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MOLECULAR EVOLUTION OF DENGUE TYPE 2 VIRUS IN THAILAND

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Abstract. Dengue is a mosquito-borne viral infection that in recent years has become a major international public health concern. Dengue hemorrhagic fever (DHF), first recognized in Southeast Asia in the 1950s, is today a leading cause of childhood death in many countries. The pathogenesis of this illness is poorly understood, mainly because there are no laboratory or animal models of disease. We have studied the genetic relationships of dengue viruses of serotype 2, one of four antigenically distinct dengue virus groups, to determine if viruses obtained from cases of less severe dengue fever (DF) have distinct evolutionary origins from those obtained from DHF cases. A very large number (73) of virus samples from patients with DF or DHF in two locations in Thailand (Bangkok and Kamphaeng Phet) were compared by sequence analysis of 240 nucleotides from the envelope/nonstructural protein 1 (E/NS1) gene junction of the viral genome. Phylogenetic trees generated with these data have been shown to reflect long-term evolutionary relationships among strains. The results suggest that 1) many different virus variants may circulate simultaneously in Thailand, thus reflecting the quasispecies nature of these RNA viruses, in spite of population immunity; 2) viruses belonging to two previously distinct genotypic groups have been isolated from both DF and DHF cases, supporting the view that they arose from a common progenitor and share the potential to cause severe disease; and 3) viruses associated with the potential to cause DHF segregate into what is now one, large genotypic group and they have evolved independently in Southeast Asia for some time.

Dengue virus, a mosquito-borne *Flavivirus*, is responsible for a growing number of human infections worldwide, mainly in tropical, urban, and periurban areas, as a function of the distribution of its vector. The majority of cases present with classic dengue fever (DF), a systemic, self-limited illness, but some individuals develop a more severe form of the disease, dengue hemorrhagic fever (DHF), with plasma leakage and thrombocytopenia: mortality rates are approximately 5%. There are four closely related, but antigenically distinct dengue serotypes (types 1-4); infection by one serotype virus does not protect against infection by a second serotype virus. Based on epidemiologic and laboratory observations, it has been shown that cross-reactive immune responses, including infection-enhancing antibodies, contribute to the higher frequency of DHF in children with sequential infections or infants born to dengue-immune mothers[1, 2] The lack of in vitro and in vivo models of severe dengue disease have hampered studies of the pathogenesis and definition of factors involved in producing DHF. Thus, it is presently unclear whether viral virulence factors contribute to severe dengue; the association of the introduction of specific genetic types of dengue serotype 2 and the appearance of DHF in the Americas has suggested that this may be the case. Two genetic variants (or genotypes) of serotype 2, which seem to have originated in Southeast Asia, have been isolated from DHF patients in Mexico and South America[3]; it is therefore important to have a better understanding of the molecular evolution of dengue viruses in Southeast Asia, a probable source of virulent variants to other continents.

Thailand has had a long history of dengue virus transmission, with all four dengue virus serotypes causing periodic outbreaks, with high numbers of DF and DHF cases reported annually since the 1950s.[1, 4] Dengue serotype 2 viruses have been associated most frequently with DHF in this country (Nisalak A, unpublished data); this group of viruses has therefore been the subject of previous genetic variation studies.[5, 6] We have studied the genetic relationships of 73 dengue viruses of serotype 2 from two areas of Thailand, Bangkok and Kainphaeng Phet, isolated over a 32-year period, using limited sequencing and phylogenetic analyses, to determine if there are specific associations between virus genetic type and severity of clinical presentation. This evolutionary study has also helped us understand the transmission patterns of virus variants through a population that is being infected regularly in a hyperendemic disease cycle.

MATERIALS AND METHODS

Viruses. Dengue viruses used in this study were selected from frozen stocks or from plasma collected during ongoing prospective clinical studies (a total of 73 from Thailand);[7, 8] virus aliquots were used to infect C6/36 mosquito cells and were identified as dengue serotype 2 by indirect fluorescent antibody tests with type-specific monoclonal antibodies.[9] Virus isolation and passage histories are given in Table 1; viruses or nucleic acids described here were isolated or amplified from human plasma, except strain PM33974, which was isolated from a mosquito pool.

Extraction of RNA and reverse transcriptase–polymerase chain reaction (RT-PCR). Nucleotide sequences for six of the 77 dengue type 2 viruses described here were obtained previously by primer-extension sequencing from viral-extracted RNA;[10] the remainder were amplified by RT-PCR. Sequences obtained by these two methods have shown no differences over the 240-nucleotide-long envelope/nonstructural protein 1 (E/NS1) region used for phylogenies.[3]

For 71 of the viruses shown here, a 100–200-μl aliquot of cell culture supernatant or human plasma was treated with 1.0 ml of Trizol (GIBCO-BRL, Gaithersburg, MD); RNA extractions were done according to the manufacturer's protocol. The RNA was resuspended in water and one-fifth of this sample was added to Superscript II RT buffer (50 mM

Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3) (GIBCO-BRL), 10 mM DTT, and 50 pM of an antisense primer (D2/2578: 5′-TTACTGAGCGGATTCCACAGATGCC-3′) that hybridizes to all known dengue serotype 2 viruses.[10] The solution was heated to 90°C for 90 sec, and cooled on ice. Each of the four deoxynucleotides were added (1 mM) along with 40 U of RNasin (Promega, Madison, WI) and 200 U of reverse transcriptase enzyme (Superscript II; GIBCO-BRL). The mixture was incubated for 1 hr at 42°C and cooled on ice. To this mixture both PCR primers (D2/2578 and D2/2170V: 5′-ATGGCCATTTTAGGTGACACAGCC-TGGGA-3′, sense) were added (150 pM), PCR buffer at pH 10.0 (60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$), deoxynucleotides at 0.2 mM, and the volume brought up to 50 μl with water. Amplitaq enzyme (Perkin Elmer, Foster City, CA) was added last (5 U) and the solution overlaid with mineral oil. The samples, including a positive PCR control (Perkin Elmer), a negative control (water), and a dengue type 2 RNA control, were placed in a thermal cycler for 30 cycles at 94°C for 60 sec, 55°C for 2 min, and 72°C for 3 min. The samples were kept at 4°C after amplification. The entire sample was electrophoresed in a 2% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) and stained with 1 μg/ml of ethidium bromide for visualization of bands. This procedure produced a 408-basepair (bp) product for sequencing; the 240-nucleotide fragment for genetic comparison is comprised within this product. Specific amplification of RNA from virus strains belonging to five previously described genotypes[10] were obtained in this manner.

Polymerase chain reaction fragment sequencing. Bands of the correct size were cut from agarose gels and equilibrated in β-Agarase buffer (10 mM Tris-HCl, 1 mM EDTA, pH 6.5) (New England Biolabs, Beverly, MA). Agarose was digested with 6 U of β-Agarase enzyme (New England Biolabs) and the DNA fragments were precipitated and washed in ethanol, according to the manufacturer's instructions. The DNA was resuspended in water and one-fourth of this sample used in dideoxynucleotide sequencing reactions. Sequencing was done manually using the Sequenase 2.0 Kit (USB, Amersham, Cleveland, OH), according to the manufacturer's instructions, including pyrophosphatase and α-^{35}S-dATP. Two primer-extension reactions using the D2/2578 primer and D2/2452 (5′-CCACATTTCAGTTCTTT-3′) provided clear results and overlap at the 240-nucleotide site used for comparisons (map sites 2311-2550).[11]

Sequence analysis and phylogenies. Nucleotide sequences for six of the strains shown here were reported previously[10] and the remainder were submitted to GenBank; accession numbers are given in Table 1. Sequences for the homologous region of serotype 1 virus (strain D81-135, Thailand, 1981), serotype 3 virus (strain H87, Philippines, 1956), and serotype 4 virus (strain 814669, Dominica, 1981) were obtained from GenBank (accession numbers M32925, M93130, and M14931, respectively). Alignments were done using encoded amino acids; none of the viruses compared here had additions or deletions in this region of the genome. Phylogenetic analyses were done using the phylogenetic analysis using parsimony (PAUP) program, with uniform character weights, tree bisection-reconnection branch-swapping, and a heuristic search for most parsimonious trees.[12] Sequences from representatives of the other three serotypes (1, 3, and 4) were used as an outgroup to root the trees. The reliability of the inferred trees was estimated using the bootstrap method, with 100 replications.[13]

RESULTS

The maximum parsimony analysis of nucleotide sequences resulted in an evolutionary tree, with branching patterns proportional to genetic relatedness among strains. The most reliable tree for all of the samples described in Table 1 is shown in Figure 1. All dengue serotype 2 viruses segregated into one large group consisting of samples from different geographic locations, with those from Guinea (1981), Sri Lanka (1985), and Puerto Rico (1969) each representing a distinct genotypic group, as inferred from the bootstrap values (range = 100–52). Samples from Thailand fell into two groups that had previously been classified as distinct;[10] bootstrap values for these two groups were less than 50 and their separation is no longer supported. The inclusion of many more variants of these genotypic groups has blurred their separation (see Discussion). Therefore, the total number of dengue genotypic groups determined by this method have been reduced from five to four. Other than this observation, there were no major differences in the branching patterns of the evolutionary tree generated by maximum parsimony (PAUP; shown here) and that generated previously, using another algorithm, the NUCLDIFF program, which is based on pairwise distances.[10]

The results obtained here do not differ substantially from those obtained by others when comparing full E gene or NS1 gene sequences of dengue type 2 viruses from Thailand.[14–16] The major branching patterns were the same for those viruses that were included in both studies; however, bootstrap values of statistical significance of branching patterns were not available for previous studies. Other studies of genetic variation of dengue type 2 viruses in Thailand using oligonucleotide fingerprinting techniques[5, 6] also agree with results reported here. Because of the large number of strains analyzed in one report,[6] it was easier to make comparisons; there were a total of nine strains in common with our study. The genetic relationships represented in the phylogenetic tree shown here were most similar to those reflected in the dendrogram generated with fingerprinting data. Because fingerprinting surveys approximately 10% of the viral genome,[17] we are confident that the comparison of sequences from the E/NS1 junction yields reliable estimates of genetic relationships among these viruses.

As can be seen in Figure 1, many variants of dengue type 2 viruses have been transmitted in Thailand over a 32-year period. There was no segregation of viruses according to location in Thailand; the two areas from which specimens were collected, Bangkok and Kamphaeng Phet, are 360 km apart. Samples obtained during the same year (e.g., 1980 or 1994) fall into different genetic clusters although they are from the same city (Bangkok). This variation, although significant in some cases (e.g., 11 of 240 nucleotides or 4.6% divergence between PUO-218 and PUO-280, and 22 of 240 or 9.2% between PUO-218 and D80-141, all from 1980; or 7 of 240, 2.9% divergence between CO562 and CO576, from 1994) confirms the quasispecies nature of RNA viruses during natural transmission.[18] A previous study had shown that the genetic relationships demonstrated by the compari-

TABLE 1
Dengue type 2 viruses compared by sequence analysis

Strain	Passage history*	Location†	Year	Clinical status‡	Accession no.§
NGC	Monk.1, mosq.1, C6/361	New Guinea	1944	DF	M32941
16681	MK2 1, C6/36 5	Bangkok, Thailand	1964	DHF	M32947
PR159	PGMK 6	Puerto Rico	1969	DF	M32953
D79-014	MK2 2, C6/36 1	Bangkok, Thailand	1979	DF	U87320
D79-069	MK2 2, C6/36 1	Bangkok, Thailand	1979	DHF	U87319
PUO-218	MK2 2, C6/36 1	Bangkok, Thailand	1980	DF	U87331
PUO-280	MK2 2, C6/36 1	Bangkok, Thailand	1980	DF	U87377
D80-038	MK2 2, C6/36 1	Bangkok, Thailand	1980	DHF	U87366
D80-100	MK2 2, C6/36 1	Bangkok, Thailand	1980	DHF	U87339
D80-141	MK2 2, C6/36 1	Bangkok, Thailand	1980	DHF	U87321
D80-159	MK2 2, C6/36 1	Bangkok, Thailand	1980	DHF	U87322
PM33974	Mosq.1, C6/36 1	Republic of Guinea	1981	–	M32962
D81-004	C6/36 1	Bangkok, Thailand	1981	DF	U87368
D81-081	C6/36 1	Bangkok, Thailand	1981	DHF	U87340
D82-033	C6/36 1	Bangkok, Thailand	1982	DHF	U87365
D82-137	C6/36 1	Bangkok, Thailand	1982	DF	U87341
D83-516	Ts 1, C6/36 2	Bangkok, Thailand	1983	DF	M32967
D84-015	Ts 1, C6/36 2	Bangkok, Thailand	1984	DF	U87323
D84-087	Ts 1, C6/36 2	Bangkok, Thailand	1984	DHF	U87369
D84-237	Ts 1, C6/36 2	Bangkok, Thailand	1984	DHF	U87371
975	C6/36 3	Sri Lanka	1985	DF	M32966
D86-004	Ts 1, C6/36 1	Bangkok, Thailand	1986	DHF	U87324
D86-337	Ts 1, C6/36 1	Bangkok, Thailand	1986	DF	U87325
D87-1036	Ts 1, C6/36 1	Bangkok, Thailand	1987	DHF	U87372
D87-1372	Ts 1, C6/36 1	Bangkok, Thailand	1987	DF	U87326
D88-007	Ts 1, C6/36 1	Bangkok, Thailand	1988	DF	U87373
D88-065	Ts 1, C6/36 1	Bangkok, Thailand	1988	DHF	U87374
D89-633	Ts 1, C6/36 1	Bangkok, Thailand	1989	DF	U87367
D89-1092	Ts 1, C6/36 1	Bangkok, Thailand	1989	DHF	U87327
D90-206	Ts 1, C6/36 1	Bangkok, Thailand	1990	DF	U87376
D90-276	Ts 1, C6/36 1	Bangkok, Thailand	1990	DHF	U87330
D91-104	Ts 1, C6/36 1	Bangkok, Thailand	1991	DF	U87328
D91-157	Ts 1, C6/36 1	Bangkok, Thailand	1991	DHF	U87329
D91-409	Ts 1, C6/36 1	Bangkok, Thailand	1991	DHF	U87332
D91-419	Ts 1, C6/36 1	Bangkok, Thailand	1991	DHF	U87335
KD91-068	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1991	DHF	U87342
KD91-113	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1991	DHF	U87343
KPH-13773	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1991	DF	U87334
KPH-15008	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1991	DF	U87333
KD92-021	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1992	DHF	U87358
KD92-097	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1992	DHF	U87338
KD92-207	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1992	DHF	U87351
KPH-63652	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1992	DF	U87363
D92-030	Ts 1, C6/36 1	Bangkok, Thailand	1992	DHF	U87336
D92-110	Ts 1, C6/36 1	Bangkok, Thailand	1992	DHF	U87364
D92-129	Ts 1, C6/36 1	Bangkok, Thailand	1992	DF	U87359
D92-287	Ts 1, C6/36 1	Bangkok, Thailand	1992	DF	U87337
KD93-390	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1993	DHF	U87375
KD93-439	Ts 1, C6/36 1	Kamphaeng Phet, Thailand	1993	DHF	U87344
K0005/94	None	Kamphaeng Phet, Thailand	1994	DHF	U87346
K0006/94	None	Kamphaeng Phet, Thailand	1994	DF	U87347
K0011/94	None	Kamphaeng Phet, Thailand	1994	DHF	U87352
K0027/94	None	Kamphaeng Phet, Thailand	1994	DHF	U87360
K0060/94	None	Kamphaeng Phet, Thailand	1994	DF	U87361
K0074/94	None	Kamphaeng Phet, Thailand	1994	DF	U87349
K0097/94	None	Kamphaeng Phet, Thailand	1994	DHF	U87348
K0142/94	None	Kamphaeng Phet, Thailand	1994	DHF	U87350
K0146/94	None	Kamphaeng Phet, Thailand	1994	DF	U87353
C0257/94	None	Bangkok, Thailand	1994	DF	U87370
C0385/94	None	Bangkok, Thailand	1994	DHF	U87345
C0441/94	None	Bangkok, Thailand	1994	DHF	U87387
C0501/94	None	Bangkok, Thailand	1994	DF	U87354
C0507/94	None	Bangkok, Thailand	1994	DF	U87362
C0562/94	None	Bangkok, Thailand	1994	DHF	U87355
C0576/94	None	Bangkok, Thailand	1994	DHF	U87356
C0590/94	None	Bangkok, Thailand	1994	DHF	U87357
K0047/95	None	Kamphaeng Phet, Thailand	1995	DHF	U87383
K0049/95	None	Kamphaeng Phet, Thailand	1995	DHF	U87384
K0079/95	None	Kamphaeng Phet, Thailand	1995	DHF	U87382

TABLE 1
Continued

Strain	Passage history*	Location†	Year	Clinical status‡	Accession no.§
C0059/95	None	Bangkok, Thailand	1995	DHF	U87378
C0084/95	None	Bangkok, Thailand	1995	DHF	U87379
C0195/95	None	Bangkok, Thailand	1995	DF	U87385
C0235/95	None	Bangkok, Thailand	1995	DHF	U87380
C0371/95	None	Bangkok, Thailand	1995	DF	U87386
C0390/95	None	Bangkok, Thailand	1995	DHF	U87381
C0452/95	None	Bangkok, Thailand	1995	DHF	U87388
C0477/95	None	Bangkok, Thailand	1995	DHF	U87389

* Monk. = rhesus monkey; Mosq. = whole mosquito, species unknown; C6/36 = *Aedes albopictus* cell line; MK2 = LLC-MK2 monkey kidney cell line; PGMK = primary green monkey kidney cell line; Ts = *Toxorynchites splendens* mosquito; None = acute plasma of patient.
† City and/or country.
‡ DF = dengue fever; DHF = dengue hemorrhagic fever; – = *Aedes africanus* isolate.
§ GenBank accession number.

son of this 240-nucleotide region are maintained independent of passage level of virus strains (e.g., PR159 and its attenuated derivative PR159S1, which had undergone 23 cell culture passes, were identical).[10] The results obtained here confirm this because there was no segregation of strains based on whether their sequences were determined from passaged virus or by RT-PCR of template from plasma. Because nucleotides from this region of the genome encode amino acids that are apparently not under immune selection (they are internal on the virion structure),[10] the phylogenies reflect only genetic relationships among strains and are probably not reflective of pathogenesis differences (i.e., they do not encode antigenic sites that may be involved in virulence or immune enhancement of infection). Therefore, these relationships should be interpreted carefully, as a reflection of virus evolution only.

The wide distribution of samples from DF and DHF patients seen in Figure 1 confirms that viruses from this area of the world (Southeast Asia) have the potential to cause severe disease and they share a common progenitor. In fact, results presented here and elsewhere suggest that viruses belonging to this Southeast Asian genotypic group have been responsible for the emergence of DHF in the Americas,[3] beginning with the 1981 epidemic in Cuba.[10] All dengue type 2 virus samples from DHF patients studied to date in our laboratories have fallen into this genotypic group, regardless of where or when they were obtained.

DISCUSSION

The wide range of samples we included in this study allowed us to observe the swarms of virus variants that originate from a common progenitor (i.e., the branches or clades of variants within a genotypic group) and the elimination of the division between the two genotypes from Southeast Asia (i.e., the two genotypic groups containing viruses from Thailand). The fact that these viruses did not segregate into branches with a linear progression of mutations over time (i.e., some variants reverted to previous consensus sequences) suggests that there is a large amount of plasticity in the E/NS1 region of the genome but we can still observe long-term evolutionary trends. Thus, as we analyze more samples of dengue type 2 viruses from around the world, we will probably have sampled enough natural variants to reflect the true plasticity of the dengue genome and our arbitrary grouping of variants into four genotypes will become blurred, although probably maintaining some segregation due to differing biologic properties (i.e., phenotype). We have no evidence to support that one variant is more stable, as a population, over another, but we are probably seeing only those mutants that are more successful at surviving because they have been isolated from acutely infected hosts and are being transmitted. These results point to the need for evolutionary studies of dengue populations in single individuals (humans and mosquitoes) to gain a better understanding of the rates of mutation or plasticity of the viral genome and the influence of host factors on that population of variants.

In terms of one of the most important questions concerning dengue pathogenesis, there was no segregation of DF- versus DHF-associated viruses on the evolutionary tree. There was no clear-cut evolutionary divergence or branching of DF versus DHF isolates; it is apparent that these viruses in Thailand share the same ancestor or progenitors. Thus, this virus lineage is capable of continuously generating variants with the potential to cause severe disease. This observation, based on comparing an area of the genome that is not involved in immune recognition/stimulation, can be expected since we were using these sequences as markers for long-term evolutionary trends. Current work is aimed at comparing the full sequence of the dengue type 2 virus genome from DF and DHF patients with carefully obtained clinical and immunologic data. This might lead to conclusions about the common or consensus structure of the protein(s) to which most individuals seem to respond immunologically. This structure may lead to the generation of antibodies that seem to be directly involved in the production of severe dengue pathogenesis.[19–21] Also, the definition of DHF-associated sites that do not code for proteins would suggest that other viral replication regulators might be involved in the pathogenesis of this disease. However, the association of virulence with specific nucleotide differences in the dengue type 2 genome will require the accumulation of a significant amount of indirect evidence since there are currently no in vitro or in vivo models of DHF.

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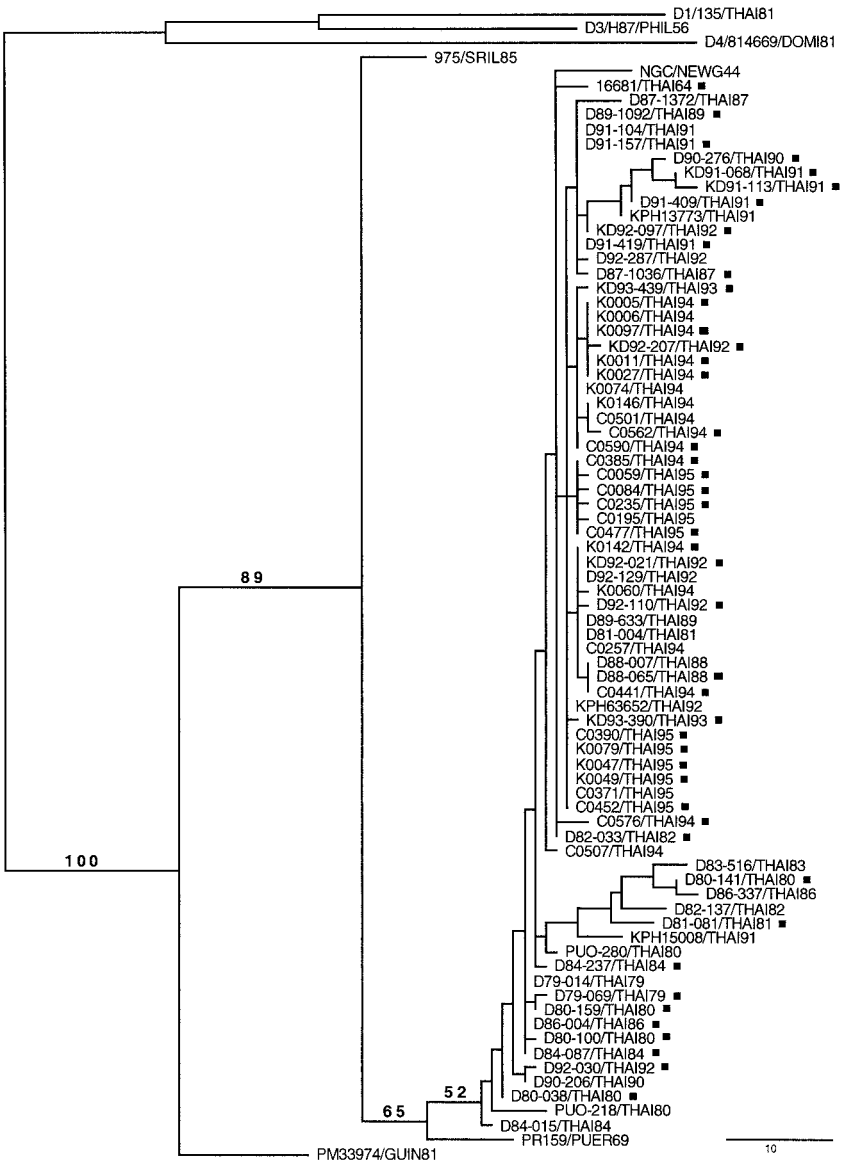

FIGURE 1. Phylogenetic tree generated by phylogenetic analysis using parsimony of nucleotide sequences from the envelope/nonstructural protein 1 junction of 77 dengue type 2 viruses and representatives of types 1, 3, and 4. Viruses are listed by strain number followed by abbreviation for country and year (see Table 1). Branch lengths (proportional to the bar, which equals 10) represent the number of nucleotide substitutions between viruses over the 240-nucleotide-long sequence used for comparisons. Bootstrap values are shown above the branches that connect the genotypic groups of dengue type 2. Black squares denote samples obtained from dengue hemorrhagic fever patients.

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