Asia, Australia, Africa, and Latin America. All of the strains isolated from imported cases from

TABLE 2
Summary of genotype distributions of DENV strains isolated from imported cases in Taiwan during 2008–2010

Serotype	DENV-1			DENV-2			DENV-3			DENV-4		Total
	I	II	III	Asian 1	Asian/American	Cosmopolitan	I	II	III	I	II	
Indonesia	24	14	0	0	0	38	19	0	0	0	25	120
Vietnam	72	0	0	12	0	0	0	8	0	3	0	95
Philippines	0	3	0	0	0	17	17	1	0	1	1	40
Thailand	22	0	0	10	0	0	0	3	3	0	0	38
Malaysia	9	0	1	0	0	8	1	0	3	0	0	22
Cambodia	5	0	0	5	0	0	0	1	0	0	0	11
Singapore	3	0	0	0	0	6	0	0	0	0	0	9
India	0	0	3	0	0	2	0	0	1	1	0	7
Myanmar	3	0	0	1	0	0	0	2	0	1	0	7
Bangladesh	0	0	0	0	0	0	0	2	0	0	0	2
Laos	0	0	0	2	0	0	0	0	0	0	0	2
Tonga	0	1	0	0	0	0	0	0	0	0	0	1
Pakistan	0	0	0	0	0	0	0	0	0	1	0	1
Peru	0	0	0	0	0	0	0	0	0	0	1	1
St. Vincent	0	0	0	0	1	0	0	0	0	0	0	1
Honduras	0	0	1	0	0	0	0	0	0	0	0	1
Total	138	18	5	30	1	71	37	17	7	7	27	358

FIGURE 1. A phylogenetic tree of dengue virus type 1 (DENV-1). The phylogenetic tree is based on the complete E-gene sequences of 85 strains of DENV-1 including 57 Taiwan isolates from imported and indigenous dengue cases during 2008–2010. The tree was constructed by the maximum likelihood method and the general time-reversible model. Bootstrap support values greater than 75 are shown. The Taiwan isolates from imported cases during 2008–2010 are designated in boldface. The Taiwan isolates from indigenous cases are designated in boldface and solid triangle (▲). Viruses were identified by using the nomenclature of serotype/country/strain/year of isolation/GenBank accession number. The scale bar on the left indicates substitutions per site.

FIGURE 2. A phylogenetic tree of dengue virus type 2 (DENV-2). The phylogenetic tree is based on the complete E-gene sequences of 75 strains of DENV-2 including 54 Taiwan isolates from imported and indigenous dengue cases during 2008–2010. See the legend of Figure 1 for other details.

the Philippines, Indonesia, Malaysia, Singapore, and India belong to this genotype. The Asian/American genotype consists of viral strains from Southeast Asia and Latin America. No Asian/American genotype strains were found from imported cases from Southeast Asia during 2008–2010, only a strain imported from Saint Vincent and the Grenadines in 2010, which is clustered with strains from the countries of the Caribbean. Similarly, no Asian genotype 2 strains were found from imported cases during 2008–2010.

Phylogenetic analysis of DENV-3 isolates. The DENV-3 strains isolated from imported cases fell into three genotypes (Table 2; Figure 3). Genotype I contains viruses from South-

east Asia and the Pacific islands, including viral strains imported from Indonesia, Malaysia, and the Philippines. Interestingly, a strain from Malaysia (D3/Malaysia/0911aTw/2009) was clustered with strains from the Philippines. The DENV-3 genotype II is composed of viral strains from Southeast Asia, including strains imported from Thailand, Vietnam, Cambodia, and Myanmar. DENV-3 strains from Bangladesh also belong to genotype II and are closely related to strains from Myanmar. The DENV-3 genotype III contains viruses from the Indian subcontinent, Africa, and Latin America. Notably, a few DENV-3 strains isolated from imported cases from Thailand and Malaysia belong to this genotype. The DENV-3

FIGURE 3. A phylogenetic tree of dengue virus type 3 (DENV-3). The phylogenetic tree is based on the complete E-gene sequences of 63 strains of DENV-3 including 38 Taiwan isolates from imported and indigenous dengue cases during 2008–2010. See the legend of Figure 1 for other details.

genotype III strains from Thailand are clustered with strains from Malaysia, India, and Bhutan.

Phylogenetic analysis of DENV-4 isolates. DENV-4 is found at low frequency in Asia. The DENV-4 viruses isolated from imported cases fell into two genotypes, I and II (Table 2; Figure 4). Genotype I contains two major clusters, representing viruses imported from the Philippines, and viruses imported from Vietnam, Myanmar, India, and Pakistan. A strain from Pakistan in 2009 is closely related to viral strains from India. Genotype II also consists of two major clusters: one is composed of viral strains from Southeast Asia and the South Pacific, including viral

strains imported from Indonesia and the Philippines during 2008–2010, and the other contains viral strains from the Americas, including a strain imported from Peru in 2010. This strain is closely related to viruses from Venezuela.

Multiple dengue epidemics in Taiwan, 2008–2010. Table 3 lists the major dengue outbreaks and epidemic viral strains circulating in Taiwan between 2008 and 2010. Our results showed that three different DENV strains, 2 DENV-1 strains (D1/Taiwan/806KH0810a/2008 and D1/Taiwan/811KH0807a/2008) and a DENV-2 strain (D2/Taiwan/830KH0809a/2008), were co-circulating in Kaohsiung City between July and

FIGURE 4. A phylogenetic tree of dengue virus type 4 (DENV-4). The phylogenetic tree is based on the complete E-gene sequences of 44 strains of DENV-4 including 23 Taiwan isolates from imported and indigenous dengue cases during 2008–2010. See the legend of Figure 1 for other details.

December of 2008. These strains are closely related to viral strains from Vietnam, Thailand, and Cambodia. In addition, a DENV-1 strain (D1/Taiwan/111TP0808a/2008) caused a small outbreak in Taipei City, with at least 20 confirmed dengue cases. This strain belongs to genotype I and is closely related to viral strains from Vietnam. In 2009, three major dengue out-

breaks occurred in Taiwan. A DENV-2 strain (D2/Taiwan/900PT0910a/2009) caused a dengue outbreak in Pintung County with at least 70 confirmed cases. This strain belonged to the Asian genotype 1 and is closely related to viral strains from Vietnam. A DENV-3 strain (D3/Taiwan/812KH0908a/2009) caused an overwinter dengue outbreak in Kaohsiung

TABLE 3
Major dengue epidemics in Taiwan between 2008 and 2010*

Year	Epidemic area	Serotype	Genotype	Epidemic dengue virus strain	No. confirmed indigenous dengue cases
2008	Kaohsiung City	DENV-1	Genotype I	D1/Taiwan/806KH0810a/2008/JQ403518	488
	Kaohsiung City	DENV-1	Genotype I	D1/Taiwan/811KH0807a/2008/JQ403517	
	Kaohsiung City	DENV-2	Asian genotype 1	D2/Taiwan/830KH0809a/2008/JQ403522	
	Taipei City	DENV-1	Genotype I	D1/Taiwan/111TP0808a/2008/JQ403516	
2009	Southern Taiwan	DENV-3	Genotype I	D3/Taiwan/812KH0908a/2009/JQ403525	848
	Pingtung County	DENV-2	Asian genotype 1	D2/Taiwan/900PT0910a/2009/JQ403523	
	Changhua County	DENV-1	Genotype I	D1/Taiwan/511CH0909a/2009/JQ403519	
2010	Kaohsiung City	DENV-3	Genotype I	D3/Taiwan/812KH0908a/2009/JQ403525	1,592
	Tainan City	DENV-4	Genotype II	D4/Taiwan/700TN1009a/2010/JQ403526	
	Kaohsiung City	DENV-2	Cosmopolitan genotype	D2/Taiwan/807KH1008a/2010/JQ403524	
	Tainan City	DENV-1	Genotype I	D1/Taiwan/718TN1008a/2010/JQ403521	
	New Taipei City	DENV-1	Genotype I	D1/Taiwan/248TP1010a/2010/JQ403520	

* Boldface indicates the same epidemic strain circulated in Taiwan between 2009 and 2010.

City between July 2009 and January 2011, with at least 1,400 dengue cases. It was the first overwinter outbreak of DENV-3 infection that occurred in Taiwan since the 1940s. This strain belongs to genotype I of DENV-3 and is closely related to viral strains from Malaysia and the Philippines. Another DENV-1 strain (D1/Taiwan/511CH0909a/2009) caused a small dengue outbreak in Changhua County in Central Taiwan. This DENV-1 strain belonged to genotype I and is closely related to viral strains from Thailand. In Kaohsiung City in 2010, a DENV-2 strain (D2/Taiwan/807KH1008a/2010) also caused an outbreak with at least 380 dengue cases. This DENV-2 strain belongs to the Cosmopolitan genotype and is closely related to viral strains from the Philippines. Two strains, a DENV-4 strain (D4/Taiwan/700TN1009a/2010) that belongs to genotype II and a DENV-1 strain (D1/Taiwan/718TN1008a/2010) that belong to genotype I, caused dengue outbreaks in Tainan City in 2010 with at least 400 dengue cases. In addition, a small outbreak occurred in New Taipei City in 2010 and was caused by a DENV-1 strain (D1/Taiwan/248TP1010a/2010) that belongs to genotype I. Phylogenetic analyses showed that these local DENV strains that caused local dengue epidemics in Taiwan were more likely to be introduced from Southeast Asian countries, including Vietnam, Thailand, Cambodia, the Philippines, and Indonesia (Figures 1–4).

DISCUSSION

Although Taiwan has local dengue outbreaks every year, dengue is not considered endemic. Molecular epidemiological studies provided substantial evidences that multiple DENV strains imported into Taiwan were responsible for local dengue outbreaks each year. In addition, phylogenetic studies of DENV strains isolated from imported dengue cases showed that similar strains, clustered in the same clade of a specific genotype, were often repeatedly isolated from the same country each year. The results suggest that the majority of these DENV strains remain stable in their geographic distribution. Through extensive laboratory-based surveillance, especially fever screening at airports, increasing numbers of imported dengue cases (104, 109, 179, 226, 204, and 304 imported dengue cases during 2005–2010, respectively) were identified, and the imported DENV strains were isolated. We previously reported the molecular characterization of DENV strains imported into Taiwan during 2003–2007. The results showed that although the geographic distributions of genotypes of the DENV-3 strains isolated from Southeast Asian countries remain unchanged, the introduction and local expansions of epidemic DENV-1, DENV-2, and DENV-4 strains into new areas in Asia were observed.

With continuous surveillance during 2008–2010, we isolated a total of 358 DENV strains including 161 DENV-1, 102 DENV-2, 61 DENV-3, and 34 DENV-4 strains. Among these, 120 and 95 strains were isolated from dengue patients imported from Indonesia and Vietnam, respectively. Genotype analysis revealed that the DENV-1 genotype I and the DENV-2 Cosmopolitan genotype were the predominant viral strains. Most important, the DENV-3 genotype III strains were found to newly emerge in Southeast Asian countries, including Thailand and Malaysia, whereas the DENV-2 Asian genotype 2 and the Asian/American genotype strains were not found in Southeast Asian countries.

Phylogenetic analysis of envelope protein gene sequences of DENV isolated from imported cases suggests that the

DENV-1 genotype I was the dominant genotype circulating in Southeast Asia during 2008–2010. The DENV-1 genotype I strains circulating in Vietnam and Thailand were highly divergent, dispersed throughout a variety of phylogenetic locations and clustered into neighboring country strains, suggesting extensive introduction and expansion of strains between Southeast Asian countries. It is also interesting to note that a strain imported from Tonga in 2008 belongs to the DENV-1 genotype II and was clustered with strains from the southern Pacific region, including French Polynesia, which caused dengue outbreaks in 2001, suggesting that these strains may have been circulating in these areas for several years.²⁴

Recent reports showed that the Asian genotype I of DENV-2 had displaced the American/Asian genotype as the predominant DENV-2 lineage in the southern Vietnam.²⁵ Our results also showed that all the DENV-2 strains imported from Vietnam belong to the Asian genotype 1. Notably, neither the Asian genotype 2 nor the Asian/American genotype of DENV-2 strains were found in imported dengue cases during 2008–2010, suggesting a low prevalence of these two genotypes in the Southeast Asian region in recent years.

The DENV-3 genotype III is composed of viral strains from Indian subcontinent, East Africa and Latin America and strains of this genotype are seldom found in Southeast Asia. However, recent reports showed that the DENV-3 genotype III strains were introduced into Bhutan and southern China and caused dengue outbreaks in recent years.^{26,27} Interestingly, we found some DENV-3 strains imported from Malaysia and Thailand during 2008–2010 also belonged to genotype III. Three strains from Malaysia (D3/Malaysia/1012aTw/2010, D3/Malaysia/1009aTw/2010, and D3/Malaysia/0811aTw/2008) and a strain from Thailand (D3/Thailand/1009aTw/2010) were clustered with strains from Singapore and Sri Lanka. In addition, two strains from Thailand (D3/Thailand/1008aTw/2010 and D3/Thailand/1002aTw/2010) were clustered with strains from India and Bhutan, indicating that the DENV-3 genotype III had expanded its geographic distribution and migrated east toward Southeast Asian countries.

A relatively low prevalence of the DENV-4 strain was found in Southeast Asia, with the exception of Indonesia, during 2008–2010. DENV-4 strains from Indonesia belonged to genotype II and were similar to epidemic strains of dengue outbreak in Indonesia in 2004.²⁸ Also note that these Indonesian strains were clustered with strains sampled from Solomon in 2007, Easter Island of Chile in 2009 and China in 2010, indicating viral strains within this cluster may have high transmission capacity to expand their geographic range.^{14,27} Interestingly, a strain imported from Pakistan (D4/Pakistan/0907aTw/2009), which belonged to DENV-4 genotype I, is closely related to epidemic strains of dengue outbreak in western and southern India in recent years. These strains might be associated with the increased severity and higher transmission rate circulating in India.^{29,30} A strain imported from Peru in 2010 (D4/Peru/1002aTw/2010) is similar to the epidemic strain that caused a dengue outbreak in Peru in 2008.³¹

Our results suggested that genotype distributions of the majority of the DENV strains circulating in each of the Southeast Asian countries remained stable. For example, genotype distributions of the DENV-1 and the DENV-4 strains from Vietnam, Thailand, Cambodia, Laos, and Myanmar remain unchanged, as do the DENV-2, DENV-3, and DENV-4 strains from Indonesia and Malaysia. Distinct clades were

formed in phylogenetic trees of all four DENV serotypes of strains from the Philippines in accordance with the distinct geographical location of this country. In addition, the DENV strains from India, Bangladesh, and Pakistan were often clustered together in the phylogenetic trees. These results indicate that genotype distribution of DENV depends largely on the geographical location and associations with the ecological factors. However, discordant genotype distributions were also observed, such as how the DENV-1 genotype I strains spread to new territory in Malaysia and Indonesia and the DENV-3 genotype III spread to Southeast Asia, thus indicating that multiple introductions and expansions of viral strains may result in genotype co-circulation or a shift in some of these countries. Although multiple introductions of DENV strains were often observed in Southeast Asian countries, our results suggest that it might be very difficult for a foreign DENV strain to establish and maintain a transmission cycle in endemic areas in Southeast Asia. In contrast, it might be relatively easy for an imported DENV strain to spread and induce an outbreak in a country where dengue is not endemic, but where the environment is suitable for viral transmission, such as Taiwan.

Because of increased international trade and travel, it is expected that DENV strains will be introduced into Taiwan from the neighboring Southeast Asian countries more frequently. In recent years in Taiwan, multiple dengue outbreaks occurred that were caused by all four serotypes of DENV. Phylogenetic analyses showed that the genotype I of DENV-1 strains is the predominant genotype/serotype of DENV circulating in Taiwan during 2008–2010 (Table 3). Three local DENV-2 strains, two belonging to the Asian genotype 1 and one belonging to the Cosmopolitan genotype, caused dengue outbreaks in Kaohsiung City and Pingtung County. A genotype I of DENV-3 strain and a genotype II of DENV-4 strain caused local outbreaks in southern Taiwan. These results indicate that the patterns of imported DENV strains observed in the travelers are connected with the overall patterns of dengue dynamics in Taiwan and suggest that multiple dengue epidemics that occurred in Taiwan may be caused by continuous introductions of multiple DENVs from the neighboring Southeast Asian countries.

In this study, we used molecular epidemiology methods to monitor DENV strain distributions and movements, and the results provide an update of our epidemiological knowledge of DENV infection in endemic areas, especially Southeast Asia. The DENV strains isolated from these imported dengue cases and the availability of the DENV genome sequence database will contribute to a better understanding of the global expansion and genetic evolution of dengue, providing useful data for disease surveillance, laboratory diagnoses, pathogenesis investigation, and vaccine development.

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