Phylogenetic Study of Dengue-3 Virus in Taiwan with Sequence Analysis of the Core Gene

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Dengue virus serotype 3 (dengue-3) has been classified into five genotypes (I–V) by phylogenetic analysis based on different viral genes. To investigate the genetic variability and evolutionary character of the dengue-3 isolates in southern Taiwan from 2005 to 2006, we analyzed the 290 nucleotides of the core (C) gene of 12 dengue-3 isolates and compared them with the published C gene sequences of global dengue-3 strains available in GenBank, including four isolates from 1998 and one isolate from 1999, from Taiwan. The dengue-3 viruses from 2005 to 2006 were not from continuous spread of an epidemic strain or re-emergence of the 2005 strains in the 2-year period because there was a 5.4–6.2% difference in the 290 nucleotides of the C gene and different genotypes between the 2005 and 2006 strains. Most of the nucleotide changes, compared with a prototype dengue-3 virus, H87, occurred in the third codon position and were non-synonymous mutations occurring naturally in the C gene. In addition, there was no consistent difference in the 290 nucleotides of the C gene between eight dengue fever and two dengue hemorrhagic fever isolates from 2006. The phylogenetic analysis indicates that the isolates from the 1998, 1999 and 2006 Taiwan dengue-3 epidemics are phylogenetically related and belong to genotype III. It was noted that the 2005 Taiwan dengue-3 isolates belong to another genotype. This molecular epidemiology study of dengue-3 viruses in Taiwan helps to elucidate whether there is a continuation of outbreaks in consecutive years, re-emergence of endemic dengue virus, or introduction of strains from other countries.

Key Words: core gene, dengue-3 virus, phylogenetic analysis
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Dengue is one of the most serious and prevalent mosquito-borne viral threats to humans worldwide [1,2]. Infection with dengue virus (DV) generally leads to fever, headache, pain in various parts of the body, prostration, rash, lymphadenopathy, leukopenia and more seriously, dengue hemorrhagic fever (DHF), which can progress to dengue shock syndrome (DSS) and death [3]. Dengue viruses, of the genus *Flavivirus*, are classified into four antigenically distinct serotypes (dengue 1–4) [3]. They have a single-stranded, positive sense RNA genome of about 11,000 nucleotides [4]. To date, dengue-3 has been classified into five genotypes (I–V) by phylogenetic analysis based on different viral gene regions, including the envelope gene and prM/M gene. The same grouping results were

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Table 1. Isolates of dengue-3 virus from Taiwan described in the study

Isolate designation	Year of isolation	Clinical status	Fever	Skin rash	GenBank accession no.	Genotype
0503-TW	2005	DF	Y	Y	EF584513	–
0504-TW	2005	DF	Y	N	EF584514	–
0601-TW	2006	DF	Y	N	EF584515	III
0611-TW	2006	DF	Y	Y	EF584516	III
0613-TW	2006	DF	Y	Y	EF584517	III
0615-TW	2006	DF	Y	Y	EF584518	III
0617-TW	2006	DF	Y	N	EF584519	III
0622-TW	2006	DF	Y	N	EF584520	III
0623-TW	2006	DHF	Y	Y	EF584521	III
0629-TW	2006	DHF	Y	N	EF584522	III
0630-TW	2006	DF	Y	Y	EF584523	III
0638-TW	2006	DF	Y	Y	EF584512	III

DF = dengue fever; DHF = dengue hemorrhagic fever; Y = yes; N = no.

obtained when analyzing different genes of dengue-3 viruses [5–7].

In Taiwan, historical epidemics of dengue in 1902, 1915, 1922, 1927, 1931 and 1942–1943 have been documented. After a 37-year period without dengue cases from 1943 to 1981, a dengue-2 outbreak occurred in the Liu Chiu District in 1981 [8]. Since the 1987 outbreak of dengue-1 infections, cases of dengue infection have been reported every year. Cases of dengue-3 infection were reported in 1988, 1991–92, 1994–96, 1998, 1999, 2001, 2005 and 2006 [9,10]. Silent transmission of dengue viruses in Taiwan has also been reported [11]. However, dengue virus strains may be introduced into Taiwan with increasing international travel.

Nucleotide sequence analysis of DV was used to define genetic variation between strains of the same serotype, follow geographical movement of the strains, and determine the evolutionary origin of epidemic dengue viruses [12–16]. To investigate the genetic variability and evolutionary character of the dengue-3 strains isolated in southern Taiwan from 2005 to 2006, we analyzed the 290 nucleotides of the core (C) gene of 12 dengue-3 isolates and compared them with the published sequences of global dengue-3 strains available in GenBank, including four isolates from 1998 and one isolate from 1999, from Taiwan.

Materials and Methods

Serum specimens

Serum specimens were collected from patients clinically suspected of DV infection in two Kaohsiung hospitals in 2005 and 2006. These serum samples were separated and stored at –80°C until use. The clinical characteristics of these patients, including isolate designation, year of isolation, clinical status, fever and skin rash, are shown in Table 1. The definition of dengue fever (DF) and DHF were made according to the WHO clinical definition [17]. These specimens were all confirmed as dengue-3 infection by culture in C6/36 cell, reverse transcription–polymerase chain reaction (RT-PCR) and serologic methods (ELISA—IgG and IgM). All cases were considered to be indigenous according to the patient's history because none of these patients or their families had a history of travel to other countries within the previous 2 weeks.

Nested RT-PCR and sequencing

RNA was extracted from the patient's serum using the QIAamp viral RNA purification kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). The outer primers D1 (5′-ATCAATATGCTGAAACGCGCGAGAAACCG-3′) and D2 (5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′) were used for the first amplification of the nucleotide sequence from position 131 to 642. The inner primers of D1 and TS3 (5′-TAACATCATCATGAGACAGAGC-3′) were used for the second amplification of the nucleotide sequence from position 131 to 420. RT-PCR was performed in a 50 μL mixture composed of 5 μL of RNA, 1 μL of 20 μM of D1 and D2 primer, 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 1 μL of 25 mM dNTP (Promega).

The following RT-PCR program was used: 37°C for 1 hour, 94°C for 5 minutes, followed by 35 cycles

Table 2. Dengue-3 strains from GenBank used in the phylogenetic analysis described in this study

Virus strain	GenBank accession no.	Clinical status	Location of isolation	Year of isolation	Genotype
98TW407	DQ675528	DF	Taiwan	1998	III
98TW414	DQ675529	DF	Taiwan	1998	III
98TW434	DQ675530	DF	Taiwan	1998	III
98TW503	DQ675531	DF	Taiwan	1998	III
99TW628	DQ675533	DF	Taiwan	1999	III
PF89/27643	AY744677		French Polynesia	1989	I
PF89/320219	AY744678		French Polynesia	1989	I
PF90/3050	AY744679		French Polynesia	1990	I
PF90/3056	AY744680		French Polynesia	1990	I
PF90/6056	AY744681		French Polynesia	1990	I
PF92/2956	AY744682		French Polynesia	1992	I
PF92/2986	AY744683		French Polynesia	1992	I
PF92/4190	AY744684		French Polynesia	1992	I
PF94/136116	AY744685		French Polynesia	1994	I
H87	M93130		Philippines	1956	V
80-2	AF317645		China	1980	V
1243	AY099337		Martinique	1999	III
1266	AY099336		Sri Lanka	2000	III
TB55i	AY858048		Indonesia	2004	I
TB16	AY858047		Indonesia	2004	I
PI64	AY858046		Indonesia	2004	I
BA51	AY858037		Indonesia	2004	I
den3_98	AY858039		Indonesia	1998	I
den3_88	AY858038		Indonesia	1998	I

of 95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute, and a final elongation step of 72°C for 5 minutes. A second amplification reaction was performed in a 100 μL mixture composed of 1/10 volume of first PCR product, 10 × PCR reaction buffer, 5 U of Pfu DNA polymerase, 1 μL of 25 mM dNTP and 2 μL of 20 μM of D1 and TS3 primer. The nested PCR program was: 94°C for 5 minutes, followed by 20 cycles of 95°C for 1 minute, 55°C for 30 seconds, 72°C for 1 minute, and a final elongation step of 72°C for 5 minutes. Fragments of 290 bp were obtained from the second amplification.

The 290-bp PCR products were purified using the GFX™ PCR purification kit (Amersham Biosciences, England). After purification, these PCR products were sequenced directly using the ABI Prism Ready Reaction Dideoxy Terminator Cycle Sequencing kit (Model 3730 version 3.4; Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Alignment of these sequences was undertaken using the Clustal method of DNASTAR MegAlign (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were constructed by the neighbor-joining method with MEGA 3 (Molecular Evolutionary Genetics Analysis, Version 3.0). The reliability of neighbor-joined trees was estimated by bootstrap analysis. All of the sequences studied in this study can be accessed in GenBank. Their accession numbers are shown in Table 1. Our sequences were compared to the other dengue-3 strain sequences available in GenBank, and aligned to the H87, a prototype strain of dengue-3. The data on dengue-3 strains used in this study, except the 2005 and 2006 Taiwan strains, are shown in Table 2.

RESULTS

Nucleotide and amino acid sequence analysis

The nucleotide sequences of these Taiwan dengue-3 strains, including four isolates from 1998, one isolate from 1999, two isolates from 2005 and 10 isolates from 2006 are shown in Figure 1. Differences in the 290 nucleotides of the C gene among isolates within the same year were 0% among 1998 isolates, 0.3% among 2005 isolates and 0–0.7% among 2006 isolates. In addition, nucleotide differences of 3.9%, 4.3%, 5.1–5.5% and

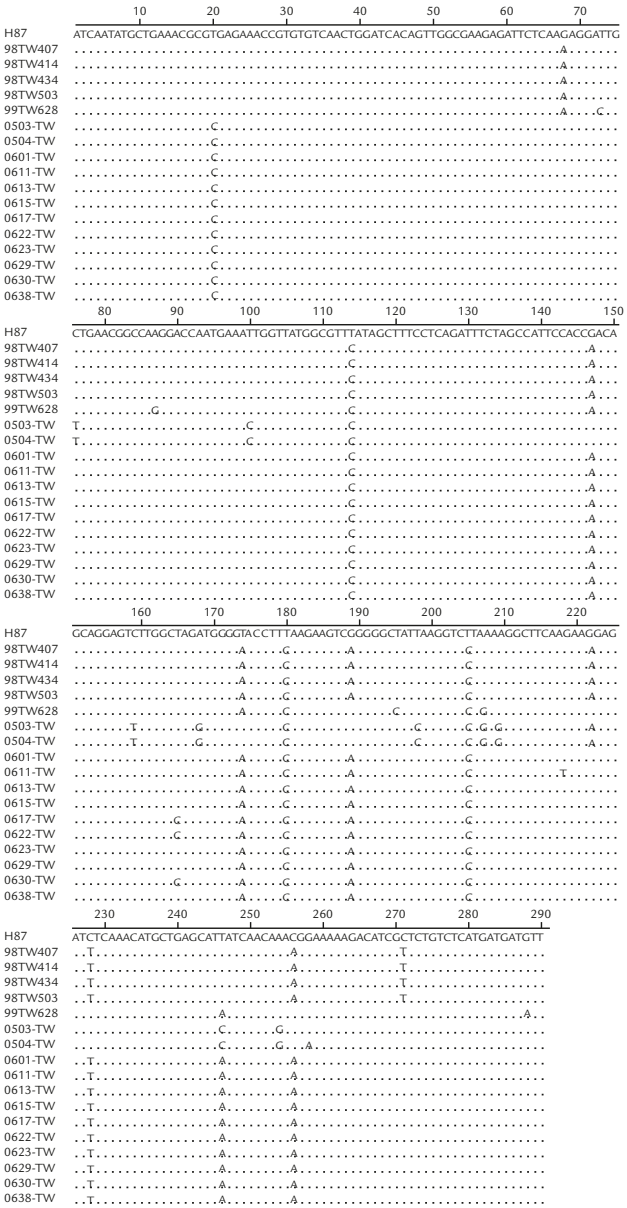

Figure 1. *Alignment of the nucleotide sequences of Taiwan dengue-3 strains and the H87 strain based on the 290 nucleotides of the C gene.*

3.5–3.9% were found in the 1998, 1999, 2005 and 2006 strains, respectively, compared with the prototype dengue-3 strain (H87). Differences of 5.1–6.2% were found between the 2005 strains and other Taiwan strains. Furthermore, no base insertions or deletions occurred in this 290-nucleotide region among these strains. The differences in nucleotide sequence among these Taiwan dengue-3 strains and H87 are shown in Table 3.

The amino acid sequences of isolates from all of the 1998 and 2005 strains and some of the 2006 strains were completely the same. The amino acid sequences encoded by the 290 nucleotides of the C gene are shown in Figure 2. Two amino acid changes occurred at the C103 (Arg → Lys) and C306 (Leu → Phe) positions in 1998 isolates. A Val → Ala change at C55 was found in 2005 and 2006 isolates. Two Lys → Arg changes were also found at C244 and C289 in 2005 strains. Except for the 0611 strain, which had one amino acid change at C253, the amino acid sequences of the proteins encoded by the C genes of 2006 epidemic isolates were the same (Figure 2).

There was no significant difference in the changes to gene and amino acid sequences between DF and DHF cases in Taiwan in 2006. Two isolates (isolate numbers 0623-TW and 0629-TW) from DHF patients who came from the same family and had symptoms on the same day had 100% homology in the 290-nucleotide C gene sequence. Both patients were dengue IgG negative and IgM positive at the symptomatic stage.

Phylogenetic analysis

The nucleotide sequences generated in this study were analyzed in combination with other dengue-3 gene sequences available in GenBank. Table 2 shows the data on all other dengue-3 strains analyzed in the study, including clinical status, location and year of isolation and GenBank accession number. A phylogenetic tree consisting of 17 Taiwan strains and 19 strains from other countries was constructed and is shown in Figure 3. The genetic relationships among worldwide isolates were inferred by the neighbor-joining method based on analysis of the 290-nucleotide C gene sequences in GenBank. The phylogenetic analysis showed that the 1998, 1999 and 2006 isolates belong to genotype III, a group that also includes the 1243 strain (isolated in Martinique in 1999) and the 1266 strain (isolated in Sri Lanka in 2000). The dengue-3 isolates in 2005 (0503 and 0504) formed a clearly distinct genogroup, which differed from genotypes I, III and V on the basis of analysis of the 290 nucleotides of the C gene.

Discussion

Nucleotide sequence analysis of DV can be used to define genetic variation between strains of the same serotype, follow geographical movement of the strains, and thereby determine the evolutionary origin of

Table 3. Comparison of the nucleotide sequence (290 nucleotides) of the C genes of Taiwan dengue-3 strains and H87

		% Nucleotide divergence																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	H87																		
2	98TW407	3.9																	
3	98TW414	3.9	0.0																
4	98TW434	3.9	0.0	0.0															
5	98TW503	3.9	0.0	0.0	0.0														
6	98TW628	4.3	3.9	3.9	3.9	3.9													
7	0503-TW	5.1	6.2	6.2	6.2	6.2	6.2												
8	0504-TW	5.5	6.6	6.6	6.6	6.6	6.6	0.3											
9	0601-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8										
10	0611-TW	3.9	2.1	2.1	2.1	2.1	3.9	5.8	6.2	0.3									
11	0613-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8	0.0	0.3								
12	0615-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8	0.0	0.3	0.0							
13	0617-TW	3.9	2.1	2.1	2.1	2.1	3.9	5.8	6.2	0.3	0.7	0.3	0.3						
14	0622-TW	3.9	2.1	2.1	2.1	2.1	3.9	5.8	6.2	0.3	0.7	0.3	0.3	0.0					
15	0623-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8	0.0	0.3	0.0	0.0	0.3	0.3				
16	0629-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8	0.0	0.3	0.0	0.0	0.3	0.3	0.0			
17	0630-TW	3.9	2.1	2.1	2.1	2.1	3.9	5.8	6.2	0.3	0.7	0.3	0.3	0.0	0.0	0.3	0.3		
18	0638-TW	3.5	1.7	1.7	1.7	1.7	3.6	5.4	5.8	0.0	0.3	0.0	0.0	0.3	0.3	0.0	0.0	0.3	

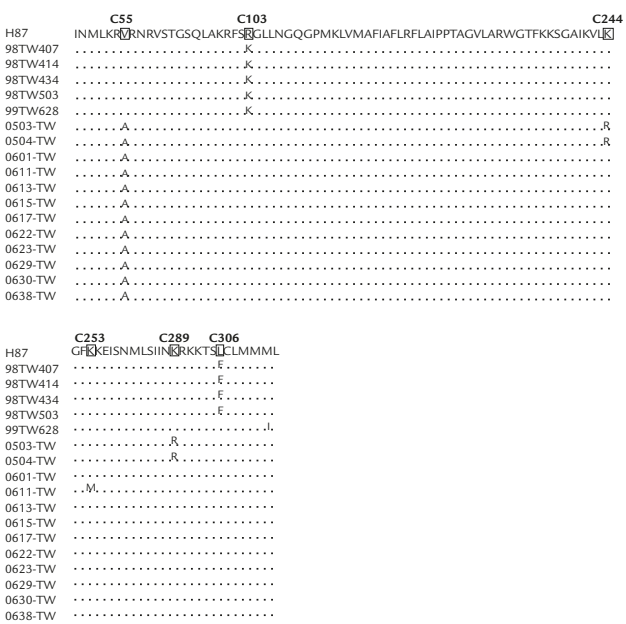

Figure 2. *Alignment of the amino acid sequences encoded by the 290 nucleotides of the C genes of Taiwan dengue-3 strains and the H87 strain.*

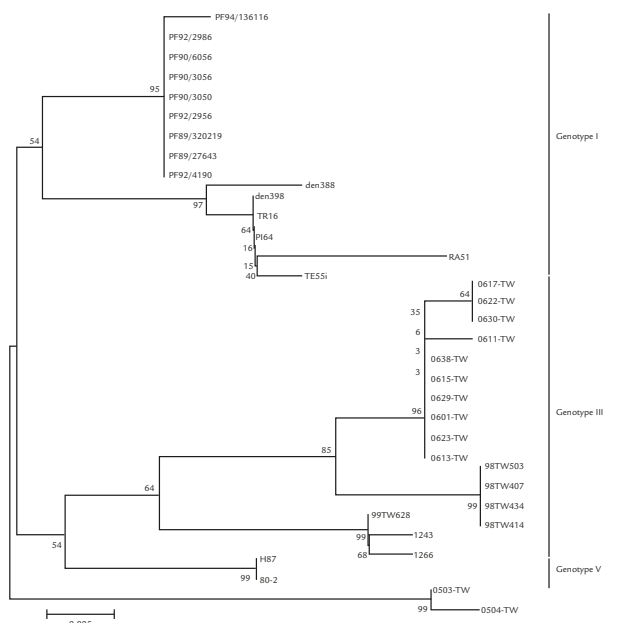

Figure 3. *Phylogenetic analysis of the C genes from 36 dengue-3 viruses. Viruses are listed by strain name in the GenBank database and genotypes are indicated. Bootstrap values are given as percentages and correspond to 1,000 replications.*

epidemic DVs [12–16]. Our study of the C gene of the dengue-3 viruses in circulation in recent years in Taiwan showed that isolates of each year had the same amino acid sequences, except for one isolate from 2006. Differences in the amino acid sequences encoded by the C gene existed among genotype III isolates from 1998, 1999 and 2006, in combination with the new genotype in 2005, suggesting that the prevalent virus changed every year. Whether the changes among the genotype III isolates in each year were from spontaneous mutations or due to the introduction of new strains each year could not be concluded in this study. However, dengue-3 viruses from 2005 to 2006 were not from continuous spread of an epidemic strain or re-emergence of the 2005 strains in the 2-year period because there was a 5.4–6.2% difference in the 290 nucleotides of the C gene between 2005 and 2006 strains, and 2005 strains had a different genotype from the 2006 strains. Most changes in nucleotides, compared with H87 virus, occurred in the third codon position and were non-synonymous mutations occurring naturally in the C gene.

In contrast to DV serotypes 1, 2 and 4, there were relatively few dengue-3 sequence data available in GenBank. The five genotype (I–V) classification is based on phylogenetic analysis of the envelope gene and prM/M gene [5–7]. Phylogenetic study of the C gene has been undertaken for DVs not of serotype 3 [18,19]. The results of our C gene phylogenetic analysis show the same grouping as the results of E gene analysis on dengue-3 strains [7]. However, studies on long sequences, such as the E gene (1,450 nucleotides) or the PrM/M/E gene (2,550 nucleotides), require virus culture and cloning. Our method of C gene (290 nucleotides) analysis had an advantage of rapid genotyping because typing results can be obtained by direct PCR sequencing of blood specimens. Phylogenetic analysis of the C genes of these Taiwan dengue-3 strains and other global strains showed that the 1998, 1999 and 2006 Taiwan strains belong to genotype III. Genotype III also included the 1243 and 1266 strains, which were isolated in Martinique in 1999 and Sri Lanka in 2000, respectively.

The 2005 isolates (0503 and 0504) formed a clearly distinct genogroup from the 1998, 1999 and 2006 Taiwan strains, which obviously differed from genotypes I, III and V in the C gene analysis. To determine whether the Taiwan dengue-3 isolates in 2005 were of genotypes II, IV or a new genotype requires further study of other genes and comparison with global isolates of known genotypes. The genotype changes in dengue-3 virus in Taiwan from 1998 to 2006 showed a different pattern from the observation in Thailand where there were extinctions and rapid emergence of strains of dengue-3 virus during an inter-epidemic period, possibly due to a genetic bottleneck condition [6].

Regarding the pathogenesis of DHF/DSS, (1) immune enhancement caused by repeat or co-infection with a second dengue virus of different serotype [20], and (2) variation in viral virulence, where the more severe disease was due to the greater virulence of specific strains [13,21], has been proposed. Previous studies have shown that mutations in the dengue genome may be associated with changes in virulence [22,23]. Although the possibility that the determinant of virulence is not located in the C gene cannot be excluded, our results showed that there were no consistent differences in the sequence of the 290 nucleotides of the C gene between eight DF and two DHF isolates in 2006. Two isolates (isolate numbers: 0623-TW and 0629-TW) from DHF patients who came from the same family and had symptoms on the same day had 100% homology in the 290-nucleotide sequence. This implied that the two patients were infected by the same dengue-3 strain in which viral virulence may play a role in the mechanism of DHF. Because both of the patients had primary dengue infection, an immune enhancement effect is unlikely to be the cause.

In summary, this study indicates that isolates of the 1998, 1999 and 2006 Taiwan dengue-3 epidemics are phylogenetically related and belong to the genotype III group of viruses. It was also noted that the 2005 Taiwan dengue-3 isolates (0503 and 0504) belong to another genotype. Phylogenetic analysis is helpful to elucidate whether there is a continuation of outbreaks in consecutive years, to recognize the possibility of re-emergence of endemic DV, and to identify the introduction of strains from other countries.

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分析核心基因以探討台灣第三型登革熱病毒的親源關係之研究

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登革熱是一種以蚊子為媒介的全世界最重要之傳染病。目前為止，根據病毒不同基因的親源關係分析，第三型登革熱病毒被分為五個基因型，即第一至第五基因型。為了探討 2005 和 2006 年於南台灣分離的第三型登革熱病毒株基因變異和分子演化的特性，我們分析 12 株病毒之核心基因的 290 個核苷酸序列，並和基因資料庫中各國第三型登革熱病毒株 (包括 4 株 1998 和 1 株 1999 台灣分離株) 做比較。我們發現 2005 和 2006 年病毒株在此段核苷酸序列，有 5.4－6.2% 的差異，且由親源關係分析可知這 2 年病毒株分別屬於不同的基因型，因此可以推論 2005 年和 2006 年的第三型登革熱病毒並不是同一流行株所造成的連續性傳播。另外，和第三型登革熱病毒之原始株 (H87) 比較，可知這些核苷酸變異多發生在遺傳密碼的第三個核苷酸位置，而呈現非同義的變異。除此之外，我們的結果顯示，2006 年的 8 個登革熱和 2 個出血性登革熱病毒株，在此 290 個核苷酸序列中並沒有明顯的差異性。另外，根據親源關係分析的結果，可知 1998，1999 和 2006 年的台灣第三型登革熱病毒株是屬於第三基因型。值得注意的是，2005 年 2 個台灣病毒株明顯地屬於不同的基因型。由此台灣登革熱第三型病毒的分子流行病學分析，我們可以清楚地瞭解各年之間的登革熱流行，是否為連續性或是地方性登革熱病毒的再次出現，或者是其他國家的境外移入株導致的流行。

關鍵詞：核心基因，第三型登革熱病毒，親源關係分析
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