mortality is still around 1%. Despite this mortality rate is lower than before, it is still higher compared to the mortality rate in other South East Asia country such as Thailand (0.2%) (WHO/SEARO, 2007). The other problem in Indonesia is the changing epidemiology of DHF. The outbreak trend of DHF has become irregular with high inter-epidemic background (Setiati, 2007). These problems can be caused by social, demographic, climate factors, as well as host, vector and viral factor.

This study is to address the viral factor to contribute in the understanding of epidemiology and pathogenesis study of dengue virus. In this study, from March 2006 to April 2008 a total of 238 plasma spesimen from children and adults at the Cipto Mangunkusumo Central Hospital in Jakarta were examined for dengue viruses. Dengue serotypes were determined by RT-PCR. We sequenced the envelope region of one DENV-1, two DENV-2 and four DENV-3. DENV-3 sequenced in this study belong to genotype I . NS-1 gene of DENV-3 from 3 strains were sequenced and compared with 11 other sequence data from GenBank,

METHODS

1. Source of the viruses, serology and RT-PCR

Specimens were collected from hospitalized patients with suspected dengue infection in Jakarta within the period of February 2006 to march 2007. Serum samples were serologically examined by Hemaglutination inhibition test and rapid immunochromatographic assay (PanBio Inc., Brisbane, Australia). The sera were also tested by RT-PCR (Lanciotti, 1992) to see the presence and type of virus.

2. Virus isolation

Virus isolation was done by inoculating 50 ul of 1:10 dilution serum samples onto C6/36 cell line in 24 well plates (Yamada et al, 2002). Dengue viruses were propagated in C6/36 cells at room teperature for 7 days. The culture supernatants were harvested and centrifuged at 900 g for 5 minutes and then filtered through the syringe driven millex GV with 0.22 um filter unit (Millipore, Co. Bedford MA USA). Culture supernatants were collected and checked for the presence of dengue virus by Hemagglutination assay and RT-PCR (Lanciotti, 1992).

3. Determination of viral nucleotide sequences

To genotypically characterize the isolated viruses, nucleotide sequencing will be done. RNA were extracted from 140 ul of plasma using Viral RNA Isolation Kit (Qiagen, GmbH, Germany) or Viral RNA Isolation Kit (Roche Applied science, Mannheim Germany) according to the manufacturer's instruction. Complementary DNA (cDNA) strands were reverse-transcribed using Super Script II First Strand Synthesis System with Random hexanucleotide primer according to the manufacturer`s instructions (Invitrogen). Amplification of DNA fragments were performed according to the annealing temperature corresponding primers used and length of the expected product. After amplification and purification, the DNA were sent to the DNA sequencing facility at the Eijkman Institute, Jakarta. The sequences were determined using Taq Big Dye Deoxy Terminator Cycle sequencing kits.

RESULTS AND DISCUSSION

Patient characterization

From March to December 2006, 141 patient plasma were collected, consisting of 22 plasma from children (< 14 years old) and 119 plasma from adult (≥ 14 years old). From February to April 2007, 97 patient plasma samples were collected, consisting of 97 plasma from children and 50 plasma from adult. Of those samples, only 116 samples of 2006 have paired sera and 38 samples of 2007 have paired sera with 3-7 days interval. Hemaglutinition inhibition test were only done to 2006 specimens (table 2). For samples of year 2007, only RT-PCR and rapid chromatographic assay were done (table 3).

From HI results (table 1) can be seen that most of the patients had secondary infection (66.4%), 2 (1.7%) patients were negative, and 7 patients (7.7%) were indeterminate because the interval of their sera is less than 7 days.

Rapid immunochromatographic assay to 2007 samples showed that 11 samples were from primary infection, 66 samples were of secondary infection, and 19 samples were negative.

Tabel 1. Hemaglutination inhibition test result of samples isolated in 2006

Infection	N	%
Primary	5	4,3
Secondary	77	66,4
Primary/secondary	9	7,7

Presumptive	14	12,1
Indeterminate	9	7,7
Negative (not dengue)	2	1,7
Total	**116**	**100**

Table 2. Rapid immunochromatographic assay of year 2007 specimens

Age	Primary	Secondary	Negative	Total
< 14 y.o.	6	37	4	47
≥ 14 y.o	5	29	15	50
Total	11	66	19	47

Virus isolation

Viral isolation using C6/36 cell line was attempted. The results were unsatisfying. Viral isolation were successful only for several specimens. Attempt to isolate more viruses is now being done.

RT-PCR for determination of dengue serotype

In 2006, from 141 samples tested, 86 samples were RT-PCR positive. Among the positives, the most frequent was DEN-2 (50.0%), followed by DEN-3 (29.1%), DEN-1 (17.4%), and rarely DEN-4 (3,5%). In 2007, from 97 samples tested, 43 samples were RT-PCR positive. Among the positives, the most frequent was DEN-2 and DEN-3 (46.5% each), followed by, DEN-1 (4.7%), and rarely DEN-4 (2.3%) (Table 3.).

Table 3. Reverse Transcriptase PCR (RT-PCR) results of year 2006 and 2007 specimens.

		RT-PCR Positive					
Year	**Age**	**DEN-1**	**DEN-2**	**DEN-3**	**DEN-4**	**n positive/ n tested**	**%**
2006	< 14 y.o.	4	1	4	2	11/22	50.0%
	≥ 14 y.o.	11	42	21	1	75/119	63.0%
2007	< 14 y.o.	0	13	10	0	23/47	48.9%
	≥ 14 y.o.	2	7	10	1	20/50	40%

Table 4. Serotypes of dengue viruses and clinical grade in children and adult in 2007

Year	DV SEROTYPE*	Total number		Clinical grade (n)				
<14 y.0		n	%	DF	DHF-1	DHF-2	DHF-3	DSS
	Negative	24	51.0%	4	0	5	11	4
	DV-1	0	0	0	0	0	0	0
	DV-2	13	27.7%	0	1	5	7	0
	DV-3	10	21.3%	1	2	0	7	0

	DV-4	0	0	0	0	0	0	0
	Total	47	100%	5	3	10	25	4
≥14 y.o	Negative	30	60%	2	21	7	0	0
	DV-1	2	4%	0	1	1	0	0
	DV-2	7	14%	0	4	2	0	0
	DV-3	10	20%	10	3	7	0	0
	DV-4	1	2%	0	0	1	0	0
	Total	50	100%	12	29	18	0	0

* Virus serotypes based on RT-PCR

NUCLEOTIDE AND AMINO ACID SEQUENCE ANALYSIS

Envelope region of DENV-1, DENV-2 and DENV-3

In this study envelope genes of one DENV-1. two DENV-2 and four DENV-4 strins were sequenced (Table 3.). Sequencing of year 2007 strains is now in progress. Phylogenetic analysis of DENV-1 was done according to the genotype classification by Goncalves (Goncalves, *et al.,* 2002). Thirty six strains including 2 strains from Indonesia and 30 other strains data obtained from Genbank were included in the analysis. The DENV-1 sequenced in this study was identified as genotype IV, together with the strains of 1980 and 2002 (Figure 1.).

Phylogenetic analysis of DENV-2 was done according to the genotype classification by Huang (Huang, *et al.,* 2007). Forty-three strains data were included in the analysis.

Two strains sequenced in this study, as well as 5 other strains from Indonesia and 36 from other countries data obtained from Genbank were included. The DENV-2 sequenced in this study were identified as cosmopolitan genotype, together with the strains of 1998 and 2004 (Figure 2.).

Phylogenetic analysis of DENV-3 was done according to the genotype classification by Zhang (Zhang, *et al.,* 2005). Thirty-seven strains data were included in the analysis. Four strains sequenced in this study, as well as 9 strains from Indonesia and 24 other strains data obtained from Genbank were included. The DENV-3 sequenced in this study were identified as genotype I (Figure 3.).

Pairwise sequence homology analysis of DENV-1 showed that the homology of nucleotide sequences among strains analyzed were in range of 90.8 to 98.8%, while the homology of amino acids were 96.8 to 100%which showed that the mutations are mostly silent. Among Indonesian strain, the homology of nucleotide were 94.9 to 99.0% and of amino acid were 98.3 to 99.0%.

Pairwise sequence homology analisis of DENV-3 was only done to compare strains from Thailand and Indonesia. Among Indonesia isolates, homology of nucleotide were inrange of 94.5 to 99.4% and of amino acids were 97.7 to 100%.

NS-1 region of DENV-3.

Since NS-1 is becoming important as a target of dengue diagnostic tools, we analyse the NS-1 region of strains DS 0002/06, DS 029/06 and DSA 02/06 and compared them with 14 other strains data from the GenBank. The results showed that isolates from Thailand belong to one cluster, and strains from Indonesia belong to one other cluster.

The highest nucleotide homology was between strain 98901517 (DHF) and strain 98901437 (DSS), and the lowest was between strain KPS/551 (DF) and strain DS 029/06 (DHF).

We also analysed four B-cell epitopes reported by Roehrig et al.(1997) and Falconar et al.(1997), i.e. epitopes LD2 (aa 25-33 : VHTWTEQYK), epitope 24A (aa 61-69: TRMENLLWKQ), epitope LX1 (aa111-119 or 113-121: LKYSWKTWGKA) and epitope 24C (aa. 299-307 or 301-309: RTTTVSGKLIH). We found that those four epitopes were conserved in all 14 strains analyzed.

Relation of amino acid sequence with disease severity

With our limited data we cannot see any specific mutations related to severe cases.

Table 5. Strains of DENV sequenced in this study

No	Strain	Year isolated	Clinical manifestation	Serotype
1.	DS27/06	2006	DHF-I	DEN-1
2	DS31/06	2006	DHF-I	DEN-2
3	DS24/06	2006	DF	DEN-2
4	DS 002/06	2006	DF	DEN-3
5	DS 029/06	2006	DHF-II	DEN-3
6	DSA 02/06	2006	DSS	DEN-3
7	17/04	2004	DHF-II	DEN-3

Figure 1. A Phylogenetic tree of DENV-1. Maximum likelihood trees showing the phylogenetic relationships among 36 strains of DENV-1. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the scheme of Goncalves, *et al.*, 2002. The name of isolates refer to country of origin, year of isolation and also diseases severity if available in GeneBank.

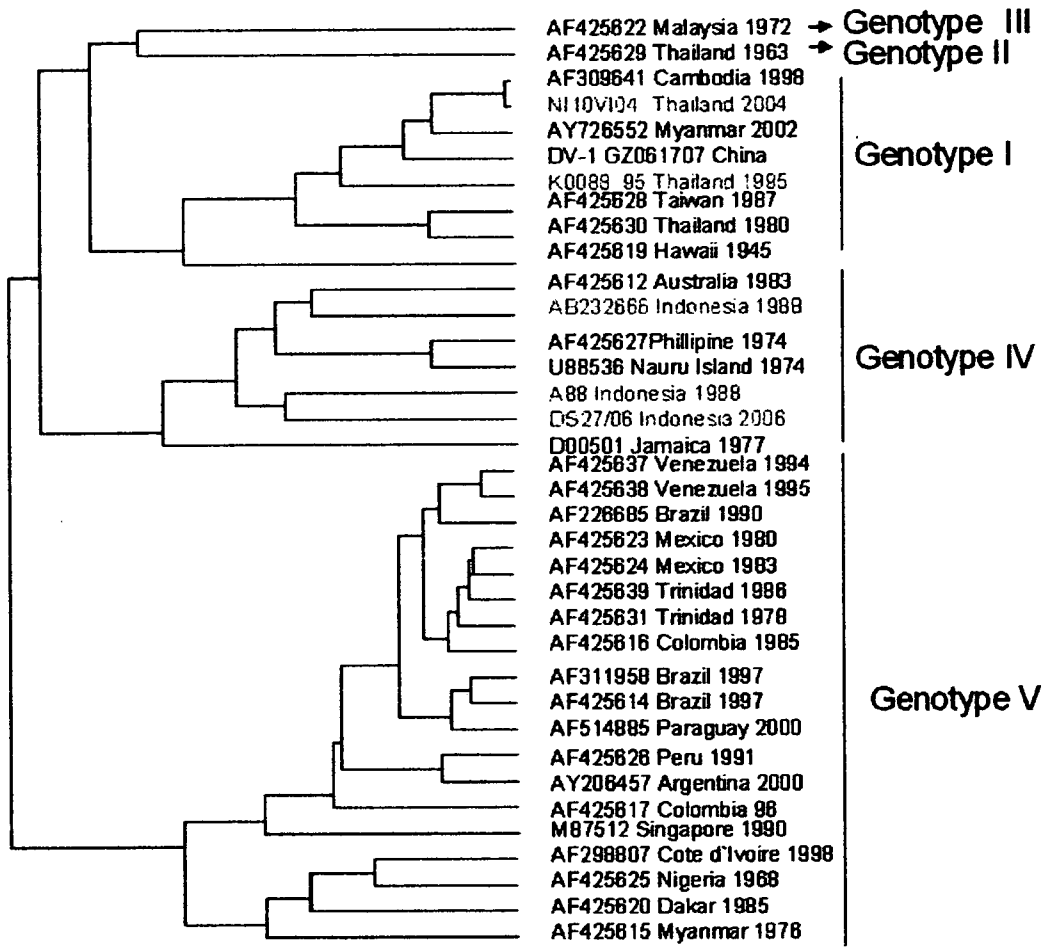


Figure 2. A Phylogenetic tree of DENV-2. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the

scheme of Zhang, *et al.*, 2005. Roman numerals denote the different genotypes of DV-3. An asterisk (*) indicates the strains sequenced in the present study. The name of isolates refer to country of origin, year of isolation and also diseases severity if available in GeneBank.

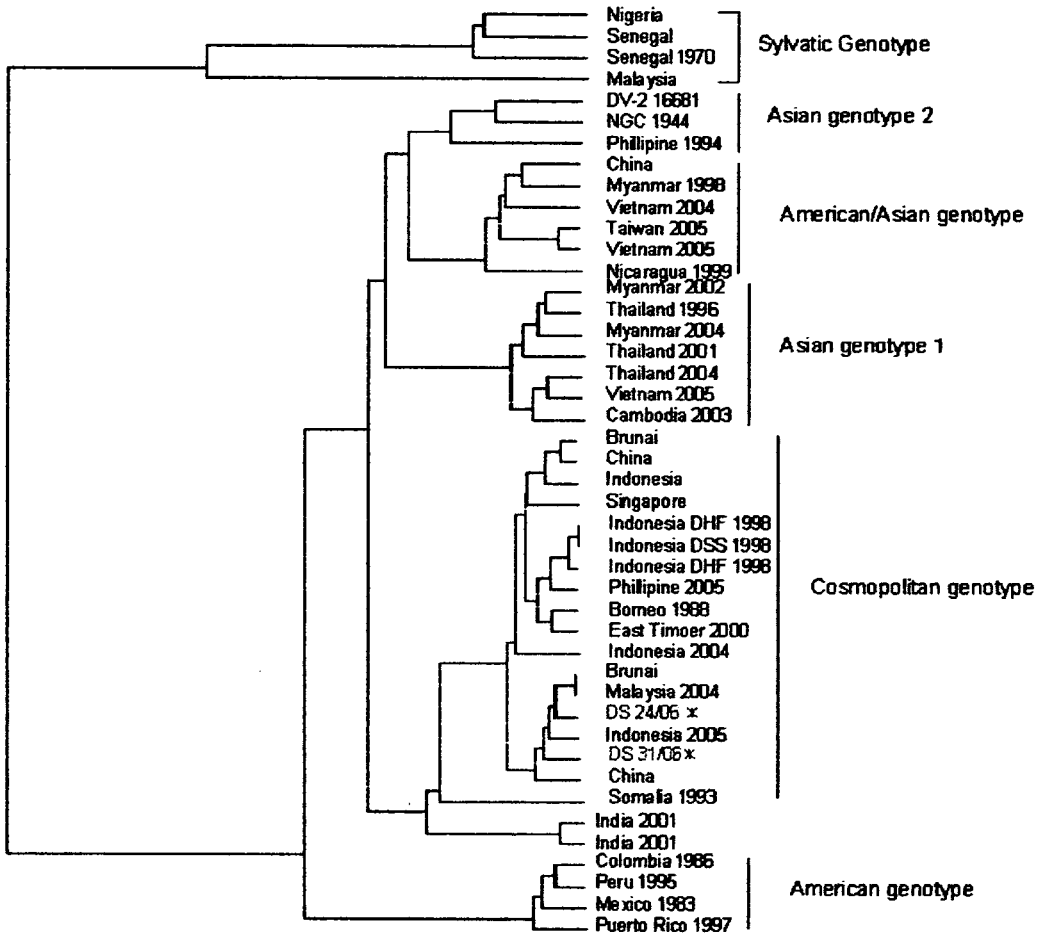


Figure 3. A Phylogenetic tree of DENV-3. The analysis was based on nucleotide sequences of the E gene. The genotype clusters are labeled according to the scheme of Zhang, *et al.*, 2005. The name of isolates refer to country of origin, year of isolation and also diseases severity if available in GeneBank.

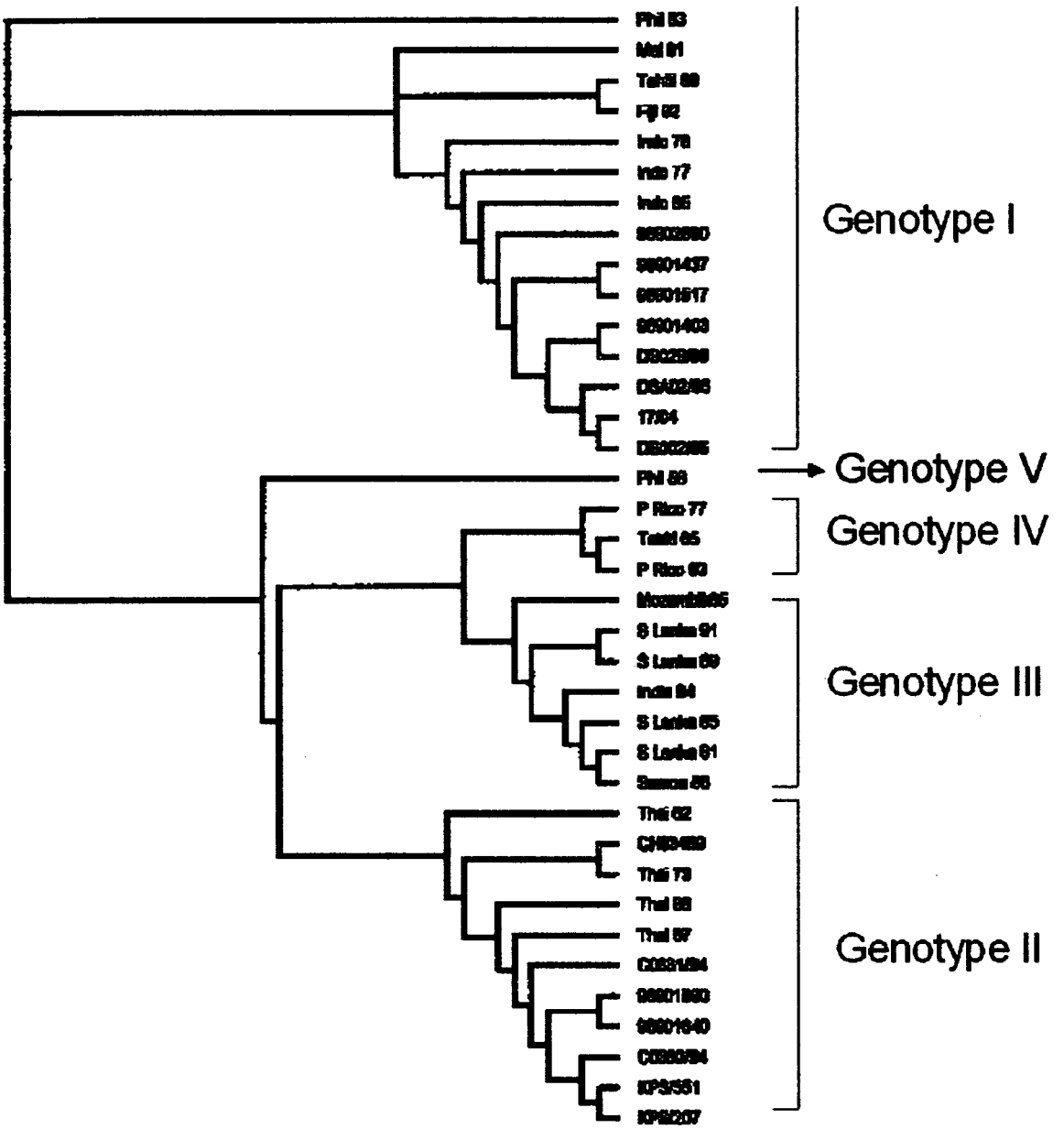
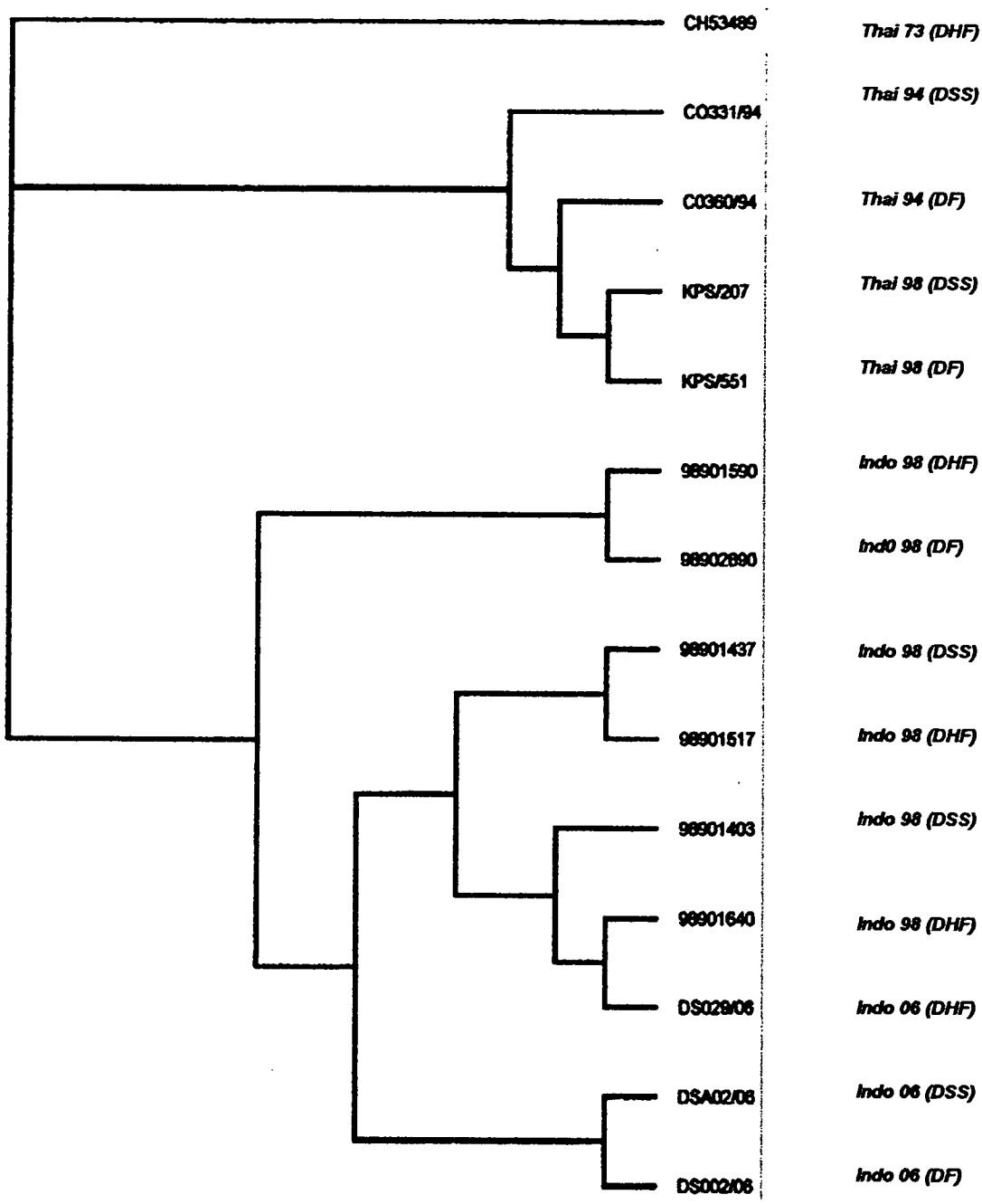


Figure 4. Phylogenetic tree of NS-1 region of DENV-3


CH53489
Thai 73 (DHF)
CO331/94
Thai 94 (DSS)
C0360/94
Thai 94 (DF)
KPS/207
Thai 98 (DSS)
KPS/551
Thai 98 (DF)
98901590
Indo 98 (DHF)
98902690
Indo 98 (DF)
98901437
Indo 98 (DSS)
98901517
Indo 98 (DHF)
98901403
Indo 98 (DSS)
98901640
Indo 98 (DHF)
DS029/06
Indo 06 (DHF)
DSA02/06
Indo 06 (DSS)
DS002/06
Indo 06 (DF)

Pairwise sequence homology of DENV-1

% identity of Nucleotide	% identity of polypeptide																	Genotype
	Indo 06	Indo 02	Indo 88	Vene 84	Thai 04	Thai 95	Thai 80	Aus 83	Trin 86	Chin 06	Ivory 85	Hawai 45	Colm 96	Myan 02	Myan 76	Sing 90	Malay 72	
Indo06		98.4	99.0	98.0	97.8	97.6	98.0	98.0	98.2	97.8	97.2	97.8	97.6	97.8	97.6	97.6	97.8	IV
Indo02	94.9		98.3	98.3	97.2	97.0	97.4	98.0	98.0	97.2	97.0	97.2	97.4	97.2	97.4	97.0	98.0	IV
Indo88	96.4	95.6		98.2	97.4	97.2	97.6	98.0	98.2	97.4	97.2	97.8	97.6	97.4	97.6	97.6	98.0	IV
Vene94	91.2	91.1	91.2		97.2	97.0	97.0	99.6	97.2	98.4	97.8	99.0	98.0	97.2	99.0	98.6	98.0	V
Thai04	91.1	91.4	91.6	91.9		99.4	98.6	97.0	97.6	100	96.8	97.2	97.0	100	97.4	97.4	97.6	I
Thai95	91.7	90.9	91.7	91.3	97.3		98.4	96.8	97.4	99.4	96.6	97.0	96.8	99.4	97.2	97.2	97.4	I
Thai80	92.0	91.6	92.2	91.9	97.2	97.1		97.0	97.8	98.6	96.8	97.4	97.2	98.6	97.2	97.2	97.8	I
Aus83	95.6	96.8	96.0	91.5	92.5	92.2	92.5		97.2	97.0	96.2	96.8	96.6	97.0	96.6	96.8	97.2	IV
Trin86	91.9	91.8	91.7	98.7	92.4	91.7	92.3	92.3		97.6	98.8	98.0	99.4	97.6	99.4	99.0	98.4	IV
Chin06	91.9	91.3	91.5	91.7	98.3	97.4	96.8	92.3	92.2		96.8	97.2	96.7	100	97.4	97.4	97.6	I
Ivory85	90.8	90.3	91.0	94.9	91.3	91.1	91.7	91.0	95.3	91.7		96.8	98.2	96.8	98.6	97.8	97.2	
Hawai45	93.3	92.6	93.5	93.5	95.2	94.5	95.6	93.6	94.0	94.6	92.9		97.4	97.2	97.6	97.4	97.6	I
Colm96	91.2	91.2	91.0	97.4	91.5	91.0	91.7	91.4	98.2	91.3	94.1	93.0		97.0	99.2	98.4	97.8	I
Myan02	92.2	91.1	91.9	91.4	98.8	97.3	96.9	92.3	91.9	98.3	91.3	94.9	91.0		97.4	97.4	97.6	I

Myan76	91.4	91.4	91.7	96.2	92.6	92.3	92.6	91.9	96.7	92.7	95.9	93.8	95.8	92.1		98.4	97.8	V
Sing90	91.7	90.9	91.4	96.5	93.1	92.5	92.7	92.0	97.2	92.9	94.0	93.6	96.3	92.5	95.4		97.4	V
Malay72	92.1	92.3	92.4	92.6	92.5	92.0	93.0	93.1	93.3	92.3	92.5	94.3	92.4	92.1	93.2	92.3		III

	% identity of polypeptide														
% identity of Nucleotide	CH 53489 (DHF)	DS 002/06 (DF)	DS 029/06 (DHF)	DSA 02/06 (DSS)	17/04 (DHF)	CO360 /94 (DF)	KPS/ 551 (DF)	9890 2890 (DF)	9890 1640 (DHF)	9890 1590 (DHF)	9890 1517 (DHF)	CO331 /94 (DSS)	KPS/ 207 (DSS)	9890 1403 (DSS)	9890 1437 (DSS)
CH53489 (DHF)		97,7	97,9	97,7	98,1	97,7	98,1	98,5	97,9	97,9	97,9	98,1	97,9	97,9	97,9
DS 002/06 (DF)	93,5		99,7	99,5	99,5	96,5	97,3	99,1	97,7	97,9	99,7	97,7	97,1	99,7	99,7
DS 029/06 (DHF)	93,3	98,3		99,7	99,7	96,7	97,5	99,3	97,9	98,1	100	97,9	97,3	100	100
DSA 02/06 (DSS)	93,4	99,1	98,5		99,5	96,5	97,3	99,1	97,7	97,9	99,7	97,7	97,1	99,7	99,7
17/04 (DHF)	93,5	99,4	98,2	98,9		96,7	97,5	99,5	97,9	98,1	99,7	97,9	97,3	99,7	99,7
CO360/94 (DF)	97,1	92,5	92,4	92,4	92,5		98,7	97,1	98,5	98,5	96,7	97,9	98,5	96,7	96,7
KPS/551 (DF)	97,0	92,6	92,6	92,6	92,6	99,3		97,9	99,3	99,3	97,5	98,7	99,7	97,5	97,5
98902890 (DF)	93,9	97,2	97,2	97,4	97,2	93,0	93,1		97,9	98,1	99,3	98,3	97,7	99,3	99,3
98901640 (DHF)	95,8	94,5	94,8	94,5	94,5	97,2	97,5	94,0		99,5	97,9	98,7	99,1	97,9	97,9
98901590 (DHF)	95,8	94,7	94,9	94,6	94,7	97,3	97,6	94,3	99,6		98,1	98,5	99,1	98,1	98,1
98901517	93,9	99,0	98,6	98,8	98,9	92,9	93,1	97,7	94,8	94,9		97,9	97,3	100	100

(DHF)														
CO331/94 (DSS)	97,3	93,0	93,1	92,9	93,0	98,5	98,7	93,5	96,8	96,8	93,4		98,5	97,9	97,9
KPS/207 (DSS)	97,0	92,7	92,7	92,6	92,7	99,2	99,7	93,2	97,4	97,5	93,1	98,7		97,3	97,3
98901403 (DSS)	93,7	99,0	98,9	98,9	98,9	92,8	92,9	97,7	94,8	94,9	99,3	93,4	93,0		100
98901437 (DSS)	93,9	99,1	98,7	98,9	98,9	93,0	93,1	97,7	94,9	95,0	99,9	93,5	93,2	99,3	

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Table 6. Primers used for PCR and sequencing.

Primers	Nucleotide sequence
D3-846s	CCCTATTTCTTGCCCATTACA
D3-1204s	CGTGTGTAAGCATACATACG
D3-1715s	GCACTGACAGGAGCTTACAGA
D3-1996s	AGTGGTGACCAAGAAGGA
D3- NS1 1s	CCGCTGGGATCCGACATGGGGTGTGTCATA
D3-2698s	GGTCCTAGAGCAAGGGAA
D3-3078s	CTGCACATGGCCAAAATCAC
D3-1056c	TTCTCGAGGAATTCTGCTGAGGCTAGAGACTTTA
D3-1192c	CTGCTCCTCAGGTAGAAT
D3-1911c	CCTTTGTACTCAACCTTAATGA
D3-2716c	TTTCCCTTGCTCTAAGACCC
D3-3367c	AAGTGTGCACGAGCGGCAAC
D3-3833c	AGCAAATTCCATTCGCCATTTG
D1 3550c	GGATCTCATTACCTCTTCGA
D1-612s	