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Periodic re-emergence of endemic strains with strong epidemic potential—A proposed explanation for the 2004 Indonesian dengue epidemic☆

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Abstract

Indonesia experienced a severe dengue epidemic in the first quarter of 2004 with 58,301 cases and 658 deaths reported to the WHO. All four dengue virus (DENV) serotypes were detected, with DENV-3 the predominant strain. To ascertain the molecular epidemiology of the DENV associated with the epidemic, complete genomes of 15 isolates were sequenced from patient serum collected in Jakarta during the epidemic, and two historical DENV-3 isolates from previous epidemics in 1988 and 1998 were selectively sequenced for comparative studies. Phylogenetic trees for all four serotypes indicate the viruses are endemic strains that have been circulating in Indonesia for a few decades. Whole-genome phylogeny showed the 2004 DENV-3 isolates share high similarity with those isolated in 1998 during a major epidemic in Sumatra. Together these subtype I DENV-3 strains form a Sumatran-Javan clade with demonstrated epidemic potential. No newly-acquired amino acid mutations were found while comparing genomes from the two epidemics. This suggests re-emergence of little-changed endemic strains as causative agents of the epidemic in 2004. Notably, the molecular evidence rules out change in the viral genomes as the trigger of the epidemic.

Keywords: Dengue; Epidemic; Molecular epidemiology; Genome

1. Introduction

Dengue fever is a mosquito-born disease with high public health impact that is estimated to affect nearly 50 million people worldwide each year (Monath, 1994; Gubler and Clark, 1995; WHO, 2002). Dengue fever is endemic throughout most of the tropical areas of the world, coincident with the distribution pattern of its mosquito vectors. Dengue disease can manifest in the form of the mild dengue fever (DF), or the more severe and potentially fatal dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) which has a fatality rate as high as 10–15% depending on the availability of healthcare (Gubler, 2002). Currently there is no vaccine or therapeutic agent available against dengue fever.

The causative agent of dengue fever, the dengue virus (DENV), is transmitted to humans by infected females of the mosquito vectors *Aedes aegypti* and *Aedes albopictus*. DENV is a single-stranded positive-sense RNA virus of the genus *Flavivirus*. The ~10.7 kb DENV genome encodes three structural (capsid, pre/membrane and envelope) and seven

☆ The GenBank accession numbers of the sequences reported in this paper are AY858035–AY858050, AY858983, AY662691 and AY947539.

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non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in a single open reading frame. DENV is divided into four antigenically-related serotypes denoted as DENV-1, DENV-2, DENV-3 and DENV-4. Each serotype is sufficiently different that infection with one does not provide complete cross protection for the other three. In a scheme first proposed by Rico-Hesse (1990), DENV can be further divided into intra-serotypic categories called interchangeably as *subtypes* or *genotypes* based on their nucleotide sequence data. Subtype determination via phylogenetic means are often used to infer the phylogeny and to monitor the spread of virus strains (Chungue et al., 1995; Rico-Hesse et al., 1997; Messer et al., 2003).

South East Asia has been a focal point of dengue activity since 1950s when DHF was first described. Indonesia, the largest country in the region, has experienced periodic outbreaks of dengue since 1968 with increasing numbers of infections and severity (Sumarmo, 1987). Major dengue epidemics occurred in Indonesia in 1998, during which 72,133 cases and 1414 deaths were reported, and again in 2004 with more than 58,301 cases and 658 deaths in the first 4 months of the year (WHO, May 11, 2004). In the 2004 epidemic, running from the start of January until early May, cases were reported in all provinces across the Indonesian archipelago. The densely populated capital city of Jakarta, with a population of over 16 million people, was the hardest hit in terms of reported cases and deaths.

Availability of viral genome sequence data will no doubt contribute to a better understanding of the molecular evolution and epidemiology of DENV, especially in a country with a long history of dengue infections such as Indonesia. To this end, we sequenced the genome of 15 DENV isolates collected from hospitals around Jakarta during the 2004 epidemic. In addition, two samples collected during previous epidemics in Jakarta in 1988 and 1998 were also sequenced to ascertain the ancestry of the DENV associated with the 2004 epidemic. Surprisingly, Indonesia does not seem to experience importation of DENV strains despite its proximity to busy international waterway that is the Malacca Strait, and to Thailand, the other country with a long history of dengue infections. Rather, our results point to periodic re-emergence of endemic strains with a demonstrated epidemic potential as the most likely cause of the 2004 epidemic.

2. Methods

2.1. Virus sample collection and preparation

Sixty nine patient serum samples were collected at eight hospitals in the Greater Jakarta area during the epidemic. Blood samples were taken from patients showing symptoms of dengue fever, as part of routine surveillance, after administering a consent form designed by the Center for Research and Program Development on Disease Control in Jakarta. The presence of acute dengue infection was confirmed by serological tests at the CDC in Jakarta and the serum samples were stored at −80 °C until use. The two historical DENV-3 samples collected in Jakarta from 1988 (den3_88) and 1998 (den3_98) were provided by the US Naval Medical Research Unit No 2 (NAMRU-2).

2.2. Virus propagation, RNA extraction and virus typing using RT-PCR

Virus samples were propagated in C6/36 *Aedes albopictus* gut cells and viral RNA was extracted using QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's protocol. Serotype of the isolates was inferred via RT-PCR prior to sequencing. Dengue viral RNA was first reverse-transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen) and random hexamers, followed by amplification using *Taq* DNA polymerase (Roche). Serotyping was performed using a slightly modified version of the multiplex RT-PCR protocol (Lanciotti et al., 1992) and uses primers described by Seah et al. (1995) that distinguishes the four dengue serotypes by PCR product size.

2.3. Primer design

Four consensus sequences, one for each serotype, were derived from alignments of published dengue genomes in GenBank. Known recombinant and artificially-mutated strains were excluded. For DENV-4, partial sequences were included in the alignment as only two complete genomes were publicly available at the time. Forward and reverse primers were designed using Vector NTI Suite 9 (Invitrogen Bioinformatics) to give overlapping sequence traces. Criteria of primers design include low number of degenerate positions, no degeneracy in the final three bases, a T_m between 55 and 65 °C and a GC content of 35–60%. The binding positions for the primers on the consensus sequence are shown in Appendix A and the sequence of the sequencing and amplification primers are shown in Appendix B.

2.4. Viral cDNA amplification, sequencing, assembly and annotation

cDNA templates were generated from viral RNA using five serotype-specific priming primers at 2 pmol each with the SuperScript III reverse transcriptase. Due to the linear nature of the viral genome the 5′ and 3′ extremes of the cDNA are primer sequence. The cDNA template was then amplified using *Pfu* Turbo DNA polymerase (Stratagene) in five separate reactions to generate five slightly overlapping PCR fragments. The PCR fragments were separated on 1% agarose gels, excised, and the DNA purified using the QIAquick® PCR Purification Kit (QIAGEN). The purified PCR fragments, ranging from 2.1 to 2.8 kb, were then sent for automated capillary sequencing using 50 serotype-specific sequencing primers. Sequencing was done with a 3730xl DNA Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Contigs were assembled using SeqScape version 2.5 (Applied Biosystems) and the assembled genome sequences were aligned to other relevant DENV

sequences using ClustalW-MPI (Li, 2003), followed by manual editing of the alignments.

2.5. Strain nomenclature

Details of the genomes sequenced in this study are listed in Table 1. The first two alphabets of the strain names refer to the identity of the hospitals from which the samples were collected. The eight hospitals were abbreviated as follows: BA for Budi Asih and PH for Persahabatan, two hospitals in the city (*kota*) of East Jakarta. Similarly, SW (Sumber Waras) is in West Jakarta, KJ (Kodja) and PI (Infectious Disease Sulianti Saroso) in North Jakarta, SC (St Carolus) in Central Jakarta, while both FW (Fatmawati) and TB (Tebet) are in South Jakarta. The genome sequences were deposited in the GenBank database with the accession numbers AY858035–AY858050 and AY858983. Both AY662691, a 2003 DF-causing strain from Singapore, and AY947539, the H241 prototype DENV-4, were previously sequenced in 2003 at NITD. Sequences and annotation of the reported genomes are also available at the DengueInfo database (http://www.dengueinfo.org/) (Schreiber et al., 2007).

2.6. Phylogenetic analysis of the DENV genomes

All phylogenetic trees were constructed from aligned nucleotide sequence data using the maximum likelihood (ML) method implemented in PAUP* (Swofford, 2002), with the best-fit model of nucleotide substitution (GTR+G) selected by Akaike Information Criterion (AIC) implemented in ModelTest (Posada and Crandall, 1998). Branch topology was verified by generating 1000 bootstraps using TBR branch-swapping and the scores on tree nodes represent the number of bootstrap replicates (presented in percentage) supporting each node. The length of the tree branches is proportional to the number of nucleotide changes. All trees are midpoint rooted and strains sequenced in this study are underlined for clarity.

Strain datasets used to construct the phylogenetic trees for the purpose of subtype determination were selected to provide good coverage of the known diversity of the four dengue serotypes. Subsequently, published complete genomes of DENV were collected from the DengueInfo database (Schreiber et al., 2007) to build genome trees for each of the serotypes. Information on the year of isolation, country of origin, and clinical outcome of the strains (DF, DHF or DSS) was collected, when available, from GenBank records or through personal communication with submitters of the records.

2.7. Clade-specific mutations of the DENV-3 isolates

Clades which are phylogenetically relevant to the Indonesian strains and contain sufficient amount of genome sequence data were then identified and selected for further comparative sequence analysis. Deduced amino acid sequences for subtype I DENV-3 genomes were divided into two groups: those belonging to the Sumatran-Javan lineage (including the slightly more distantly-related Timor Leste strains) and those that are not. The χ^2 test was used to identify residues that are significantly different between the two groups at the 0.05 significance level. The identified residues were submitted to SIFT (http://blocks.fhcrc.org/sift/SIFT.html), a tool that uses sequence homology to predict the effects of amino acid substitutions on protein function (Ng and Henikoff, 2001). The database used by SIFT was SWISS-PROT 51.3 and TREMBL 34.3, with the median conservation of sequences set at 3.00. Conservative and non-conservative amino acid substitutions were defined according to the BLOSUM62 matrix (Henikoff and Henikoff, 1992) with changes having a positive or neutral value in the matrix considered as conservative whereas those with a negative value considered as non-conservative. Similar analyses for the other DENV serotypes were not pursued due to a lack of closely related genome sequences for comparison (data not shown).

Table 1
The GenBank accession, strain name, serotype, year of isolation, sequence length and patient-related information of dengue genomes sequenced in this study

GenBank accession	Strain name	Serotype	Year isolated	Length	Severity	Patient age	Patient sex
AY858035	BA05i	DENV-2	2004	10723	DF	2	M
AY858036	TB61i	DENV-2	2004	10723	–	–	–
AY858037	BA51	DENV-3	2004	10707	DF	14	M
AY858038	den3_88	DENV-3	1988	10707	–	–	–
AY858039	den3_98	DENV-3	1998	10707	–	–	–
AY858040	FW01	DENV-3	2004	10706	DF + HM	33	M
AY858041	FW06	DENV-3	2004	10707	–	–	–
AY858042	KJ30i	DENV-3	2004	10707	–	–	–
AY858043	KJ46	DENV-3	2004	10706	DHF-I	18	F
AY858044	KJ71	DENV-3	2004	10707	–	–	–
AY858045	PH86	DENV-3	2004	10707	DF	15	M
AY858046	PI64	DENV-3	2004	10707	DHF-I	31	M
AY858047	TB16	DENV-3	2004	10707	DF	59	M
AY858048	TB55i	DENV-3	2004	10673	–	–	–
AY858049	SW36i	DENV-4	2004	10114	DHF-I	12	F
AY858050	SW38i	DENV-4	2004	10516	DHF-I	30	F
AY858983	SC01	DENV-1	2004	7455	DF	7	M

The disease severity for seven cases was not evaluated by the admitting hospitals. Key: DF, dengue fever; HM, haemorrhagic manifestations; DHF-I, dengue haemorrhage fever grade I according to WHO guidelines, i.e. patients with positive tourniquet test, thrombocytopenia and plasma leakage.

2.8. Site-specific selection pressures

Site-specific selection pressures acting on the DENV-3 coding sequences were determined using the HyPhy package, implemented as a Web application at http://www.datamonkey.org/ (Pond and Frost, 2005a,b), using the single likelihood ancestor counting (SLAC) method (Pond and Frost, 2005a,b) and the General Reversible Model nucleotide substitution model.

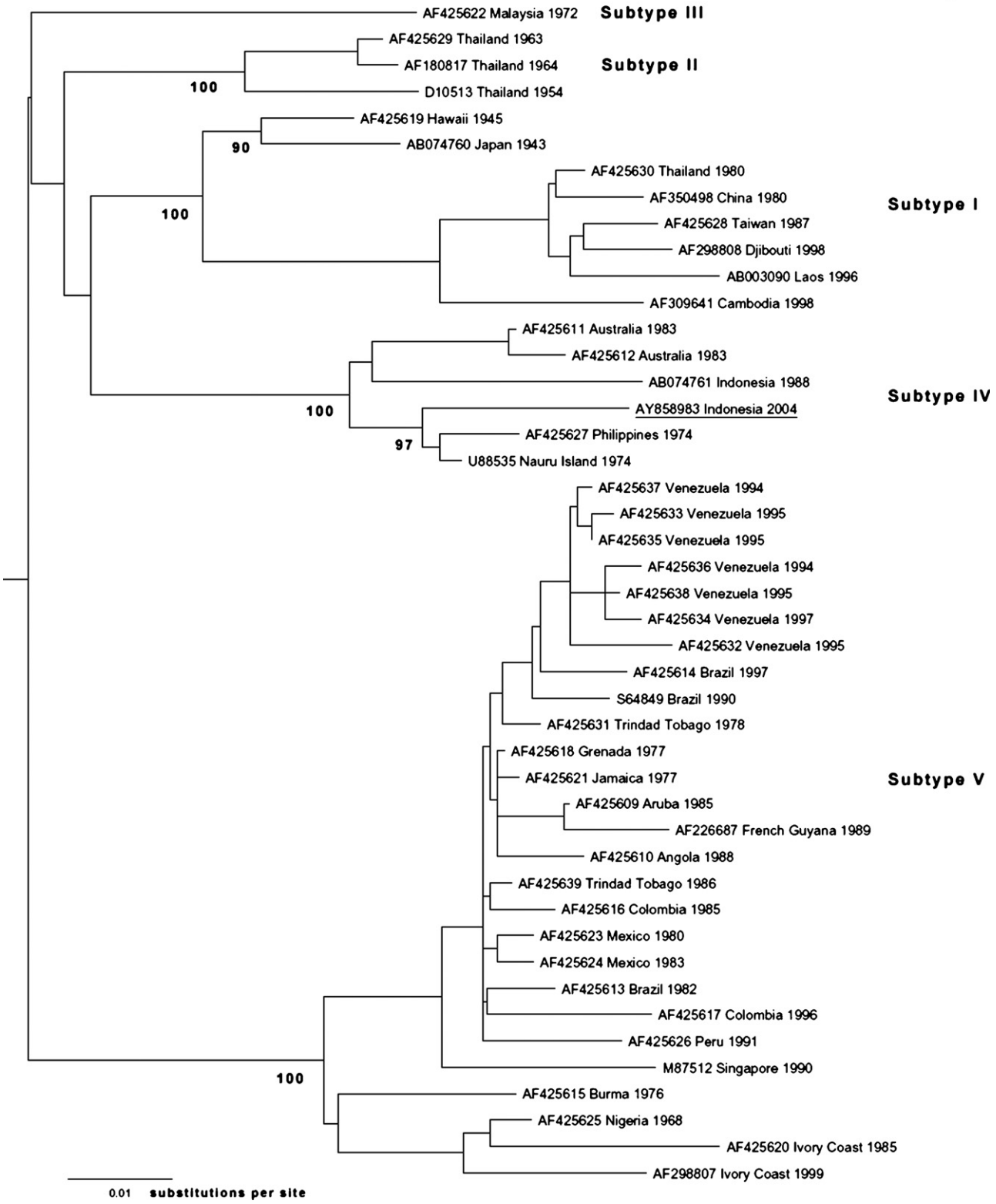

Fig. 1. Phylogenetic tree of DENV-1 based on the complete nucleotide sequences of the E gene (1485 bases). The leaves are labeled with the GenBank accession number, country of isolation, and year of isolation. The dataset used is identical to the one used by Goncalvez et al. (2002) with the addition of AY858983, which fall within subtype IV of DENV-1.

3. Results

3.1. Genome sequencing of dengue isolates

The genomes of 15 DENV isolates were successfully sequenced out of the 69 serum samples collected in Jakarta, Indonesia during the 2004 epidemic. Ten of the genomes were found to be DENV-3, along with two DENV-2, two DENV-4 and one DENV-1. In the remaining cases, there was insufficient virus in the serum samples to allow viral propagation or RNA extraction. Two historical Indonesian dengue samples collected in 1988 and 1998 (den3_88 and den3_98) were similarly

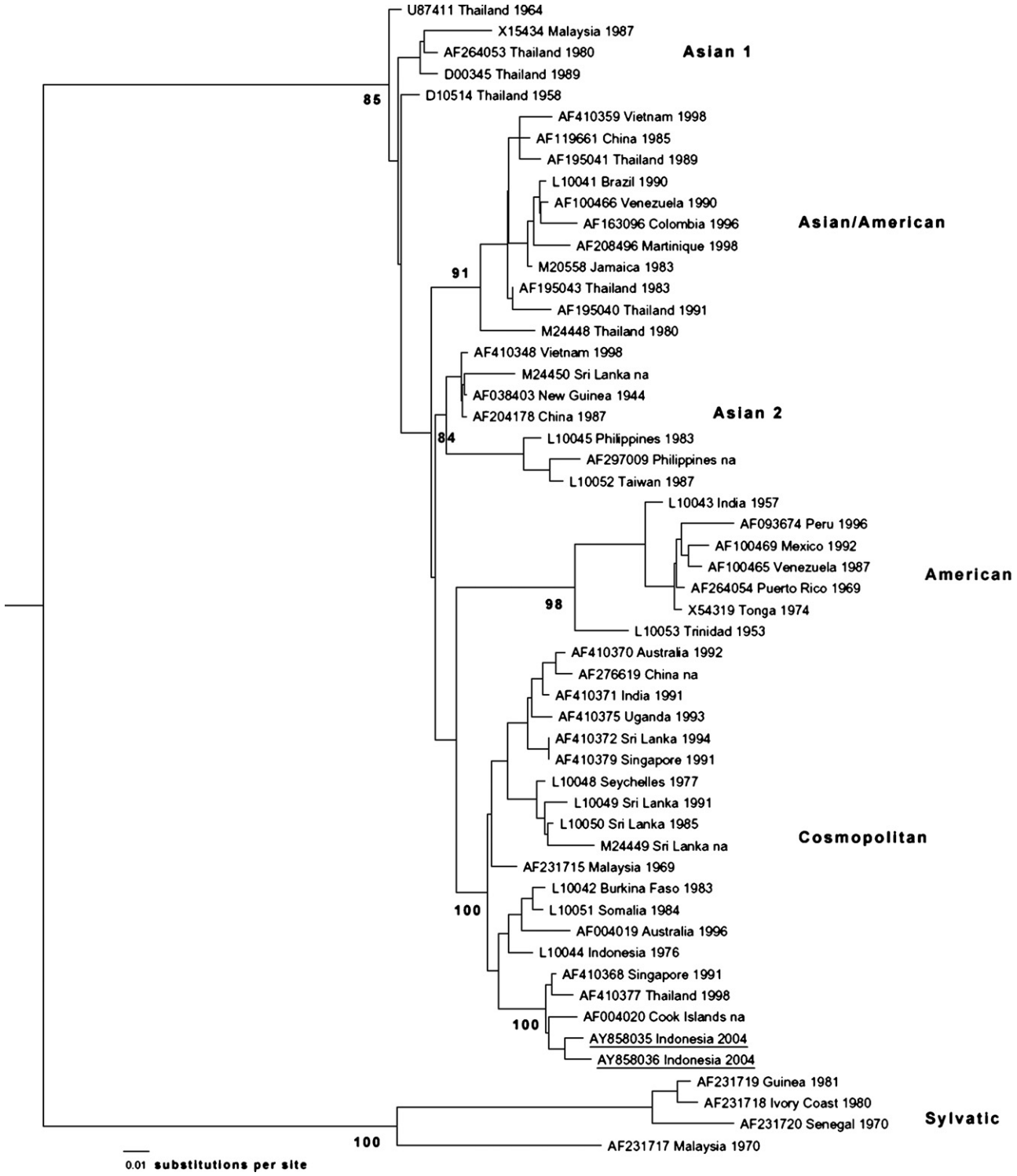

Fig. 2. Phylogenetic tree of DENV-2 based on the complete nucleotide sequences of the E gene (1485 bases). The leaves are labeled with the GenBank accession number, country of isolation, and year of isolation. The dataset used is a subset of the one used by Twiddy et al. (2002), comprising just 52 of the original 147 strains, and with the addition of AY858035 and AY858036. The two 2004 Indonesian strains sequenced in this study fall within the ''Cosmopolitan'' subtype of DENV-2.

sequenced. The two samples were selected to represent the two other major dengue epidemics reported in Indonesia in 1988 and 1998 (Corwin et al., 2001; WHO, May 11, 2004; Suwandono et al., 2006).

The final length obtained for each genome and other relevant details are listed in Table 1. The solitary DENV-1 genome was sequenced apart from the last 2.7 kb which were recalcitrant to repeated amplification attempts. Similarly, approximately 550

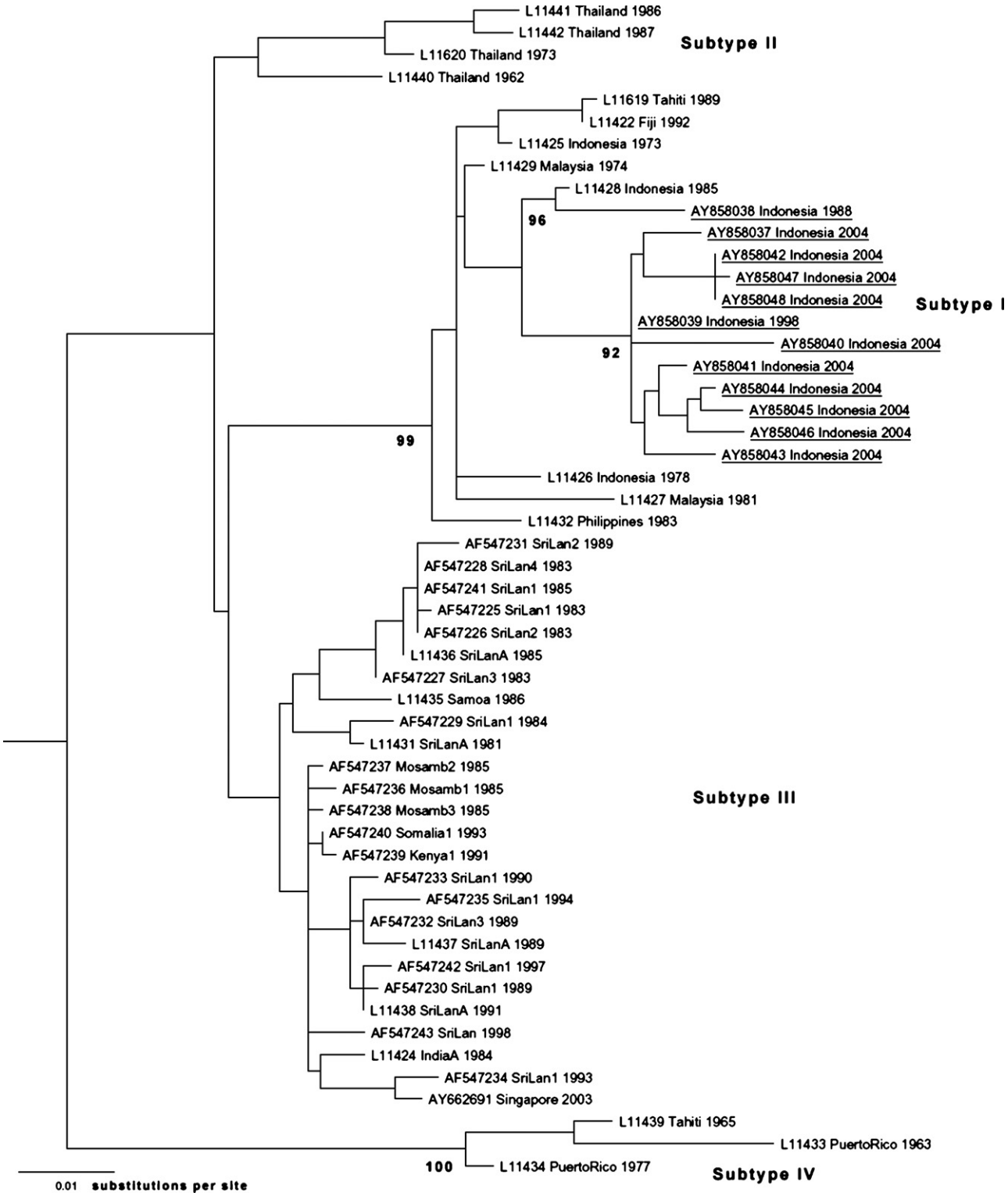

Fig. 3. Phylogenetic tree of DENV-4 based on the complete nucleotide sequences of the E gene (1485 bases). The leaves are labeled with the GenBank accession number, country of isolation, and year of isolation. The dataset used is identical to the one used by Lanciotti et al. (1997) with the addition of AY858049, AY858050 and AY947539. The two 2004 Indonesian strains sequenced in this study fall within subtype II of DENV-4.

bases at the 5′ end for SW36i could not be obtained. Due to the small number of published DENV-4 genomes in GenBank, it was not possible to design redundant primers that encompass the variability typically seen within a dengue serotype. For this reason, the two DENV-4 isolates were subsequently sequenced using a primer walking approach.

3.2. Phylogeny of the DENV-1, DENV-2 and DENV-4 isolates

Phylogeny of the sequenced DENV-1, DENV-2 and DENV-4 isolates were inferred based on the nucleotide sequences of the complete envelope (E) gene. This choice is due to the availability of a greater diversity of E gene sequences for these serotypes compared to genome sequences.

The E gene phylogenetic tree of DENV-1 places the solitary DENV-1 isolate in this study, SC01 (AY858983), in subtype IV as described by Goncalvez et al. (2002) (Fig. 1). This group primarily contains isolates from Australia and the West Pacific islands (Indonesia and the Philippines included) – a result that suggests SC01 is an endemic strain in Indonesia.

The E gene phylogenetic tree for DENV-2 (Fig. 2) places BA05i (AY858035) and TB61i (AY858036) in the Cosmopolitan subtype according to the classification scheme proposed by Twiddy et al. (2002). This subtype has previously been associated with DHF/DSS (Leitmeyer et al., 1999) and as the name suggests, is a diverse lineage that contains viruses from India, Southeast Asia, Africa, the Middle East and Australia. This means the two DENV-2 strains are likely to be regionally endemic strains.

The E gene phylogenetic tree for DENV-4 (Fig. 3) shows both SW36i (AY858049) and SW38i (AY858050) as belonging to subtype II, a genetic lineage that contains viruses from Indonesia, the South Pacific and the Western hemisphere (Chungue et al., 1995; Lanciotti et al., 1997). Another Indonesian strain isolated in 1973 is also found in the clade

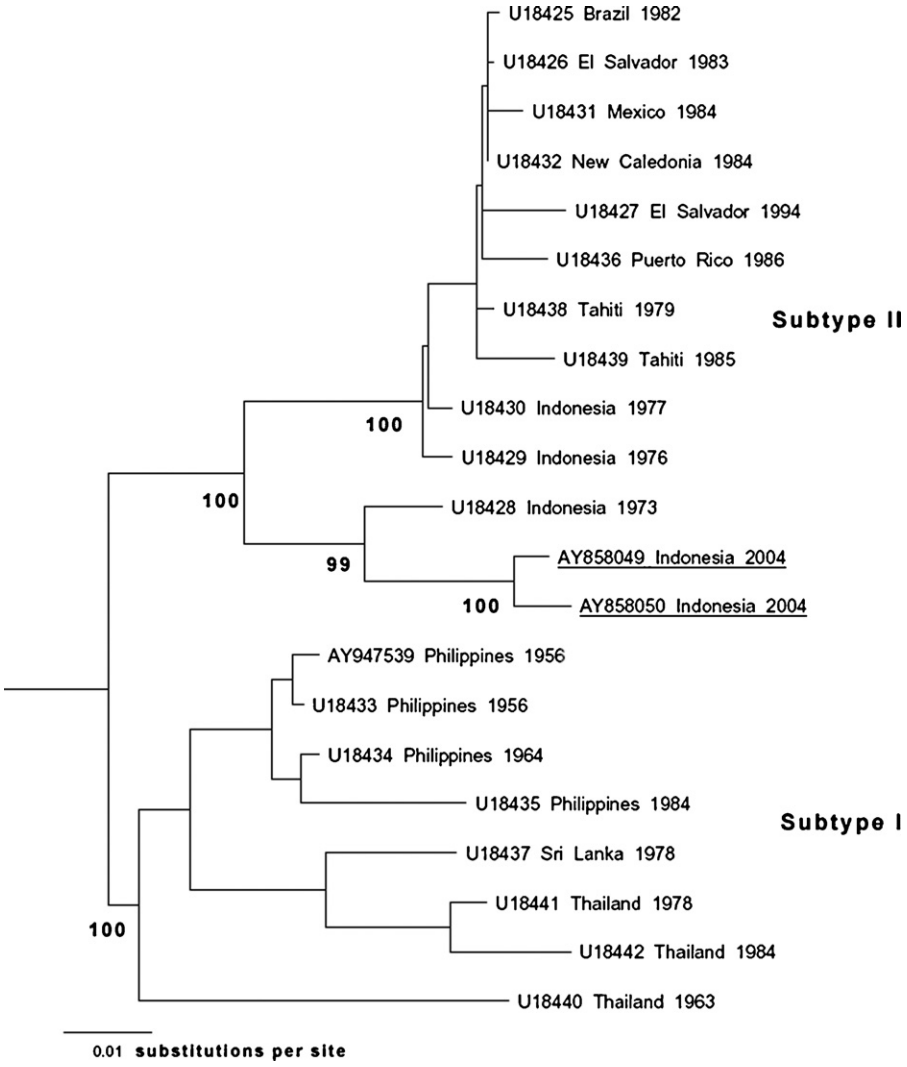

Fig. 4. Phylogenetic tree of DENV-3 based on a 705-base segment covering the pre-M/M and a part of the E gene (from position 437 to 1141 of the DENV-3 genome). The leaves are labeled with the GenBank accession number, strain name/country of isolation, and year of isolation. The dataset used is identical to the one used by Messer et al. (2003) with the addition of AY858037-AY858048 and AY662691. All Indonesian strains sequenced in this study fall within subtype I of DENV-3 whereas the AY662691 strain from Singapore fall within subtype III.

that contains the two 2004 strains, thereby confirming the two as being endemic in Indonesia.

3.3. Diversity and phylogeny of the DENV-3 isolates

Fig. 4 shows the phylogenetic tree constructed from a dataset containing the 40 DENV-3 isolates described by Messer et al. (2003), but with additional sequences from a DF-causing strain isolated in 2003 in Singapore (AY662691) and the newly-sequenced Indonesian DENV-3 isolates. This tree is based on a 705-base segment covering pre-M/M and a portion of the E gene. All DENV-3 strains from Indonesia fall into subtype I, according to the classification described by Lanciotti et al. (1994), a lineage that can be traced all the way back to strains isolated in the same region in the early 1970s. In contrast, the strain from nearby Singapore clusters with subtype III strains which are commonly associated with viruses found in Eastern Africa and South Asia. The 2004 Indonesian strains can be further divided into two distinct clades, but there is no spatial clustering by the location of the admitting hospitals. The amount of data available is insufficient to attempt any correlation between genetic variation and the reported disease severity.

After the intra-serotypic classification of the DENV-3 strains had been established, all published DENV-3 complete genomic sequences were collected to construct a whole-genome phylogeny. The whole-genome phylogenetic tree for 30 subtype I genomes (Fig. 5) shows a distinct cluster that groups strains isolated from 1988, 1998 and 2004 in Indonesia, as well as strains isolated in a subsequent outbreak in Timor Leste in 2005 (WHO, 2005). This Sumatran-Javan clade is potentially a viral lineage that possesses a superior level of evolutionary

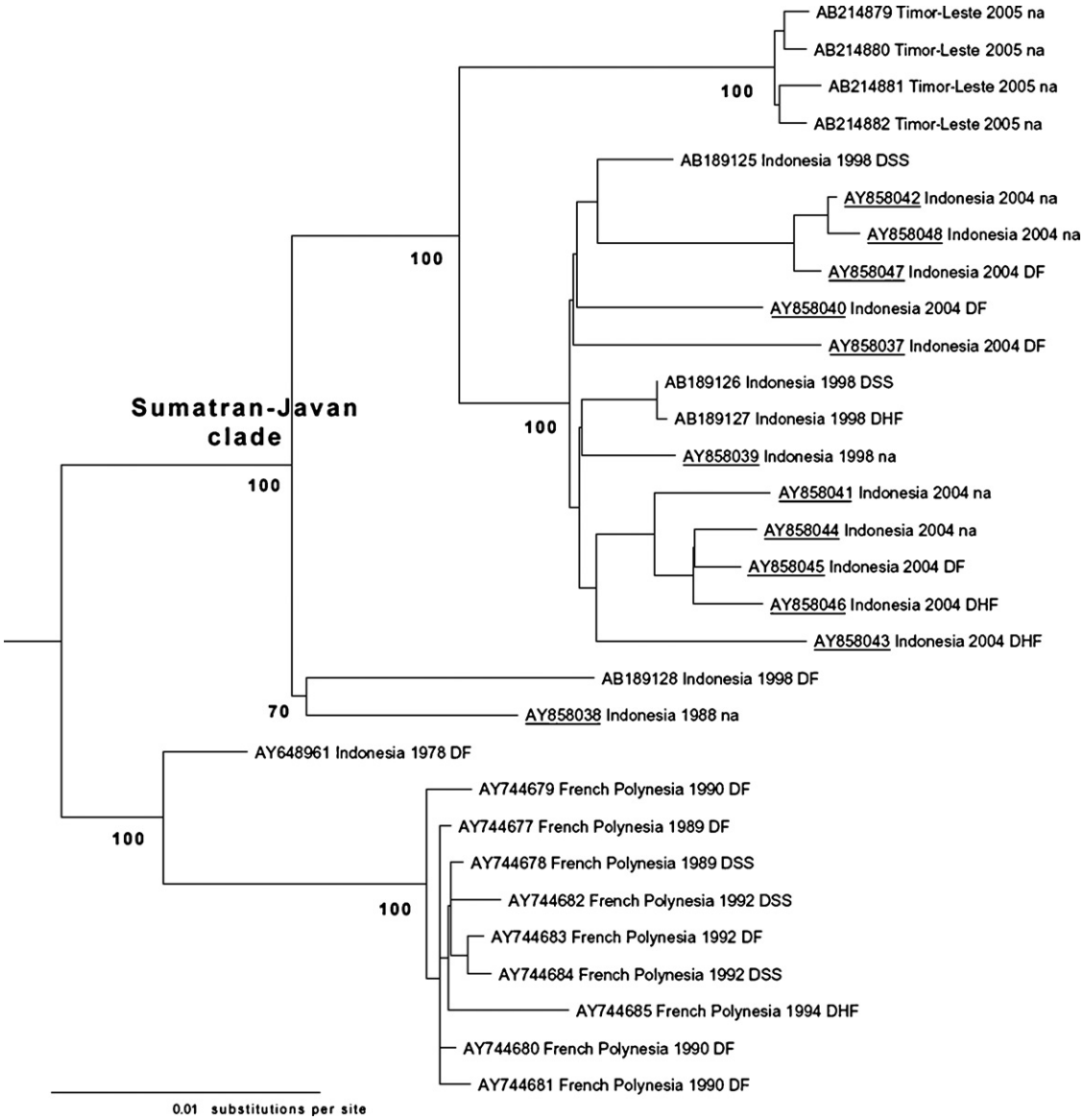

Fig. 5. Phylogenetic tree based on 30 subtype I DENV-3 whole-genome nucleotide sequences. The tree leaves are labeled with the GenBank accession number, country of isolation, year of isolation and known clinical severity. The branch topology for the Indonesian strains sequenced in this study is similar to the prM-E tree in Fig. 4.

fitness and epidemic potential, as evident by its sustained transmission since 1970s and being implicated in major dengue epidemics in 1988, 1998 and 2004. In contrast, the other clade within subtype I does not have a similar epidemic-causing history, and has not been implicated in any epidemic since the early 1990s. The ability to cause DHF/DSS is not restricted to the Sumatran-Javan lineage and is observed in both lineages within subtype I (Fig. 5), suggesting that disease severity and epidemic potential are likely to be governed by separate discrete factors.

3.4. Clade-specific mutations of the DENV-3 isolates

The availability of complete genome sequences made it possible to identify amino acid mutations that are specific to the Sumatran-Javan lineage, which could be the viral genetic elements that contribute to its continuing circulation in the region and its epidemic potential. Comparative analysis identified 24 amino acid residues that are significantly different between the Sumatran-Javan lineage and the other subtype I DENV-3 strains at the 0.05 significance level (Fig. 6).

The impact of amino acid changes on protein function were subsequently predicted using SIFT (Ng and Henikoff, 2001) to identify residues that could be conferring competitive fitness advantage to the Sumatran-Javan lineage. Within the PrM protein an A107T substitution located downstream of the furin cleavage site and retained in the mature virus was identified. Two non-conservative mutations were found in the E protein: a leucine typically found at position 124 of the E protein in strains within the Sumatran-Javan clade is replaced by a serine (L124S) outside the clade; the second mutation (S301L) is located within domain III of the E protein which is believed to be involved in antigenicity (Roehrig, 2003). There were also mutations within the non-structural proteins but none that are chemically non-conservative and predicted as not tolerated by SIFT (Table 2). Using the single likelihood ancestor counting (SLAC) method (Pond and Frost, 2005a,b) we determined site-specific selection pressures acting on the all available DENV-3 coding sequences. While many sites seem to be under a negative selection pressure, no evidence of positive selection was found. Unfortunately the Sumatran-Javan clade is too small to confidently detect any evidence of positive selection within this group.

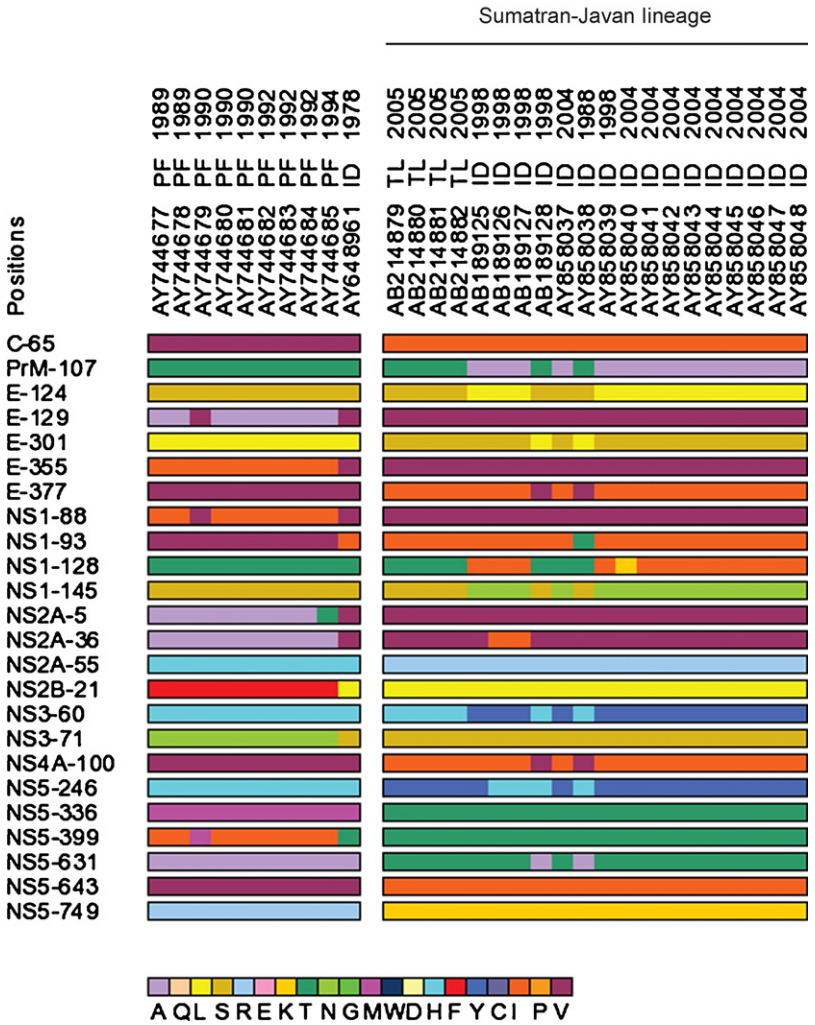

Fig. 6. Amino acid residues that are significantly different between the Sumatran-Javan lineage and other subtype I DENV-3 strains ($p < 0.05$, chi-square). AY858037-AY858048 are the strains sequenced in this study. Amino acids are color-coded as shown in the key beneath the main diagram.

Table 2
Amino acid residues that potentially confer competitive fitness advantage to the Sumatran-Javan DENV-3 lineage when compared to other subtype I DENV-3 genomes

Viral protein	Mutation	Functional domain	Amino acid variation	SIFT prediction
C	I65V	Helix α3	Conservative	Not tolerated
PrM/M	A107T	-	Conservative	Tolerated
E	L124S	Domain II	Non-conservative	Not tolerated
E	V129A	Domain II	Conservative	Tolerated
E	S301L	Domain III	Non-conservative	Not tolerated
E	V355I	Domain III	Conservative	Not tolerated
E	I377V	Domain III	Conservative	Tolerated
NS1	V88I	-	Conservative	Tolerated
NS1	I93V	-	Conservative	Tolerated
NS1	I128T	-	Non-conservative	Tolerated
NS1	N145S	-	Conservative	Tolerated
NS2A	V5A	-	Conservative	Not tolerated
NS2A	V36A	-	Conservative	Not tolerated
NS2A	R55H	-	Conservative	Not tolerated
NS2B	L21F	-	Conservative	Not tolerated
NS3	Y60H	Protease	Conservative	Tolerated
NS3	S71N	Protease	Conservative	Tolerated
NS4A	I100V	Transmembrane Segment 2	Conservative	Tolerated
NS5	Y246H	Methyltransferase	Conservative	Tolerated
NS5	T336M	Polymerase	Non-conservative	Tolerated
NS5	T399I	Polymerase	Non-conservative	Tolerated
NS5	T631A	Polymerase	Conservative	Tolerated
NS5	I643V	Polymerase	Conservative	Tolerated
NS5	K749R	Polymerase	Conservative	Tolerated

The two most likely candidates are shaded in grey. Mutations predicted by SIFT as "Tolerated" are less likely to have an impact on the fitness level of the virus. Threshold of intolerance used by SIFT is 0.05.

A similar comparison of the deduced amino acid sequences involving only the 16 Indonesian DENV-3 strains from the epidemics in 1988, 1998 and 2004 did not detect any specific mutation that could have served as the trigger of the epidemic in 2004 (Fig. 7). Only 98 sites out of 3390 (2.89%) of the DENV-3 polyprotein reported any mutation, and this number drops to 87 if den3_88 from 1988 is removed from the analysis. From the pattern of mutations observed these differences are likely to be the result of random mutations that confers little or no evolutionary advantage.

4. Discussion

The genome sequences of three serotypes of DENV were successfully obtained using a genome sequencing strategy that can be used on patient serum samples. Success with DENV-4 sequencing using this approach is expected in the future as more DENV-4 genomes become available for better RT-PCR primer design. Based on the obtained sequences, we have determined the molecular characteristics and phylogenetic relationships of the DENV strains isolated during the 2004 epidemic in Jakarta, Indonesia.

The phylogenetic trees for each of the four serotypes show that the viruses isolated in the 2004 epidemic cluster within subtypes that have been circulating in South East Asia for at least 30 years. The strongest evidence of these being endemic strains comes from the phylogenetic data for DENV-3. All 12 Indonesian DENV-3 strains sequenced in this study fall into subtype I which is recognized as comprising strains from Southeast Asia and the South Pacific islands. The close

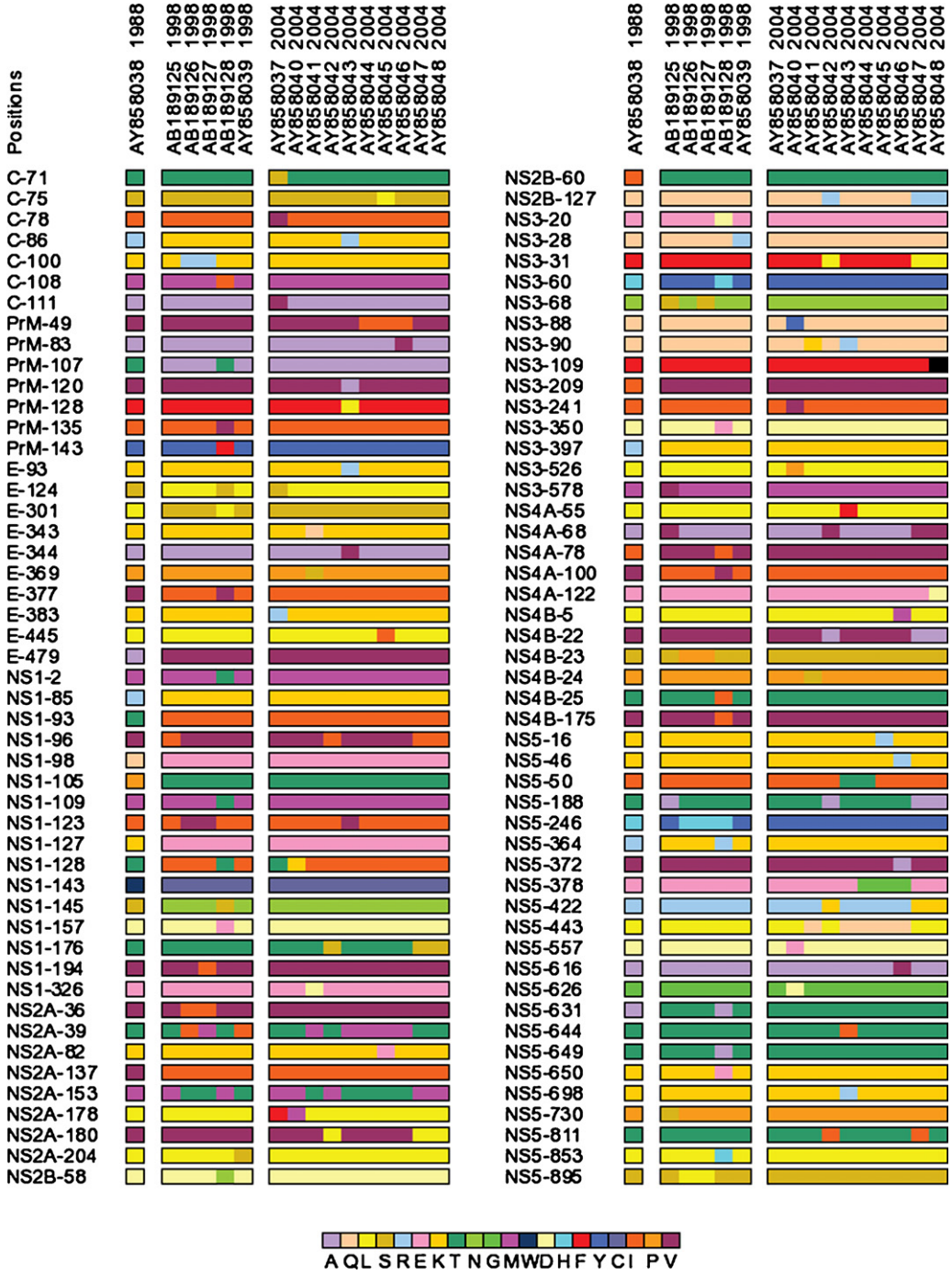

Fig. 7. Amino acid differences among the Indonesian DENV-3 strains isolated in 1988, 1998 and 2004. The sequences are sorted in chronological order followed by accession number, starting from the left. Only 98 positions out of 3390 of the DENV-3 polyprotein reported any mutations, and none could be suggested as the trigger for the epidemic in 2004. Amino acids are color-coded as shown in the key beneath the main diagram.

phylogenetic relationship observed between the strains isolated from Sumatra in 1998 and those from Jakarta in 2004 indicates that the 2004 epidemic might have had its origins in strains derived from viruses circulating in Sumatra in 1998, or possibly in a common ancestor of the Sumatran strains (Fig. 5). Sumatra is one of the largest Indonesian islands and Jakarta on the nearby island of Java is the capital city of Indonesia, therefore frequent transmission by travelers is likely.

The identified Sumatran-Javan clade links the newly sequenced DENV-3 strains directly to the strains implicated in the epidemic on the island of Java in 1988, on the island of Sumatra 6 years earlier and those that subsequently caused an outbreak in Timor Leste in 2005. This clearly suggests these viruses possess an inherent epidemic potential. Since viruses from this particular lineage have been implicated in causing four epidemics in the past two decades, it is very likely these viruses could yet cause another dengue epidemic in Indonesia

in the near future if they remained in circulation. We then examined the clade-specific mutations of the Sumatran-Javan DENV-3 isolates for potential viral genetic markers that could be the trigger of the epidemics. However, comparative study of the deduced polyprotein sequences established that re-emergence of little-changed endemic strains, and not newly-acquired amino acid mutations by the DENV-3 strains, as the most likely cause of the dengue epidemic in 2004 (Fig. 7).

According to a parallel serological study involving 272 hospitalised patients, all four dengue serotypes were detected during the 2004 epidemic with DENV-3 being the predominant circulating serotype (Suwandono et al., 2006). Similar serological result was reported for the 1998 epidemic in south Sumatra, and the predominance of DENV-3 as a result of inherent sampling bias (on the premise that DENV-3 causes more severe illness and therefore causes more hospitalisation that facilitates sampling) has been proposed (Corwin et al., 2001). The fact that all four serotypes were found to be circulating in successive epidemics adds credence to the suggestion that advantageous amino acid mutations is not the trigger of the epidemics, otherwise this hypothetical fitter form would then dominate and become the sole serotype detected in subsequent outbreaks.

The availability of genome sequence data has made possible the attempt to identify putative viral genetic factors that contributed to the continuing circulation of the Sumatran-Javan lineage and its epidemic potential. Two candidate residues were found in the E protein (Table 2) that may account for this difference however no conclusive evidence of positive selection was found. It is clear the genome sequencing and analysis strategy broadens the search for novel functional mutants beyond the scope of most previous studies which have mainly focused on using nucleotide sequences from the three dengue structural proteins.

The sequence data obtained in this study tells little about the role of population dynamics of the four serotypes in causing epidemics, but it clearly shows that the viruses that caused the 2004 epidemic in Indonesia are local strains that have been circulating in the region for a few decades. The identified Sumatran-Javan lineage of DENV-3 apparently is robust enough for sustained transmissions and has demonstrated strong epidemic potential spanning two decades.

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Appendix A. Binding positions of RT-PCR primers used in this study

Primers for DENV-1	Binding positions (sense strand)
d1a5	8558–8577
d1a9	6551–6573
d1a13	4544–4561
d1a17	2540–2559
d1a23	10716–10735
d1s6	2201–2223
d1s10	4213–4231
d1s14	6216–6235
d1s18	8211–8232
d1s22	1–20
Primers for DENV-2	**Binding positions (sense strand)**
d2a6	8468–8488
d2a10	6477–6497
d2a14	4461–4484
d2a18	2455–2474
d2a23	10704–10723
d2s5	2182–2201
d2s9	4175–4197
d2s13	6193–6213
d2s16	7669–7692
d2s23	1–20
Primers for DENV-3	**Binding positions (sense strand)**
d3a6	8342–8361
d3a10	6339–6360
d3a14	4334–4356
d3a18	2361–2380
d3a23	10688–10707
d3s5	2035–2053
d3s8	3532–3553
d3s13	6032–6053
d3s17	8025–8046
d3s23	1–20

Primers for DENV-3Binding positions (sense strand)AF1109–131AF51927–1949BF13629–3652CF17111–1734AR12708–2727BR14133–4153CR17664–7687DR110606–10637

Appendix B. Sequencing and amplification primers used in this study

Primer name	Sequence	Serotype
d1a1	5′-ACAGCTTCCCCTGGTGTTGG-3′	DENV-1
d1a2	5′-DTCTTCCCAACTGGAYACATG-3′	DENV-1
d1a3	5′-YACRCARTCATCTCCRCTGAT-3′	DENV-1
d1a4	5′-CACTCCACTGAGTGAATTCTCTCT-3′	DENV-1
d1a5	5′-GGRATRACATCCCATGGTTT-3′	DENV-1
d1a6	5′-AGRACACGTAACGTTCTWCCTTC-3′	DENV-1
d1a7	5′-CCTACCTCCTCCTARAGATTTCA-3′	DENV-1
d1a8	5′-CAAGTCCCATCAATATAGCTGC-3′	DENV-1
d1a9	5′-CCAGTYARCACAGCTATCAAAGC-3′	DENV-1
d1a10	5′-TCTCTCYGGCTCAAAGAGGG-3′	DENV-1
d1a11	5′-CRTAGCCTGARTTCCATGATCT-3′	DENV-1

Appendix B (*Continued*)

Primer name	Sequence	Serotype
d1a12	5′-CCTCGTCCTCAATCTCTGGTAG-3′	DENV-1
d1a13	5′-TTCCACTTCYGGAGGGCT-3′	DENV-1
d1a14	5′-CCGGAAGCCATGTTGTTTT-3′	DENV-1
d1a15	5′-GCATYTTTCTRCTCCATCTGGATC-3′	DENV-1
d1a16	5′-CARCTTCCARGTYTCGTTCTT-3′	DENV-1
d1a17	5′-CCAATGGCYGCTGAYAGTCT-3′	DENV-1
d1a18	5′-AAAGGTGGYTCYGYYTCAAT-3′	DENV-1
d1a19	5′-GTTTGTGGACRAGCCATGATT-3′	DENV-1
d1a20	5′-CGTCTTCAAGAGTTCAATGTCC-3′	DENV-1
d1a21	5′-CATYGCAATRAGRGTGCACAT-3′	DENV-1
d1a22	5′-AGCTTCCGATTCGAAACTGT-3′	DENV-1
d1a23	5′-AGAACCTGTTGATTCAACAG-3′	DENV-1
d1s1	5′-TRGCTCCATCGTGGGGAT-3′	DENV-1
d1s2	5′-TTGCTYTCAGGCCAAGGACC-3′	DENV-1
d1s3	5′-AAACGTTCCGTSGCACTGGC-3′	DENV-1
d1s4	5′-TGTGTGTCGMCGAACGTT-3′	DENV-1
d1s5	5′-GCAATGCACACYGCGTTG-3′	DENV-1
d1s6	5′-GGYTCTATAGGAGGRGTGTTCAC-3′	DENV-1
d1s7	5′-GGCCCAAGGRAARAAAATG-3′	DENV-1
d1s8	5′-ACAAACAGCAGGGCCRTGGCA-3′	DENV-1
d1s9	5′-CCTAGCYYTGATGGCYACTTT-3′	DENV-1
d1s10	5′-RGCYGGSCCACTAATAGCT-3′	DENV-1
d1s11	5′-AAGAGRCTGGAACCRAGYTGGGC-3′	DENV-1
d1s12	5′-AAATGGCAGAGGCGCTCAAGGG-3′	DENV-1
d1s13	5′-ACAAAAAAYAAYGACTGGGACTAT-3′	DENV-1
d1s14	5′-ATGGRGAAAGGAACAACCAG-3′	DENV-1
d1s15	5′-GGATAGCGGCCTCYATCATACT-3′	DENV-1
d1s16	5′-GCAAARGCYACTAGAGAAGCTCAA	DENV-1
d1s17	5′-GAAACRACYAAACAYGCAGTG-3′	DENV-1
d1s18	5′-CCACYCATGAAATGTAYTGGGT-3′	DENV-1
d1s19	5′-GCCARGTGGTTATGGGGTTT-3′	DENV-1
d1s20	5′-GGATGATCTTCAGAATGAGGC-3′	DENV-1
d1s21	5′-TYATGAAGGATGGGAGGGA-3′	DENV-1
d1s22	5′-AGTTGTTAGTCTACGTGGAC-3′	DENV-1
d2a1	5′-AGGAAACGAAGGAACGCC-3′	DENV-2
d2a2	5′-ACGCCATGCGTACAGCTT-3′	DENV-2
d2a3	5′-CCGTYGTCATCCATTCATG-3′	DENV-2
d2a4	5′-TTTCTTCTGTGRCTGTCAGGTG-3′	DENV-2
d2a5	5′-TCTGCTGCCTTTTGCCTT-3′	DENV-2
d2a6	5′-CATGGTAWGCCCAYGTTTTGT-3′	DENV-2
d2a7	5′-TTCTGGCGGRRTGAAGAA-3′	DENV-2
d2a8	5′-TGACACYGCAATGGTAGTGTT-3′	DENV-2
d2a9	5′-CAATGCTATGTCTCARCATTGGTGT-3′	DENV-2
d2a10	5′-TACGCCCTTCCRCCTGCTTCA-3′	DENV-2
d2a11	5′-CCAGTGTGCACAGTCTTCATCAT-3′	DENV-2
d2a12	5′-ATGGRTCTCTRCTTCCCGG-3′	DENV-2
d2a13	5′-CACCATTACCATAAAGACCCAC-3′	DENV-2
d2a14	5′-GCCGTGATTGGTATTGATACAGGA-3′	DENV-2
d2a15	5′-GTGCAACTCACTTTCCATGC-3′	DENV-2
d2a16	5′-CGGCTGTGACCAAGGAGTT-3′	DENV-2
d2a17	5′-CCGCTGACATGAGTTTTGAGTC-3′	DENV-2
d2a18	5′-CCACTGCCACATTTCAGTTC-3′	DENV-2
d2a19	5′-GGCGRCCTAAGACATRTCTTTT-3′	DENV-2
d2a20	5′-GCCATARCCTGTCARTTCTGC-3′	DENV-2
d2a21	5′-CTGAAACCCCTTCTACAAAGTCTC-3′	DENV-2
d2a22	5′-TGTGGTTCTCCGTTACGTGT-3′	DENV-2
d2a23	5′-AGAACCTGTTGATTCAACAG-3′	DENV-2
d2s1	5′-GCAACAGCTGACAAAGAGATTCTC-3′	DENV-2
d2s2	5′-CACCACRGGAGAACAYAGAAGA-3′	DENV-2
d2s3	5′-CAGCCTAAAWGAAGAGCAGGA-3′	DENV-2
d2s4	5′-GCGAAGAAACAGGATGTTGTTG-3′	DENV-2
d2s5	5′-GGTGACACAGCCTGGGATTT-3′	DENV-2
d2s6	5′-YATGACAGGAGACATCAAAGGA-3′	DENV-2
d2s7	5′-WCAACACAACTAYAGACCAGGCT-3′	DENV-2
d2s8	5′-TGGGCGTGACTTATCTTGC-3′	DENV-2

Appendix B (*Continued*)

Primer name	Sequence	Serotype
d2s9	5′-GCATTTTRGCCAGTTCTCTCCTA-3′	DENV-2
d2s10	5′-GYGCTGTYCTAATGCATAAAGG-3′	DENV-2
d2s11	5′-YAGAGTCGTGGCAGCTGAA-3′	DENV-2
d2s12	5′-GGAAGACYTTTGATTCTGAGTATGT-3′	DENV-2
d2s13	5′-GCAGACAGAAGGTGGTGTTTT-3′	DENV-2
d2s14	5′-CCACACTGGATAGCAGCTTCAATA-3′	DENV-2
d2s15	5′-GACTYCAAGCAAAAGCAACC-3′	DENV-2
d2s16	5′-CAGGAAGTGGATAGAACCTTAGCA-3′	DENV-2
d2s17	5′-CTCTCACGRAACTCCACACAT-3′	DENV-2
d2s18	5′-RGCAGAGTGGCTKTGGAAA-3′	DENV-2
d2s19	5′-GGGACACAAGAATCACACTAGAAG-3′	DENV-2
d2s20	5′-GCCYTTYTGTTCACACCATTTCCA-3′	DENV-2
d2s21	5′-AGGAATACACAGATTACATGCCA-3′	DENV-2
d2s22	5′-GGAATGGTGCTGTTGAATCAAC-3′	DENV-2
d2s23	5′-AGTWGTTAGTCTACGTGGAC-3′	DENV-2
d3a1	5′-GGTTTCTCACGCGTTTCAG-3′	DENV-3
d3a2	5′-TTTTAACGTCCTTGGACGG-3′	DENV-3
d3a3	5′-GGATGCTAGTCTRAGATCTCTTCTG-3′	DENV-3
d3a4	5′-CTGCCTCTTTGGTCTTTCCT-3′	DENV-3
d3a5	5′-CGTTCTCTGTCCACAAGTTTCC-3′	DENV-3
d3a6	5′-GCATTRACATGTCGRGTTCC-3′	DENV-3
d3a7	5′-TCCTCGCACTTCTGTRACTTT-3′	DENV-3
d3a8	5′-TTGAACTGCACACARAACCAG-3′	DENV-3
d3a9	5′-CACCTGGYTCYTTAGACATTCCTA-3′	DENV-3
d3a10	5′-GCYGCAAARTCCTTGAATTCCT-3′	DENV-3
d3a11	5′-TTGGTCCAGCCAGGATCA-3′	DENV-3
d3a12	5′-GTGAAATGRGCCTCATCCAT-3′	DENV-3
d3a13	5′-CCTGGCATGGTTTGAAAGTT-3′	DENV-3
d3a14	5′-ACTGTGATCATTAARTTGTGGGA-3′	DENV-3
d3a15	5′-CCCCARAGCRATTCCATT-3′	DENV-3
d3a16	5′-GGCAACACCATTCGTGTATCA-3′	DENV-3
d3a17	5′-CACTTGGACACTCCGGTGT-3′	DENV-3
d3a18	5′-GATTCCTATCGCAATGCATG-3′	DENV-3
d3a19	5′-GCGTTTCKGAGACTTCTTTCTTC-3′	DENV-3
d3a20	5′-GACGGTGTATTTGAGGTTCTCA-3′	DENV-3
d3a21	5′-GGCTAGTATGGTRAACCCTGG-3′	DENV-3
d3a22	5′-CCTTCTTGAAGCCTTTYARGACCT-3′	DENV-3
d3a23	5′-AGAACCTGTTGATTCAACAG-3′	DENV-3
d3s1	5′-CAGTTTCGACTCGGAAGCTT-3′	DENV-3
d3s2	5′-CAACATGTGCACACTCATAGCC-3′	DENV-3
d3s3	5′-GACTACCATGGCTAAGAACAAGC-3′	DENV-3
d3s4	5′-GAAGAACAAAGCATGGATGGTA-3′	DENV-3
d3s5	5′-TGAACCTCCTTTTGGGGAA-3′	DENV-3
d3s6	5′-CCMAAAAGATTGGCAACAGC-3′	DENV-3
d3s7	5′-CATGGGCTATTGGATAGAAAGC-3′	DENV-3
d3s8	5′-GGTGATGAGAGGAAAATTTGGG-3′	DENV-3
d3s9	5′-GAAAACAGATTGGCTCCCAA-3′	DENV-3
d3s10	5′-CCCCCCAGAGACACAGAAAG-3′	DENV-3
d3s11	5′-CGACACCAGAGTTGGAAGAAG-3′	DENV-3
d3s12	5′-GCTCATGGAATTCAGGCAAT-3′	DENV-3
d3s13	5′-CCAGCTCTCTTTGAACCAGAAA-3′	DENV-3
d3s14	5′-CTCYTGGGACTGATGATCTTGT-3′	DENV-3
d3s15	5′-CTGATGGGTTTRGACAAAGGA-3′	DENV-3
d3s16	5′-TTTTTCTATYATGAAATCAGTTGGA-3′	DENV-3
d3s17	5′-CAACAGTGGAAGAAAGCAGAAC-3′	DENV-3
d3s18	5′-ACAAAACCATGGGATGTGG-3′	DENV-3
d3s19	5′-CTGGTTCTCGCGTGAAAAC-3′	DENV-3
d3s20	5′-GGGATGATTGCGTAGTGAAA-3′	DENV-3
d3s21	5′-TCCAGTCACAACGTGGGAA-3′	DENV-3
d3s22	5′-TGTACCTCCTTGCAAAGGACTA-3′	DENV-3
d3s23	5′-AGTTGTTAGTCTACGTGGAC-3′	DENV-3
AR1	5′-CTTGCCTTYGGTCAACACCC-3′	DENV-4
BR1	5′-CCTCGTTAAGRGGCCARGATC-3′	DENV-4
CR1	5′-GGCTTCAGTCCTGTCCACTTCTAG-3′	DENV-4
DR1	5′-ATCCATCTTGCGGCGCTCTGTG-3′	DENV-4

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