Molecular Epidemiology of Dengue Virus Serotype 2 in the Taiwan 2002 Outbreak with Envelope Gene and Nonstructural Protein 1 Gene Analysis

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The genetic relationships among dengue virus serotype 2 (DEN-2) isolates from the Taiwan 2002 epidemic were studied by sequence analysis of the envelope (E) and nonstructural protein 1 (NS1) genes. A 0–0.4% divergence among 10 isolates revealed an epidemic strain in the outbreak. Phylogenetic study demonstrated that the 2002 Taiwan isolates were of the Cosmopolitan genotype, which is different from the Asian 1 and Asian 2 genotypes of Taiwan DEN-2 isolates from 1981 to 1998 and the American/Asian genotype of 2005 Taiwan isolates. Although grouping results from both E and NS1 gene sequence analyses were the same, the usage of the NS1 gene as a sequence analysis target has not been validated for the lower bootstrap support values of branches in the phylogenetic tree. Our result showing the same genotype changes in Taiwan and Philippines isolates suggests strain transfer of DEN-2 to nearby countries resulting in the same trend of genotype change.

Key Words: dengue virus serotype 2, envelope gene, nonstructural protein 1 gene
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Dengue fever (DF) is a major infectious disease of tropical countries and the World Health Organization has estimated that approximately 50 million cases of dengue infection occur worldwide every year [1]. Although most dengue virus-infected cases are asymptomatic or show minor constitutional symptoms, it may lead to fever, headache, pain in various body parts, prostration, rash, lymphadenopathy, and leukopenia. The severe forms of dengue infections are dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which manifest with bleeding and shock [2].

Dengue virus has a positive-sense, single-stranded RNA genome of about 11,000 nucleotides [3]. The viral genome consists of three structural and seven nonstructural proteins in the order C-preM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The envelope (E) protein, which is embedded in a lipid bilayer, may mediate virus attachment and penetration into cells, and is both a target and a modulator of the host's immune response [4]. E gene sequence analysis has been used to study global genetic variations in dengue virus serotype 2 (DEN-2) [5,6] and genotypes [7,8]. Nonstructural protein 1 (NS1) is a 45-kDa nonstructural protein that resides in the plasma membrane of infected cells and is released in an oligomeric form to the

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extracellular milieu [9]. High levels of NS1 are found in the circulation of dengue virus-infected patients during the acute phase of the disease. In addition, NS1 is strongly immunogenic, and anti-NS1 antibodies play a role in protection against disease [10–12]. Despite the importance of NS1 to the dengue infectious process, genetic variations in the NS1 gene over time and in different geographic locales for DEN-2 have been less well described than those of other serotypes of dengue virus and variation in the E gene [13,14]. Nucleotide sequence analysis of dengue virus is useful for defining genetic variations between isolates of the same serotype, tracing the geographical migration of strains, and determining the evolutionary origin of epidemic DEN viruses [5,15–18]. Recently, DEN-2 isolates were classified into six genotypes distributed in different geographic localities, according to the analysis of Twiddy et al [7].

Epidemics of dengue in Taiwan have been documented in 1902, 1915, 1922, 1927, 1931, and 1942 – 1943 [19–21]. No dengue cases were reported between 1943 and 1981, until a dengue-2 outbreak occurred in Hsiao Liu Chiu District, an offshore island in southwest Taiwan [20,22]. This was followed by a large dengue-1 epidemic in 1987–1989 and a small dengue-3 epidemic in 1998 in southern Taiwan [23]. In 2002, a large dengue-2 outbreak occurred between June and December in southern Taiwan, and more than 1,200 cases, including DF and DHF cases, were reported [24]. To investigate the genetic variability and evolutionary character of the DEN-2 isolates from the 2002 Taiwan epidemic, we analyzed the genomic sequences of the E and NS1 genes of ten DEN-2 isolates and compared them with previously published sequences for Taiwan isolates and global DEN-2 viruses following the genotyping scheme proposed by Twiddy et al [7].

METHODS

Virus isolates, cell line, and clinical definition

Ten DEN-2 isolates from the Taiwan outbreak between July and December 2002 (Table 1) were obtained from the Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Taiwan. The isolates, which were originally obtained from serum samples from patients, were cultured in the *Aedes albopictus* cell line C6/36, as previously described [25]. C6/36 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 2% nonessential amino acids. The resultant viruses were analyzed in the present study. Definitions of DF and DHF were according to established criteria [26].

Table 1. Taiwan DEN-2 virus isolates in the study

Isolate designation	Clinical status	E gene GenBank accession no.	NS1 gene GenBank accession no.	GenBank accession no.	Year	Genotype
TW01-02	DF	EF016250	EF486507	EF016250	2002	Cosmopolitan
TW32-02	DF	DQ472144	DQ472144	DQ472144	2002	Cosmopolitan
TW34-02	DF	EF016251	EF486508	EF016251	2002	Cosmopolitan
TW35-02	DF	DQ472145	DQ472145	DQ472145	2002	Cosmopolitan
TW37-02	DF	DQ472146	DQ472146	DQ472146	2002	Cosmopolitan
TW47-02	DF	EF016252	EF486509	EF016252	2002	Cosmopolitan
TW54-02	DF	DQ472147	DQ472147	DQ472147	2002	Cosmopolitan
TW71-02	DF	EF016253	EF486510	EF016253	2002	Cosmopolitan
TW75-02	DHF	DQ472142	DQ472142	DQ472142	2002	Cosmopolitan
TW2523-02	DHF	DQ472143	DQ472143	DQ472143	2002	Cosmopolitan
PL046*	IU	EF540856	N/A	EF540856	1981	Asian 1
TW-L10052*	DF	L10052	N/A	L10052	1987	Asian 1
247TP9810a-Tw*	IU	DQ518644	N/A	DQ518644	1998	Asian 1
807KH9809a-Tw*	IU	DQ518645	N/A	DQ518645	1998	Asian 2
806KH0110a-Tw*	IU	DQ518630	N/A	DQ518630	2001	Cosmopolitan
704TN0510a-Tw*	IU	DQ518640	N/A	DQ518640	2005	American/Asian
Taiwan-1008DHF*	DHF	AY776328	AY776328	AY776328	IU	Cosmopolitan

*Previously published E gene sequence data for Taiwan isolates in GenBank. DF = dengue fever; DHF = dengue hemorrhagic fever; IU = information unknown; N/A = not applicable.

RNA extraction

RNA was extracted when over 50% of the cells showed specific immunofluorescence in an indirect immunofluorescence assay with dengue virus type-specific monoclonal antibodies [25]. Viral RNA was extracted using the QIAmp™ viral RNA purification kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions.

Reverse-transcriptase polymerase chain reaction and sequencing

D2E1 (5′-AAGATCAGTGGCGCTCGTTCC-3′) and D2E2 (5′-AAGATCCCGCTGCCACATTTC-3′) primers were used to amplify nucleotides 708–2480, which span the E gene. The primers D2NS1-1 (5′-TTCACGTAGC-ACCTCACTGTCT-3′) and D2NS1-2 (5′-CTACTC-GGGTCCTAAGCATTTC-3′) were used to amplify nucleotides 2343–3559, which span the NS1 gene. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed in a mixture composed of 5 μL of RNA, 1 μL of each primer (50 μM), 40 U of RNase inhibitor (Promega, Madison, WI, USA), 200 U of MMLV (Promega), 2.5 U of Pfu DNA polymerase (Protech, Taipei, Taiwan), 10 × PCR reaction buffer, and 15 mM dNTP (Promega).

The following RT-PCR program was used: 37°C for 1 hour, 94°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 55°C for 30 seconds and 72°C for 1 minute, and a final elongation step of 72°C for 5 minutes. Two fragments of 1773 bp and 1217 bp, including the entire region of the E gene (1485 bp) and the NS1 gene (1056 bp) respectively, were amplified.

Cloning and sequencing

The amplified PCR products were purified using the GFX™ PCR purification kit (Amersham Biosciences, Buckinghamshire, England). After purification, PCR products were cloned into the yT&A vector (Yeastern Biotech, Taipei, Taiwan) at room temperature for 2–3 hours. The ligation mixtures were used to transform competent JM103 cells and the transformed cells were plated on LB agar plates containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal), isopropyl-beta-D-thiogalactopyranoside (IPTG) and ampicillin. The inserts were selected by a color change (blue to white) that developed during overnight incubation at 37°C. Clones with 1773-bp and 1217-bp inserts were confirmed by PCR. Sequencing was performed using the ABI Prism Ready Reaction Dideoxy Terminator Cycle Sequencing kit (Model 3730 version 3.4; Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Our sequence results were compared with worldwide sequences from GenBank with the GenBank accession numbers shown in Tables 1 and 2. Among them, seven sequences of the E gene from Taiwan isolates in 1981, 1987, 1998, 2001 and 2005 were available. Our sequences were compared with E and NS1 gene sequences available in GenBank and to the New Guinea C strain (NGC), a prototype strain of DEN-2. Alignments of the entire E and NS1 gene sequences were undertaken using the CLUSTAL method of DNASTAR MegAlign (DNASTAR Inc., Madison, WI, USA). Two phylogenetic trees were constructed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA), version 3.0 software. The reliability of the neighbor-joined trees was estimated by bootstrap analysis with 1,000 replications.

RESULTS

Nucleotide and amino acid sequences

Differences of 5.3–5.7% and 6.5–6.9% were evident in the DEN-2 E and NS1 gene nucleic acid sequences, respectively, of the isolates gathered during the 2002 Taiwan dengue epidemic, as compared with the prototype DEN-2 strain NGC. No base insertions or deletions were found among the 10 isolates regarding E and NS1 gene sequences, although both transitions and transversions were detected. The transition:transversion ratios of the E and NS1 gene were 72:11 and 64:9, respectively. The amino acid sequence differences were 0–0.6% and 0–0.9% for the E and NS1 genes, respectively, among the 2002 DEN-2 strains, indicating that an epidemic strain caused the outbreak. With the exception of three isolates showing amino acid changes, the NS1 amino acid sequences from all 2002 isolates were the same. No significant differences in gene and amino acid sequences were evident in eight DF and three DHF samples.

Phylogenetic analysis

The genetic relationships among worldwide isolates were inferred by the neighbor-joining method based on the 1,485 nucleotides of the E gene and 1,056 nucleotides of the NS1 gene in GenBank. A divergence

Table 2. Accession number, area and year of the DEN-2 virus isolates in the phylogenetic analysis

Strain	GenBank accession no.	Area/country	Year
Cuba58a/97	AY702042	Cuba	1997
Cuba205/97	AY702060	Cuba	1997
MART-98-703	AF208496	Martinique	1998
LARD1557	AF363071	Venezuela	1997
lard6123	AF398114	Venezuela	2000
PR_19_88	DQ364555	Puerto Rico	1988
Mara4	AF100466	Venezuela	1990
JAM/95/83	DQ364484	Jamaica	1983
Jamaica/N.1409	M20558	Jamaica	1983
PR_Moca_97	DQ364509	Puerto Rico	1997
D83-061	AF195043	Thailand	1983
D91-533	AF195040	Thailand	1991
D2-D80-141	M24444	Thailand	1980
704TN0510a-Tw	DQ518640	Taiwan	2005
CTD203	AF410360	Vietnam	1998
China/04	X65240	China	1985
CTD29	AF410346	Vietnam	1998
D80-038	U87366	Thailand	1980
M1	X15434	Malaysia	1987
M2	X15433	Malaysia	1987
PUO/218	D00345	Thailand	1980
PUO/312	AF264053	Thailand	1980
D83-307	AF195035	Thailand	1983
DST87-060	U34952	Thailand	1980
ThNH-81/93	AF169688	Thailand	1993
ThNH-28/93	AF022435	Thailand	1993
ThNH-p36/93	AF022441	Thailand	1993
D2/Hu/Thailand	AB194882	Thailand	2004
807KH9809a-Tw	DQ518645	Taiwan	1998
D87-881	U34951	Thailand	1987
K0010	DQ181890	Thailand	2001
K0005	AY158336	Thailand	1994
ThD2/K0001/95	DQ181881	Thailand	1995
ThD2/K0035/96	DQ181901	Thailand	1996
CTD113	AF410358	Vietnam	1997
CTD208	AF410362	Vietnam	1998
New/Guinea/C	M29095	New Guinea	1944
Strain/43	AF204178	China	1987
D2-SL77	M24450	Sri Lanka	IU
PL046	EF540856	Taiwan	1981
PHILIP	L10045	Philippines	1983
DOH/005	AF295697	Philippines	1995
SLMC/451	AF297009	Philippines	1998
247TP9810a-Tw	DQ518644	Taiwan	1998
TW-L10052	L10052	Taiwan	1987
CI-15	AF295696	Philippines	1998
DOH97/95	AY786368	Philippines	1995
BRL/008	AF295694	Philippines	1996
SLMC/179	AF297008	Philippines	1996
BRL/020	AF295695	Philippines	1996
DOH/321	AF297005	Philippines	1995
CAMR17	AF410379	Singapore	1991
SL714	L10055	Sri Lanka	1989
CAMR11	AF410375	Uganda	1993
FJ-10	AF276619	China	1987

(Contd.)

Table 2. *Continued*

Strain	GenBank accession no.	Area/country	Year
P7-863	AF231716	Malaysia	1969
SL767	M24449	Sri Lanka	IU
SEY-42	L10047	Seychelles	1977
SEY-52	L10048	Seychelles	1977
P8-377	AF231715	Malaysia	1969
Somalia	L10051	Somalia	1984
Torres/Strait	AY706012	Australia	2003
Cook/Islands/1	AF004020	Cook Islands	1997
Indonesia	L10044	Indonesia	1976
01ST418/01	AY786393	Philippines	2001
Taiwan-1008DHF	AY776328	Taiwan	IU
806KH0110a-Tw	DQ518630	Taiwan	2001
India	L10043	India	1957
Ven2	AY158328	Venezuela	1987
Tonga/74	AY744147	Tonga	1974
Peru/539/96	AF093674	Peru	1996
Iqt2913	AY158339	Peru	1996
P8-1407	AF231717	Malaysia	1970
DAKHD10674	AF231720	Senegal	1970
DAKAr578	AF231718	Ivory Coast	1980
PM33974	AF231719	Guinea	1981

IU = information unknown.

of 6% within the studied gene region was taken as a cut-off point for virus groupings based on previous studies with the same E gene interval and short sequences at the E/NS1 junction [27,28]. Six genetic groups, each representing a distinct genotype based on E gene analysis, are shown in Figure 1. All Taiwan DEN-2 isolates were of the Cosmopolitan genotype. The 2002 Taiwan DEN-2 isolates were closest to a Philippines strain, 01St41801. Four genotypes were found among all Taiwan DEN-2 viruses. There were Taiwan isolates from 1981, 1987 and 1998 belonging to the Asian 1 genotype; isolates from 1998 of the Asian 2 genotype; isolates from 2001 and 2002 of the Cosmopolitan genotype; and isolates from 2005 of the American/Asian genotype.

There is less DEN-2 gene sequence data available for the NS1 gene than there is for the E gene. The NS1 gene phylogenetic tree for DEN-2 revealed the same grouping results as the E gene phylogenetic tree, but with a lower bootstrap support value for each branch (Figure 2).

Discussion

Our results revealed the appearance of DEN-2 isolates with the Cosmopolitan genotype in 2001 and 2002 in Taiwan, which is different from previous Taiwan isolates from 1981, 1987 and 1998 belonging to the Asian 1 or Asian 2 genotypes. The DEN-2 isolate in 2005 belonged to the American/Asian genotype. A 0–0.4% divergence among the 10 isolates of the 2002 Taiwan epidemic revealed the existence of an epidemic strain being spread in the outbreak. As Taiwan is near dengue-epidemic Asia/Pacific Rim countries, and there had been a period of no dengue cases for 37 years, the various genotypes of DEN-2 isolates in different years suggested the import of dengue virus from different places at different times, rather than a continuous clonal transmission in Taiwan. A study of Taiwan dengue isolates from 2005 indicated that the spread of DEN-2 and dengue virus serotype 3 strains from Vietnam and the Philippines caused the outbreak. This provides molecular epidemiologic evidence that the Taiwan dengue outbreak in 2005 was related to dengue virus imported from Southeast Asia [29].

All of the 2002 Taiwan DEN-2 isolates belonged to the Cosmopolitan genotype and had a much closer relationship to a Philippines strain, 01St41801, which was found to be closely related to viruses from Australia, Singapore and Thailand [30]. An earlier Taiwan strain (TW-L10052) isolated in 1987 belonged to the Asian 1 genotype and clustered with many

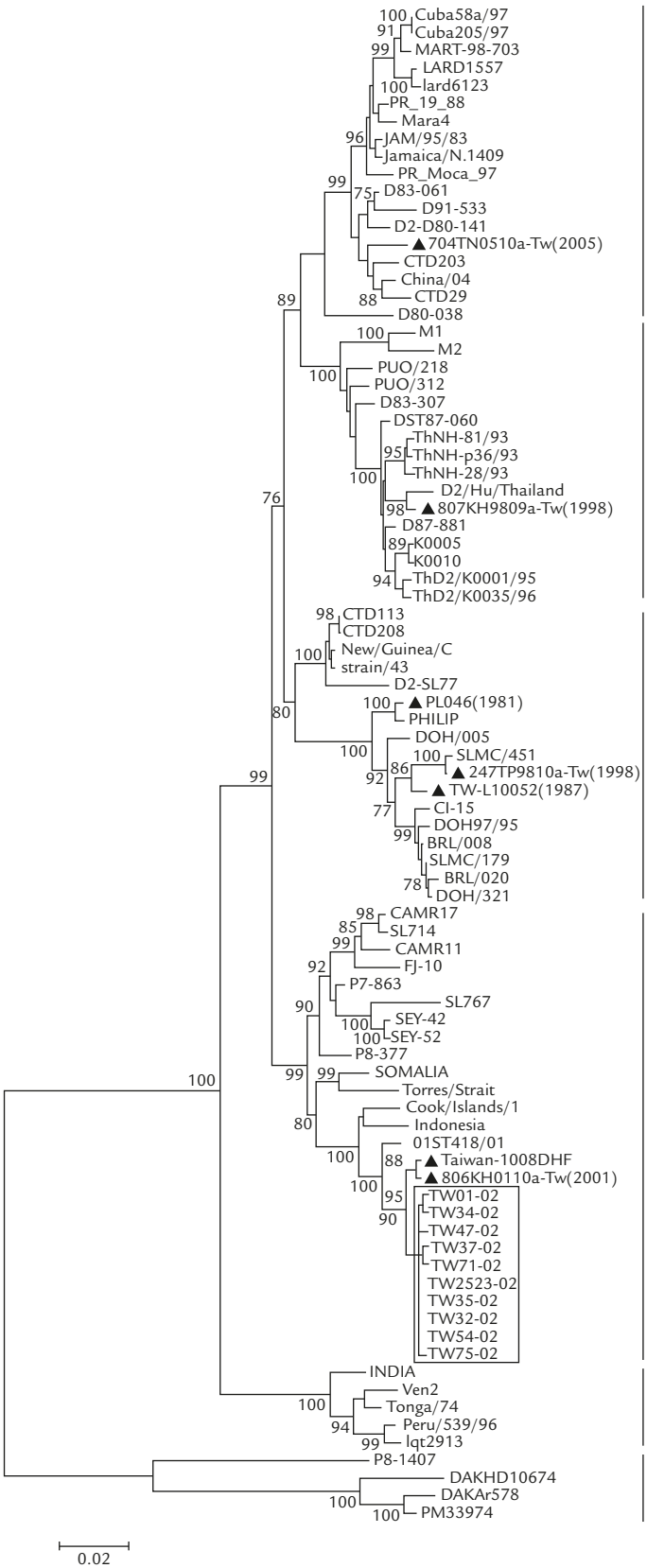

Figure 1. *Phylogenetic relationships, presented as a maximum likelihood tree, of envelope gene sequences and the genotypes of 86 DEN-2 isolates. Viruses are listed by isolate name in the GenBank database (Tables 1 and 2). The numbers on branches represent bootstrap support for each branch. The black triangles indicate Taiwan isolates. Isolates in the box indicate isolates from the Taiwan 2002 outbreak.*

Figure 2. *Phylogenetic tree of NS1 gene sequences and genotypes of 36 DEN-2 isolates. Viruses are listed by isolate name in GenBank (Tables 1 and 2). The numbers on branches represent bootstrap support for each branch.*

other Philippines strains. The shift in the prevalent genotype in Taiwan from the Asian 2 genotype to the Cosmopolitan genotype is the same as the shift in DEN-2 genotypes that occurred in the Philippines, where the Cosmopolitan genotype replaced the Asian 2 genotype as the dominant genotype between 1995 and 2002 [30]. A similar trend for DEN-2 genotypes over time in Taiwan and the Philippines suggests that the international spread of dengue virus causes the same genotype changes in nearby countries.

E gene sequencing has been widely used to assess the phylogenetic relationships among DEN-2 isolates [7,30]. In contrast, the phylogenetic significance of the NS1 gene of DEN-2 has not been extensively studied [13,14]. There were nucleotide mutations observed in the NS1 and NS5 genes, but not in other genes, in the

1997 Cuba outbreak [31], indicating the higher mutation rate of the NS1 gene during an epidemic (which could be a valuable molecular epidemiology tool during an epidemic outbreak). The difference in the sequence of the NS1 gene between the 2002 Taiwan DEN-2 isolates and the DEN-2 prototype strain is larger than the difference in the sequence of the E gene between these strains (6.5–6.9% *vs.* 5.3–5.7%).

Compared with the E gene, a relatively small amount of NS1 sequence data for DEN-2 is available in GenBank. Comparison of the phylogenetic trees from studies of the E and NS1 genes reveals a similar grouping, but with a lower bootstrap number. Our result did not validate the use of the NS1 gene for phylogenetic studies owing to the small number of sequences available and the lower bootstrap support for DEN-2. Furthermore, NS1 gene sequences obtained from different areas and at different times are necessary to identify the usefulness of the NS1 gene for phylogenetic analysis.

In summary, the same grouping result is obtained using the NS1 gene or the E gene for phylogenetic analysis of DEN-2. Our result indicates that the isolates of the 2002 Taiwan DEN-2 epidemic are clonally related and belong to the Cosmopolitan genotype. Four genotypes of Taiwan DEN-2 since 1981 by E gene analysis suggest that DEN-2 in Taiwan has mainly originated from other countries.

Continuous monitoring of the dengue virus genotype in combination with a worldwide database may help improve the understanding of viral genotype shifts locally and their relationship with worldwide epidemiology.

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分析 Envelope 與 Nonstructural Protein 1 基因以探討 2002 年台灣第二型登革熱群突發之分子流行病學

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由 **envelope (E)** 與 **nonstructural protein 1 (NS1)** 基因分析 **2002** 年台灣第二型登革熱群突發發現十株病毒株之間只有 **0–0.4%** 之差異，顯示該群突發為一流行病毒株之傳播。親源關係分析顯示 **2002** 年台灣第二型登革熱病毒為 **Cosmopolitan** 基因型，不同於 **1981** 至 **1998** 的 **Asian 1** 與 **Asian 2** 基因型以及 **2005** 年的 **American/Asian** 基因型。雖然以 **E** 基因和 **NS1** 基因分析對病毒株之分群可得到一致的結果，**NS1** 基因用於第二型登革熱病毒親源關係分析的效度因其較低之 **bootstrap** 數值而未能在本研究中獲得證實。研究發現台灣與菲律賓之第二型登革熱之主要基因型至 **2002** 年都同樣有由 **Asian 2** 基因型改變為以 **Cosmopolitan** 基因型為主，顯示病毒株的傳播可能導致臨近國家有相同基因型改變之趨勢。

關鍵詞：第二型登革熱病毒，**envelope** 基因，**nonstructural protein 1** 基因
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