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Dengue Virus Circulation and Evolution in Mexico: A Phylogenetic Perspective

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Background. Dengue is the most important arthropod-borne viral infection in the Americas. In the last decades a progressive increment in dengue severity has been observed in Mexico and other countries of the region.

Methods. Molecular epidemiological studies were conducted to investigate the viral determinants of the emergence of epidemic dengue, dengue hemorrhagic fever and dengue shock syndrome as major public health problems in Mexico. Bayesian phylogenetic analyses were conducted to determine the origin, persistence and geographical dispersion of the four serotypes of dengue virus (DENV) isolated in Mexico between 1980 and 2002. Tests for natural selection were also conducted.

Results. The origin of some, but not all, strains circulating in Mexico could be inferred. Frequent lineage replacements were observed and were likely due to stochastic events. In situ evolution was detected but not associated with natural selection. Recent changes in the incidence and severity of dengue were temporally associated with the introduction and circulation of different serotypes and genotypes of DENV.

Conclusions. Introduction of new DENV genotypes and serotypes is a major risk factor for epidemic dengue and severe disease. Increased surveillance for such introductions is critical to allow public health authorities to intervene in impending epidemics.

Key Words: Dengue, Evolution, Phylogenetic analysis, Mexico.

Introduction

Dengue has become the most important arthropod-borne viral infection of humans with about 100 million cases and 25,000 dengue-related deaths reported annually. Dengue virus (DENV) is a member of the genus *Flavivirus*, family *Flaviviridae.* There are four antigenically related serotypes (DENV-1–4), which have the same transmission cycles and cause similar diseases. Infection with one serotype confers long-term protection to that serotype but no cross protection to the other serotypes. Mosquitoes of the genus *Aedes*, most importantly *Aedes aegypti,* transmit dengue. About 2.5 billion people live in areas infested with this vector and therefore are at risk of acquiring dengue (1,2). Dengue severity ranges from asymptomatic to fatal disease. Most symptomatic cases are classified as dengue fever (DF), an acute and self-limited condition characterized by fever, generalized pains, rash, lymphadenopathy and minor hemorrhages. An increasing proportion of cases have a more serious outcome designated dengue hemorrhagic fever (DHF), which is characterized by hemostatic disorders, hepatic involvement and plasma leakage resulting from increased vascular permeability. In its extreme presentation, dengue shock syndrome (DSS), DHF is potentially fatal (1,3,4).

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Epidemic dengue and DHF increased dramatically in the last decades of the 20th century, especially in the New World. The causes of the increased incidence of DENV infection include demographic, cultural, environmental and political changes that have been extensively reviewed (2,3,5). The reasons for the dramatic increase in DHF have been the subject of much speculation. One hypothesis is that sequential epidemics are now arising from the increase in trafficking of different DENV serotypes and strains. This has lead to an increase in the frequency of secondary infections and the occurrence of the immune-enhancement phenomenon, which is a major risk factor for DHF/DSS (2,6). Another hypothesis is that increased travel and commerce have facilitated the dispersal of more fit and more virulent DENV strains (7,8). Such strains displace other, less virulent, indigenous strains that would have otherwise immunized the populations without causing significant mortality. There is evidence to support both hypotheses; however, it seems that the relative importance of these two factors is not the same everywhere. In Southeast Asia, immunological priming by a previous serotype is the most important risk factor for developing DHF/DSS (9–11). However, in the Americas, the severity has been more strongly associated with the circulation of a DENV-2 strain of Asian origin introduced in the early 1980s (12–14).

In the Americas, dengue has been present since the 17th century, but most outbreaks were isolated in time and space (3,15). During the 1950s and 1960s, the Pan American Health Organization (PAHO) led a campaign to eradicate *Ae. aegypti*, which resulted in the disappearance of DF in most of Central and South America. However, DENV-2 and DENV-3 remained in some Caribbean islands during those years (3,16). In the 1970s the PAHO campaign gradually eroded and *Ae. aegypti* reappeared in most countries. Dengue followed soon after, and in 1977 and 1981 DENV-1 and DENV-4, respectively, were introduced and caused repeated outbreaks in the region (15). DF was the most frequent clinical presentation, although a few severe cases were reported. DHF/DSS emerged abruptly in 1981 in Cuba and caused about 150,000 hospitalizations and more than 150 deaths in less than 4 months (17). An intensive eradication program stopped the outbreak, and DHF did not spread beyond Cuba. DHF reappeared in Venezuela in 1989, and soon after it was detected in other countries of South America. DHF was not diagnosed in Central America until 1994, concurrently with the reintroduction of DENV-3, which had not been detected in the Americas since 1977 (16).

In Mexico, from 1947 to the 1960s, health authorities conducted intensive campaigns to control *Ae. aegypti*, and the mosquito was declared eradicated from the country in 1963 (18). However, *Ae. aegypti* reappeared 2 years later. Major epidemics of DF caused by DENV-1 occurred on the eastern coast of Mexico during 1979 and 1980. Approximately 17,000 cases of dengue were reported in 1981 (19) (Table 1). DENV-4 caused a major epidemic in 1984 in the Yucatan peninsula; there were more than 5,000 reported cases and four fatalities, but only one case met all WHO criteria for DHF (20). In 1984 and 1985, DF was diagnosed in 25 of 32 states and DENV-1, 2, and 4 were reported throughout Mexico (15). DENV circulation decreased in the late 1980s and early 1990s but increased again in 1995 when DENV-3 was isolated for the first time and a few cases of DHF were confirmed (21). DHF appeared in epidemic proportions in 1996 and 1997. DENV-3 was the predominant serotype isolated in these years but all the other serotypes were also present. Following a period of relative calm (1999–2001), DHF increased again in 2002 (18) and more than 1000 cases per year have been reported since that year. The proportion of DHF/DSS cases out of the total dengue cases reported increased from near zero before 1994 to 1.4–4.0% during 1995–2000 to 14–28% in 2002–2004 (Table 1). The causes of the recent increase in the severity of clinical outcomes in Mexico have not been extensively studied.

Based on clinical and laboratory reports, some assumptions have been made about DENV circulation in Mexico. Because the southern states are usually the first and most

Table 1. Cases of DF and DHF reported and serotypes circulating in Mexico 1980–2005[a]

Year	DHF	DF + DHF	%DHF	Serotypes
1978	0	38	0	?
1979	0	6,187	0	?
1980	0	51,406	0	1
1981	0	17,040	0	1
1982	0	32,640	0	1, 2
1983	0	19,028	0	1, 2, 4
1984	8	27,653	0.03	1, 2, 4
1985	0	13,688	0	1, 2, 4
1986	0	19,708	0	1, 2, 4
1987	0	13,371	0	1, 4
1988	0	10,526	0	1, 4
1989	1	7,121	0.01	1
1990	8	9,524	0.08	1, 4
1991	2	5,865	0.03	1, 4
1992	0	11,925	0	1, 2, 4
1993	0	2,899	0	2
1994	30	8,102	0.37	1, 2
1995	539	36,568	1.47	1, 2, 3, 4
1996	1,456	36,538	3.98	1, 2, 3, 4
1997	980	53,541	1.83	1, 2, 3, 4
1998	372	23,639	1.57	1, 2, 3, 4
1999	212	23,194	0.91	1, 2, 3, 4
2000	67	1,781	3.76	1, 2, 3
2001	312	4,955	6.30	2, 3
2002	2,559	15,813	16.18	2, 3
2003	1,776	6,996	25.39	2, 3
2004	1,959	8,202	23.88	1, 2, 3, 4
2005[b]	2,937	15,554	18.88	1, 2, 3, 4

[a]Data from Dirección General Adjunta de Epidemiología, Secretaría de Salud, Mexico.
[b]Up to 43th epidemiologic week.

affected, it is commonly thought that dengue is periodically introduced from Central America through the southern frontier (18). Two different epidemiological patterns of DENV transmission have been identified: 1) an endemic pattern in coastal states of the Gulf of Mexico and 2) a seasonal pattern in the Pacific coast and the Yucatan peninsula (Figure 1) (18). However, two studies, one in the southeastern state of Yucatán conducted in 1986 (22) and a recent one in the western state of Colima demonstrated focal, low-level DENV transmission during inter-epidemic periods (23). Thus, the persistence of dengue in Mexico could result either from frequent reintroduction of the virus from neighboring DENV-endemic countries or from continual circulation within the country.

Phylogenetic analyses of sequence data can provide useful information concerning virus trafficking and disease emergence and persistence and on the importance of viral genotypes on the clinical outcome, providing that appropriate collections of isolates are available for analysis. Sequence analysis can also be used to investigate other important evolutionary parameters, such as nucleotide substitution rates, nucleotide diversity and natural selection (24–26). We previously attempted to determine the phylogenetic origin and epidemiological correlates of DENV strains circulating in the Yucatán peninsula of Mexico using sequences of the prM gene (27). Identification of the genotypes of DENV that circulated between 1980 and 1997 was possible and some inferences about their origin were made. However, the paucity of DENV prM gene sequences available in the GenBank database and the small size of the prM sequences analyzed generated phylogenies with limited resolution and branch support. The routes of DENV trafficking in the Western hemisphere were not resolved and definitive conclusions about the importance of viral factors in dengue severity could not be made.

In this work, we addressed the limitations of our prM analysis by sequencing and analyzing the complete E gene of isolates of all four DENV serotypes. The geographic representation of the Mexican DENV collection was expanded to at least nine states and was updated until 2002. Thus, it was possible to describe the origin, geographical dispersion and persistence of DENV strains in Mexico and other countries of the Americas. It was also possible to temporally associate recent changes in the incidence and severity of dengue with the introduction and circulation of different serotypes, genotypes and strains of DENV. Additionally, some evolutionary parameters were computed and used to derive useful information about forces acting in DENV evolution.

Materials and Methods

Viruses

Most of the Mexican viruses analyzed were originally isolated in the "Centro de Investigaciones Regionales Dr. Hideyo Noguchi" of the Universidad Autónoma de Yucatán, Mérida, Mexico. Other isolates were obtained from collections at the University of Texas Medical Branch, Galveston (UTMB), the Dengue Branch, San Juan Laboratories (SJL), Center for Disease Control and Prevention, Puerto Rico, and the Instituto Nacional de Salud Pública

Figure 1. Distribution of DENV transmission patterns in Mexico according to Navarrete et al. (18) and sites of isolation of the viruses analyzed in this study. Place of isolation was not known for 13 isolates.

(INSP), Cuernavaca, Mexico. Isolates used in the analyses (Table 2) were selected to represent most years and regions with dengue activity in Mexico. Figure 1 shows the approximate locations where the viruses were isolated. The origin of the samples was unknown for 13 isolates. All Mexican viruses analyzed were isolated from DF cases. Some sequences had been previously obtained and studied by others (28–31). Eight non-Mexican DENV strains not previously reported were also included in this study (Table 2). Isolates were between second and fourth cell culture passage in *Aedes albopictus* C6/36 cells.

Molecular Procedures

Viral genomic RNA was extracted from cell culture supernatant using a silica-based method (QIAamp viral RNA, Qiagen, Valencia, CA). RNAs were copied into cDNAs using reverse primers annealing in the NS1 gene (Table 3) and RAV-2 reverse transcriptase (Amersham, Piscataway, NJ). cDNA segments between 2,474 and 2,577 nucleotides encompassing the prM and E genes of DENV were amplified by PCR using the consensus forward primer D1 (32) and serotype-specific reverse primers (Table 3). DNA polymerase used was *Taq* DNApol (Promega, Madison, WI). PCR products were verified by 0.8% agarose gel electrophoresis. The PCR products were then purified using either the QIAquick PCR purification kit or the QIAquick gel extraction kit (Qiagen). Both strands of the amplicons were sequenced using the primers listed in Table 3 and the Big Dye Terminator Cycle Sequencing System (ABI, Foster City, CA). Sequences obtained were submitted to GenBank. Accession numbers are provided in Table 2.

Sequence Analyses

Sequences corresponding to the entire E gene of each serotype were aligned using Clustal X package (33) with sequences previously obtained and submitted to GenBank (28–31,34–37). Sequences of prototype strains of the other three serotypes were included to root the trees. Strains belonging to all major branches of previously published DENV phylogenies were included in the analyses and were selected to maximize the representation of genotypes, countries and years of isolation. Sequences that were very closely related to one another in the data set as well as those believed to represent recombinant events were removed from the analyses.

Phylogenies were estimated with maximum parsimony, distance/neighbor joining, and Bayesian analyses. Only minor differences were observed among the results produced by these methods. Only Bayesian phylogenies are provided. Separated analyses for each serotype were performed using MrBayes 3.0 program (38). They were run with a general time reversible (GTR) substitution model. Substitution rates were assumed to follow a gamma plus invariants distribution, and the α shape parameter was estimated from the data. The shape parameter was estimated independently for the third codon position. Four MCMC tree searches of a million generations each were run simultaneously, sampling one in every 100 trees and computing a 50% majority-rule consensus tree out of the last 8,000 sampled trees. The consensus trees were drawn with the TreeView program (39).

Tests for natural selection and measures of nucleotide diversity were performed independently for each serotype using the DnaSP 4.0 software package (40). Fu and Li's F* (41) and Tajima's D (42) test the null hypothesis of neutral evolution, based on the distribution of polymorphisms (43). Diversity indexes included number of variable sites between all pairs of aligned sequences (K), numbers of parsimony informative sites, and the mean pairwise nucleotide diversity (π) (43). Nonsynonymous to synonymous diversity ratio (πns/πsy) is an indicator of selective pressures at the protein level, with ratios >1, <1 and ~1 indicating positive selection, purifying selection and neutral variation, respectively (44).

Results

General data about the amount and distribution of variability in the four alignments, including parameters and statistics relevant to the phylogenetic analysis and detection of natural selection, are presented in Table 4. All measurements of diversity were consistently greatest for DENV-2 and least for DENV-4. Variation was strongly concentrated at synonymous sites as demonstrated by πns/πsy ≤ 0.060. Fu and Li's F* and Tajima's D statistics were negative in all serotypes indicating strong purifying selection (43). Parsimony informative substitutions in Mexican viruses are listed in Table 5. Bayesian phylogenetic trees are presented in Figures 2–5. The outgroups were excluded from the Figures.

DENV-1

The inferred phylogeny of serotype 1 is shown in Figure 2. There were five major branches that correspond to the previously described genotypes (28). The only known sylvatic strain of this serotype (Malaysia/72 P72) formed an independent major branch (genotype III). Genotypes I, II, and IV were comprised mostly of viruses from Asia and the Pacific. Genotype V included three different branches, containing isolates from Asia, Africa and the Americas, respectively. The American isolates formed a monophyletic group that resolved as a polytomy with four isolates branching independently and four clades originated from this polytomy. One clade is comprised of Mexican viruses isolated in the 1980s. Mexican isolates from the 1990s appear in a different clade, which also contains viruses from Aruba, French Guayana and the isolate PRS288690/Jamaica/77

Table 2. DENV isolates from Mexico and sequenced and/or analyzed in this study

	Mexican isolates sequenced in this or other studies				
Isolate ID	DENV	State	City	Year	GenBank accesion #
1298/YUCATAN-MX/80*	1	Yucatán	Mérida	1980	AF425623
1379/MEXICO/82	1	Unknown	Unknown	1982	DQ341188
1378/MEXICO/83*	1	Unknown	Unknown	1983	AF425624
1462/PUEBLA-MX/84	1	Puebla	Unknown	1984	DQ341189
1463/SONORA-MX/84	1	Sonora	Unknown	1984	DQ341190
1756/MEXICO/86	1	Unknown	Unknown	1986	DQ341191
3425/YUCATAN-MX/94	1	Yucatán	Mérida	1994	DQ341192
4642/CAMPECHE-MX/95	1	Campeche	Campeche	1995	DQ341193
4942/QUINTANA ROO-MX/95	1	Quintana Roo	Cozumel	1995	DQ341194
1421/MEXICO/83	2	Unknown	Unknown	1983	DQ341195
1482/MEXICO/84	2	Unknown	Unknown	1983	AY449675
131/SONORA-MX/92*	2	Sonora	Navojoa	1992	AY158332
3315/QUINTANA ROO-MX/94	2	Quintana Roo	Unknown	1994	DQ341196
328298/REYNOSA-MX/95*	2	Tamaulipas	Reynosa	1995	AY158338
BC17/YUCATAN-MX/96	2	Yucatán	Mérida	1996	AY449677
C932/GUERRERO-MX/97	2	Guerrero	Acapulco	1997	AY449678
C1077/GUERRERO-MX/97	2	Guerrero	Chilpancingo	1997	AY449679
Oax468/OAXACA/00*	2	Oaxaca	Juchitán	2000	AY158341
12021/YUCATAN-MX/01	2	Yucatán	Oxcutzcab	2001	AY449682
12914/YUCATAN-MX/01	2	Yucatán	Tekax	2001	AY449680
13381/YUCATAN-MX/02	2	Yucatán	Chocholá	2002	AY449683
13404/YUCATAN-MX/02	2	Yucatán	Opichén	2002	AY449685
6097/MEXICO/95*	3	Unknown	Unknown	1995	AY146763
4841/YUCATAN-MX/95	3	Yucatán	Mérida	1995	DQ341202
6584/YUCATAN-MX/96	3	Yucatán	Hunucmá	1996	DQ341203
6883/YUCATAN-MX/97	3	Yucatán	Hocabá	1997	DQ341204
6889/QUINTANA ROO-MX/97	3	Quintana Roo	Can Cún	1997	DQ341205
6896/QUINTANA ROO-MX/97	3	Yucatán	Quintana Roo	1997	DQ341206
OAXACA-MX/00	3	Oaxaca	Unknown	2000	DQ341207
1414/MEXICO/83	4	Unknown	Unknown	1983	DQ341210
1420/MEXICO/83	4	Unknown	Unknown	1983	DQ341211
1503/YUCATAN/84	4	Yucatán	Mérida	1984	DQ341212
1551/MEXICO/85	4	Unknown	Unknown	1985	DQ341213
1554/MEXICO/85	4	Unknown	Unknown	1985	DQ341214
MEXICO/91*	4	Unknown	Unknown	1991	AY152378
111/MEXICO/95*	4	Unknown	Unknown	1995	AY152304
4915/YUCATAN-MX/95	4	Yucatán	Oxcutzcab	1995	DQ341215
4959/QUINTANA ROO/95	4	Quintana Roo	Benito Juárez	1995	DQ341216
5962/YUCATAN-MX/96	4	Yucatán	Izamal	1996	DQ341217
6637/YUCATAN-MX/97	4	Yucatán	Tzucacab	1997	DQ341218

Non-Mexican isolates sequenced and analyzed in this study

Isolate ID	Type	Country	Year	
IBH11208/NIGERIA/66	2	Nigeria	1966	DQ341197
1328/PUERTO RICO/77	2	Puerto Rico	1977	DQ341198
S9/SOMALIA/93	2	Somalia	1993–4	DQ341199
620/BORNEO/88	2	Unknown	1988	DQ341200
541/NICARAGUA/99	2	Nicaragua	1999	DQ341201
S142/SOMALIA/93	3	Somalia	1993	DQ341208
PANAMA/94	3	Panama	1994	DQ341209
371813/COLOMBIA/96	4	Colombia	1996	DQ341219

*Isolates sequenced in one of the following studies: Goncalvez et al. (DENV-1) (18), Leitmeyer et al. or Armstrong and Rico-Hesse (DENV-2) (29,34), Uzcategui et al. (DENV-3) (30), Foster et al. (DENV-4) (31).

Table 3. Primers used for E gene amplification and sequencing

Primer	Sequence 5′ → 3′	Use
D1	TCAATATGCTGAAACGCGCGAGAAACCG	PCR
D1-682F	AACCGGYGAACACCGACGAGA	Sequencing
D1-1064F	GAACTCTTGAAGACGGAGGTCACGAA	Sequencing
D1-1167R	TTGTTCTTCCACCAGTGTAGCCTCTC	Sequencing
D1-1488F	GCTCACCTAGAACAGGGCTGGACTTT	Sequencing
D1-1649R	TTCTTTGCATGAGCTGTCTTGAATGT	Sequencing
D1-1972F	CCAGAATGGGAGATTGATAACA	Sequencing
D1-2125R	CGGTTGCTTCGAACATTTTCCCTATG	Sequencing
D1-2726R	ATGGGTTGTGGCCTAATCAT	RT, PCR, Sequencing
D2-739F	ATGGGATTGGAGACACGAACTGAA	Sequencing
D2-1184F	ATGAAGAGCAGGACAAAAGGTT	Sequencing
D2-1225R	CCATTTCCCCATCCTCTGTCTAC	Sequencing
D2-1540F	GAAGACAAAGCTTGGCTGGTG	Sequencing
D2-1648R	GCATGGGGATTTTTGAARGTGAC	Sequencing
D2-2028F	GGAGGTTCTGCTTCTATGTTGACT	Sequencing
D2-2120R	TCAAACATTTGGCCGATRGAACTTC	Sequencing
D2-2588R	TCTTGTTACTGAGCGGATTC	RT, PCR, Sequencing
D3-644F	CTTACATCAACATGGGTGACTTAT	Sequencing
D3-1343F	TACACCGTCATCATCACAGTG	Sequencing
D3-1390R	CTTACATCAACATGGGTGACTTAT	Sequencing
D3-1671F	AAGAAGTAGTTGTCCTTGGAT	Sequencing
D3-1953R	CATTGTGAGCTTTCCCTTGTC	Sequencing
D3-2429R	TTCTTTGCCTTTCCAGTTTAT	RT, PCR, Sequencing
D4-762F	GAGACATGGATGTCATCGGAAGG	Sequencing
D4-1240F	GGGGCAATGGCTGTGGCTTGTT	Sequencing
D4-1308R	TTGGACCAAATTGCCTGTTATCTT	Sequencing
D4-1667F	YCATGCCAAGAGACAGGATGTGAC	Sequencing
D4-1702R	GCAAGAATGCATGGCTCCTTCCTGAG	Sequencing
D4-2158R	GAATGGCCATTCGTTTTGCACCTC	Sequencing
D4-2052F	CCYTTTGGGGACAGCGCTACATA	Sequencing
D4-2649R	TGTCCTCCTTCCCAGAGAACATAGTT	RT, PCR, Sequencing

believed to represent the original DENV-1 strain introduced that year to the Western Hemisphere. The other clades and independent branches contain mostly South American viruses.

DENV-2

The phylogenetic tree for DENV-2 is shown in Figure 3. Most genotypes previously described (26) are strongly supported, but the sylvatic strains segregated into two different branches according to their Asian or African origin. The last one includes the IBH11208/NIGERIA/66, an isolate not previously reported. Most isolates of the Western hemisphere are located either in the American or the American-Asian genotypes. However, the Mexican isolates segregated into four different genotypes. Strains isolated from 1983–1995 are in the American genotype. One 1996 isolate grouped with viruses in the ''Cosmopolitan'' genotype (26). Two isolates from 1997 clustered in the Asian-2 genotype near the prototype DENV-2 NGC/New Guinea/44, the Cuban strain of 1981 and some recent isolates from Asia. Finally, all Mexican DENV-2 isolates from 2000 to 2002 grouped in the American-Asian genotype.

DENV-3

The phylogenetic tree of DENV-3 is presented in Figure 4. The tree is composed of five previously described branches (36,45). All the American DENV-3 isolates subsequent to the reintroduction of that serotype in 1994 form a monophyletic group inside genotype III. These viruses are more closely associated with African strains from Mozambique and Somalia than with the other isolates from Asia and the Pacific. Most Mexican isolates from 1995–2000 form a monophyletic group but the 4841/Yucatán-Mx/95 clusters with Panama/94 and 24/Nicaragua/94, two of the first isolates of the reintroduced strain (46,47). This separate clustering is not strongly supported. Isolates from South America and the Caribbean segregated in two independent and strongly supported clades.

DENV-4

The phylogenetic tree of DENV-4 isolates is presented in Figure 5. In agreement with a previously published work (48), there are three major branches or genotypes although genotype II has low support. Three strains isolated from rural mosquitoes or monkeys in Malaysia formed the strongly

Table 4. Diversity indexes and tests for selection in the four sequence datasets

Parameter or statistic	DENV-1	DENV-2	DENV-3	DENV-4
# of sequences in the alignment (*n*)	42	52	40	46
Length (nt) of the alignment (L)	1485	1485	1479	1485
# of variable sites (K)	441	564	367	354
# of parsimony informative sites	307	433	256	285
Nucleotide diversity (π) total	0.059	0.081	0.050	0.040
π at non-synonymous sites (πs)	0.010	0.013	0.010	0.007
π at synonymous sites (πsy)	0.212	0.307	0.178	0.148
πns/πsy diversity ratio	0.047	0.042	0.056	0.047
Fu & Li's F* statistic	−1.048	−0.840	−0.801	−0.400
Tajima' D statistic	−0.874	−0.816	−0.709	−1.190

divergent sylvatic genotype. Most strains from Asia were in genotype I (35). However, the isolate 1132/Indonesia/77 appeared in a basal position of genotype II, suggesting that it is close to the ancestor of all the American DENV-4 isolates. S44754/Tahiti/79 and 5489/New Caledonia/81 strains are also in genotype II and are very close to the Dominica/81 strain, believed to represent the original introduction of this serotype in the Americas, and to other ''early'' (1981–1984) American isolates. These early American isolates correspond to the paraphyletic ''group A'' described by Foster et al. (31). American DENV-4 isolated in 1985 or later segregated in three different branches: most strains isolated in the Caribbean islands, as well as one Venezuelan and one Costa Rican isolate, formed a branch that corresponded to ''clade B'' described by Foster et al. (31). Another branch, here named ''clade C'', is comprised of isolates from Honduras, Ecuador and Colombia. The remaining ''clade D'' included all the Mexican isolates of 1985 and thereafter as well as two isolates from El Salvador.

Discussion

Phylogenetic analysis is a powerful approach to determine the geographical and biological origin of emerging viruses as demonstrated in studies with human immunodeficiency virus (HIV) and West Nile virus (WNV) (49,50). The pioneering work by Rico-Hesse using a short segment of 240 nucleotides of DENV revealed the extent of variation and general distribution of lineages of DENV-1 and DENV-2 (12). Most recent phylogenies of DENV are based on the full E gene, which provided better resolution, allowed description of new lineages, and revealed in situ evolution in viruses isolated in close temporal and geographical proximity (28,31,37). Here we used Bayesian analysis, a phylogenetic method that estimates the most likely evolutionary

Table 5. Shared nucleotide and amino acid changes observed in Mexican clades

Serotype	Years of isolation	Position in E gene	Nucleotide change	Amino acid change
DENV-1	1980–1986	33	C→T	No
	1982–1984*	217	A→C	No
	1984–1986*	234	A→C	No
	1980–1986*	240	T→C	No
	1982–1984*	507	T→C	No
	1980–1986*	752	T→C	Val→Ala
	1984–1986*	888	G→A	No
	1984–1986*	957	T→C	No
	1984–1986*	1016	C→T	Thr→Ile
	1980–1986	1075	A→G	Thr→Ala
	1982–1984*	1252	A→G	Thr→Ala
	1984–1986*	1419	G→A	No
	1994–1995	57	G→A	No
	1994–1995	375	A→G	No
	1994–1995	394	T→C	Tyr→His
	1994–1995	960	T→C	No
	1994–1995	1013	C→T	Ser→Leu
	1994–1995	1152	A→G	No
	1994–1995	1230	A→G	No
	1994–1995	1266	C→T	No
	1994–1995	1482	A→G	No
DENV-2	1983–1984*	723	A→G	No
	1983–1984*	1178	A→G	Lys→Arg
	1983–1984*	1413	T→C	No
	1983–1995*	1374	C→T	No
	1992–1995*	285	C→T	No
	1992–1995*	381	A→G	No
	1992–1995	783	T→C	No
	1992–1995*	801	T→C	No
	1992–1995	813	G→A	No
	1992–1995*	1362	T→A	No
	1992–1995*	1386	C→T	No
	1997	157	C→T	Pro→Ser
	2000–2001	121	T→C	No
	2000–2001	753	T→C	No
	2001	137	T→C	Ile→Thr
	2001	630	G→A	No
	2001	750	C→T	No
	2002	1293	T→C	No
DENV-3	1995–2000*	48	A→T	No
	1995–2000*	282	T→C	No
	1997	492	T→C	No
DENV-4	1985–1997	210	G→A	No
	1991–1997	480	T→C	No
	1991–1995*	564	G→A	No
	1991–1995*	680	C→T	Ser→Leu
	1991–1997	918	T→C	No
	1991–1997*	1029	C→T	No
	1991–1997	1051	A→G	Ile→Val
	1991–1997*	1083	C→T	No
	1991–1997	1257	T→C	No
	1991–1997	1380	G→T	No
	1995–1997*	645	T→C	No
	1995–1997*	732	T→C	No
	1995–1997*	1428	T→C	No

*Substitution not observed in one or more isolate of these years.

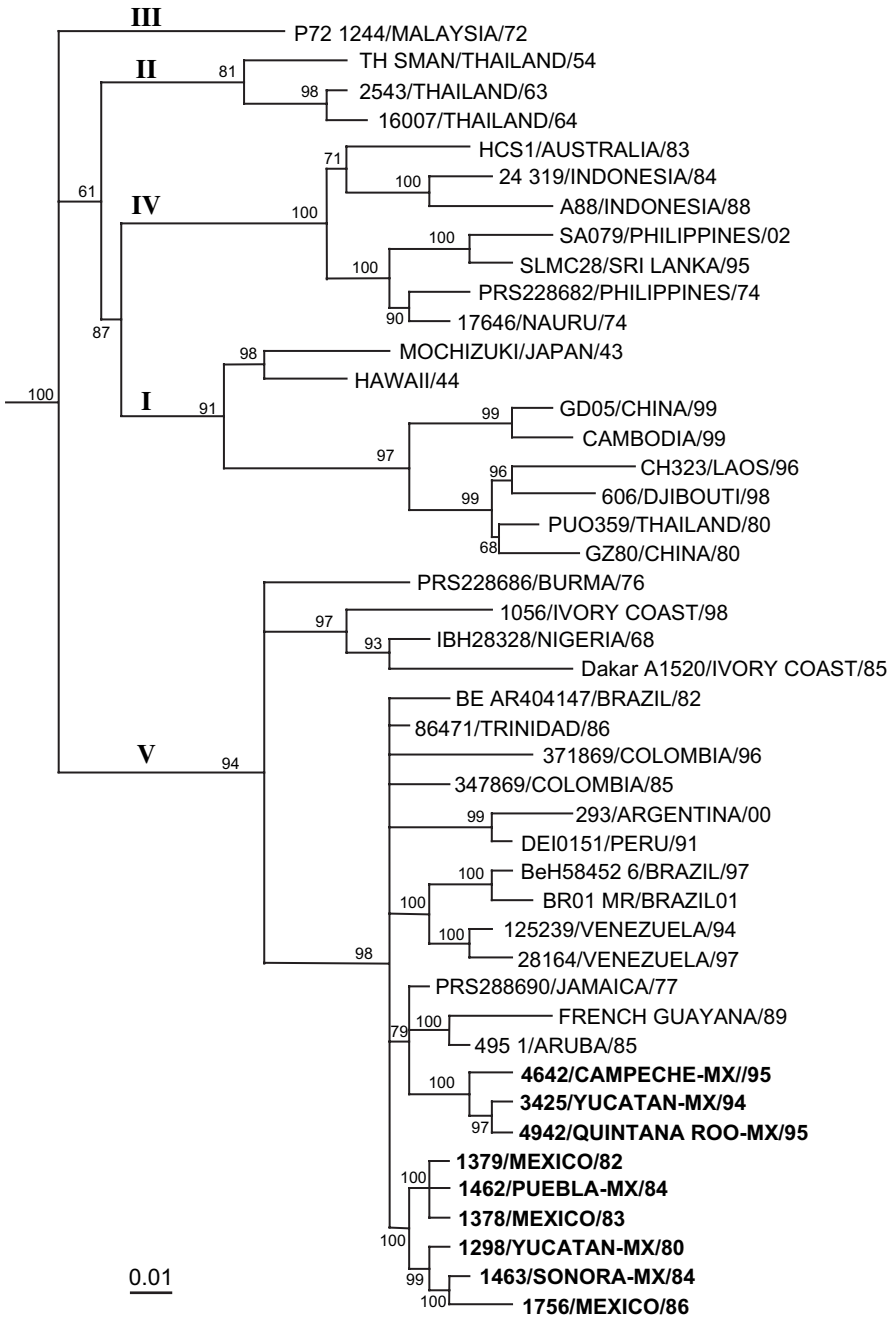

Figure 2. Bayesian phylogenetic tree derived from DENV-1 E gene sequences. Numbers near nodes are the posterior probabilities of the corresponding clade. Genotypes designated by Goncalvez et al. (28) appear as roman numerals. The tree was rooted with prototype strains of the other serotypes. Mexican viruses appear in bold.

tree under a maximum likelihood framework given a model of nucleotide substitution. It also estimates the posterior probability of each clade in the tree. The resulting probability values are less conservative estimates of branch support than bootstrap estimates, so that only values ≥95% are considered significant.

The phylogenetic analyses suggest single introductions of DENV-1 and DENV-4 into the Americas in recent decades (Figures 2 and 5). This and previous studies (51,52) indicate that American DENV-4 genotypes are derived from a strain that circulated in the Pacific during the late 1970s and early 1980s. Note the close similarity of isolates from Tahiti 1979 and New Caledonia 1981. In contrast, the origin of the American DENV-1 cannot be inferred from the phylogenetic tree (Figure 2). The long branches that connect it with African and Asian strains suggest that no sequence representative of the ancestor was included in the analysis. The published sequence of S275/Singapore/90

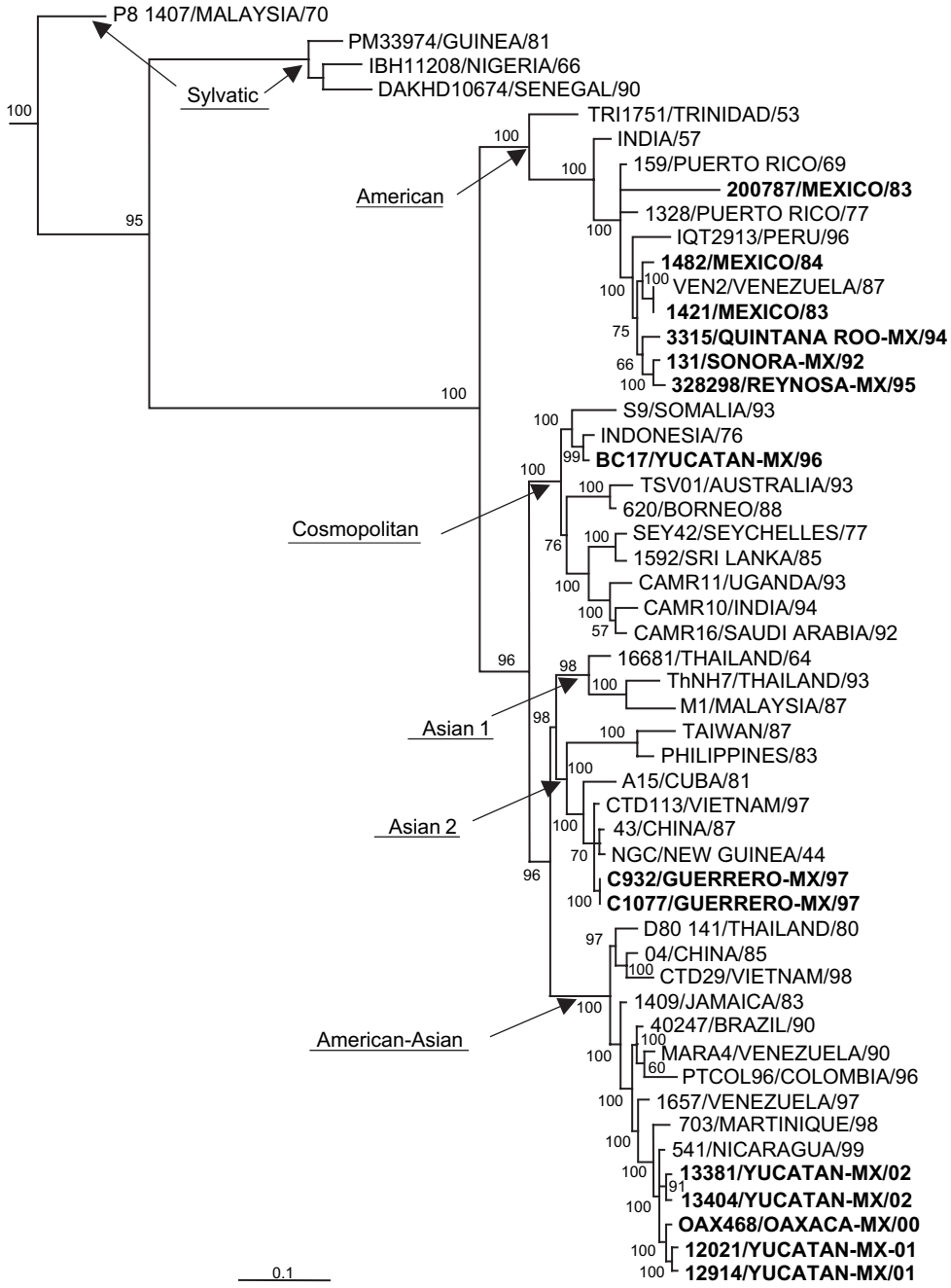

Figure 3. Bayesian phylogenetic tree derived from DENV-2 E gene sequences. Numbers near nodes indicate posterior probabilities of the corresponding clade. Genotypes designated by Twiddy et al. (26) are underlined. The tree was rooted with prototype strains of the other serotypes. Mexican viruses appear in bold.

strain is closer to the American DENV-1 than any other studied to date (28), but it was excluded here because it appears to be a recombinant virus (53). The large polytomy from which American DENV-1 strains emerge suggests that there was a very rapid radiation of that serotype in different countries and independent lineages were formed after its putative introduction in 1977.

The introduction of a new DENV-3 strain into Central America in 1994, different from that circulating during 1960s and 1970s, has been previously documented (21,46,47,54) and an Asian origin for this strain has been proposed (3,55,56). However, by including the S142/Somalia/93–94 isolate from an American soldier returning from that country (57), we found that the new American DENV-3 strain is phylogenetically closer to African strains (Figure 4) as previously noted (58). Nevertheless, this grouping is supported by only two shared-derived characters at positions 588 and 633 (data not shown) and its

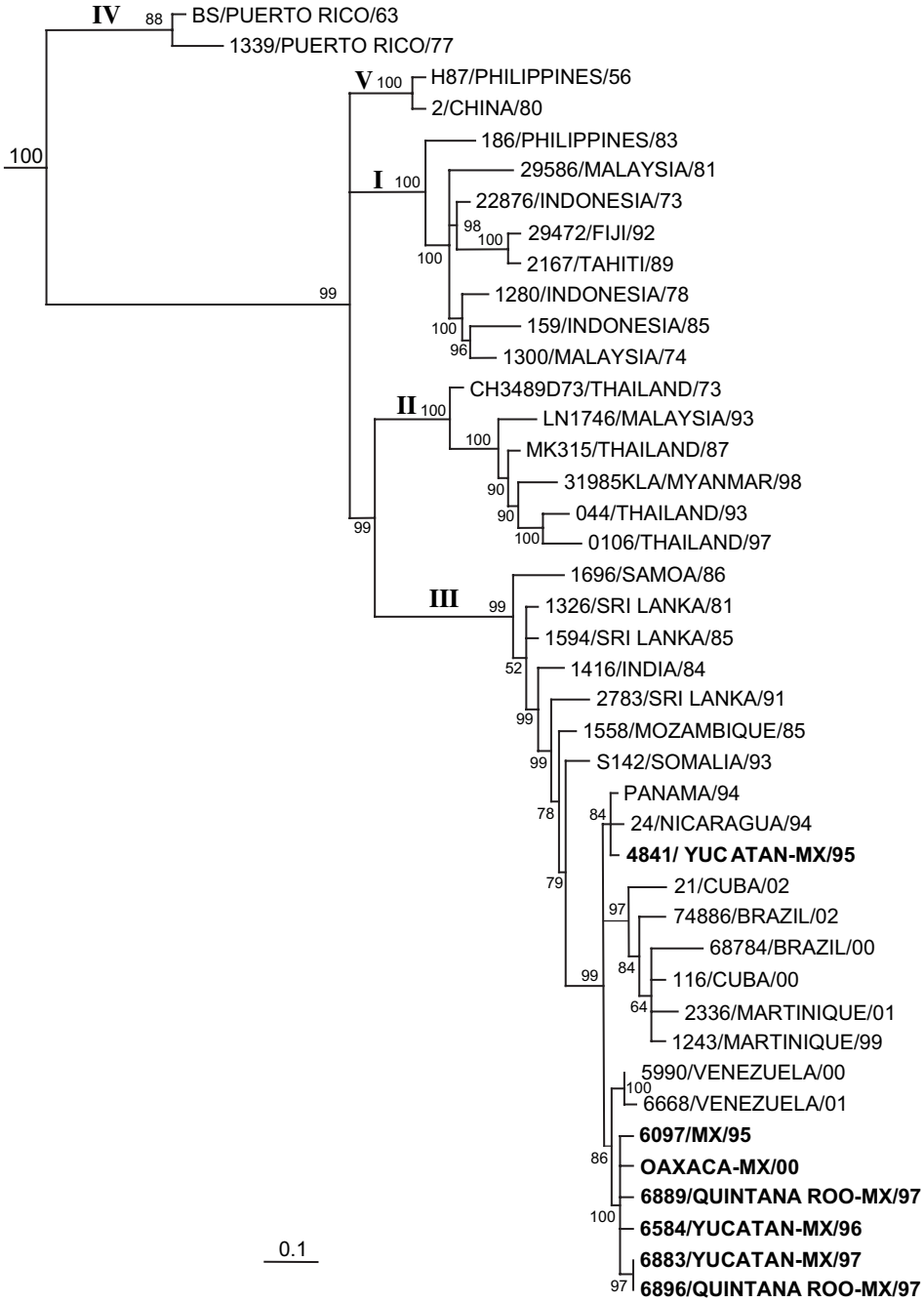

Figure 4. Bayesian phylogenetic tree derived from DENV-3 E gene sequences. Numbers near nodes are the posterior probabilities of the corresponding clade. Genotypes designated by Wittke et al. (45) appear in roman numerals. The tree was rooted with prototype strains of the other serotypes. Mexican viruses appear in bold.

posterior probability is low. A conclusive assertion about the origin of this strain requires the analysis of more African and Asian DENV-3 from the early 1990s.

The phylogeny of DENV-2 is different from the other serotypes in that several genotypes have been recently circulating in Mexico and other Central and South American countries (Figure 3). The progressive displacement of the old American DENV-2 genotype by viruses originating in Asia and the concomitant emergence of DHF had been previously documented (12,13). This displacement took place in the Caribbean during the 1980s, in northern South America and Brazil in the early 1990s and in Central America, Bolivia and Peru in the late 1990s (13,54,59,60). In Mexico the latest report of the American genotype was 1995 and the earliest of the American-Asian was 2000 (8). We found that the isolate BC17 from 1996 grouped in the Cosmopolitan

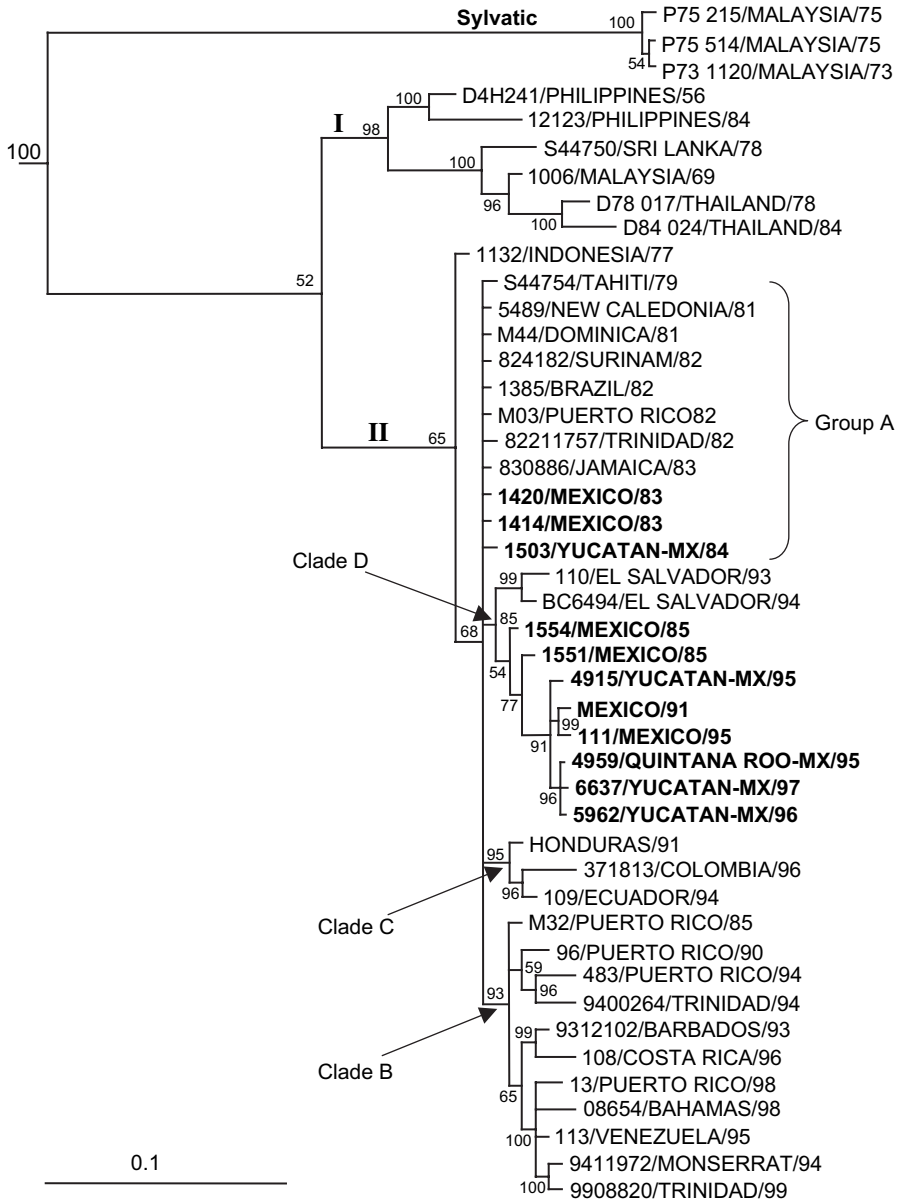

Figure 5. Bayesian phylogenetic tree derived from DENV-4 E gene sequences. Numbers near nodes are the posterior probabilities of the corresponding clade. Genotypes designated by Lanciotti et al. (35) appear in roman numerals, except the sylvatic genotype (48). Clades and groups are designated with letters following Foster et al. (31) and this work. The tree was rooted with prototype strains of the other serotypes. Mexican viruses appear in bold.

genotype also known as Indian subcontinent genotype or subtype IV (54,61). This isolate is not closely related to any virus reported in the Americas and similar isolates have not been detected thereafter, suggesting that its circulation in this hemisphere was limited. The isolates C-932 and C-1077 from samples collected in the southwestern state of Guerrero in 1997 grouped in Asian-2 genotype (26). Similar topology had been reported in viruses isolated in Mexico in the southern state of Chiapas and in the central state of San Luis Potosi in 1995 (13) as well as from samples from Cuba in 1981 (14,62), Honduras 1986 (59) and Venezuela in 1994 and 1995 (13). Similar sequences have also been reported from recent isolates from China and Vietnam (26). Rico-Hesse has argued that because of the close similarity of these sequences with the old prototype DENV-2 NGC from 1944, they could correspond to laboratory contaminations with that strain, which has been maintained as a reference virus in many laboratories around the world for more than 50 years (54). Indeed, the genetic distance among the old prototype strain and the recent isolates in this group is less than expected from the DENV substitution rate (59). However, the repeated finding of such sequences in different laboratories from different countries makes it difficult to believe that the same mistake has

happened many times. It is possible that the old NGC strain could have escaped from one or several laboratories and started recirculating in different countries. Introduction and circulation of viruses that were stored for years is possible as exemplified by the Russian flu epidemic in 1977 (63). More studies will be necessary to settle this point.

Phylogenetic analyses of isolates from DHF cases or epidemics have implicated viral factors as important determinants of clinical outcome (13). We could not test this hypothesis because of the lack of DHF-derived isolates in our collection. However, we correlated changes in the circulation of specific serotypes or genotypes with specific clinical and epidemiological outcomes at the population level. The severity of dengue in Mexico seems to have increased twice (Table 1). In the mid-1990s, epidemic DHF was reported for the first time (18,21). This could have been caused by the introduction of DENV-3, which predominated in 1996–1998 and coincided with the epidemic. However, circulation of DENV-2 non-American genotype strains in those years could also have contributed to the increase in DHF. In 2001, another increase in the proportion of DHF/DSS cases occurred (18), following the introduction of the American-Asian genotype of DENV-2, at least in the southern part of the country (64). Importantly, several DENV serotypes have been co-circulating in Mexico since the early 1980s when DHF was rare (Table 1) (18,21). This suggests that hyperendemicity, the simultaneous circulation of several serotypes, is not necessarily sufficient by itself to result in epidemic DHF in the absence of virulent strains as previously reported (65). Thus, our studies indirectly support the role of the agent in the severity of the clinical outcome.

In the last few years several phylogenetic studies have focused on the forces affecting DENV evolution in particular countries or regions (25,28,30,37,45,49,66). DENV-1 has been present in Mexico since the late 1970s and DENV-2 and -4 since the early 1980s, but their presence was not detected every year (Table 1). Phylogenetic analysis might reveal whether those years with no serotype detection correspond to local extinctions followed by reintroduction or to deficiencies in surveillance during interepidemic periods. We found that DENV circulation in Mexico is characterized by frequent episodes of introduction, expansion, apparent extinction, and reintroduction of the same or different viral genotypes. For example, DENV-1 isolates detected in 1994–1995 clustered in a different clade from those isolated between 1980 and 1986 (Figure 2); none out of 12 shared substitutions observed in isolates from the 1980s appeared in isolates from the 1990s (Table 5), suggesting that the strain prevailing in the former decade was lost by random genetic drift, perhaps during the bottleneck occurring in 1993 (Table 1). A similar situation happened with the American genotype of DENV-2, in which only one of four shared substitutions appearing during 1983–1984 was conserved in isolates from 1992–1995 (Table 5). The former strain could have disappeared during the late 1980s when no DENV-2 circulation was detected (Table 1). However, we acknowledge that sampling bias could also have produced these patterns. Analyses of a larger number of isolates are necessary to resolve this issue. DENV-4 exhibits a different history since all isolates were from the 1985–1996 cluster in the same clade, suggesting continuous within-country circulation and evolution. No isolates of this serotype were obtained in some years, e.g., 1989, 1993 and 1994.

Virus trafficking seems to have played a major role in both the incidence and the severity of the clinical presentation of dengue in Mexico and other countries, although it is clearly not the only factor involved. It is not known, for example, why the introduction of the DENV-2 American-Asian genotype into Jamaica was not accompanied by an outbreak of DHF in that country, which happened later in South and Central America and Mexico (16,60,64). Differences in the immune status and genetic background of the respective populations must be interacting with the virulence of the agent to determine the clinical outcome and the epidemiological behavior of dengue. Overall, it is likely that hyperendemicity and virulent strains of DENV act synergistically to condition epidemic DHF. Clearly, however, introduction of new DENV genotypes and serotypes is a major risk factor for epidemic dengue and severe disease. Increased surveillance for such introductions is certainly warranted.

Nucleotide diversity was highest in DENV-2, intermediate in DENV-1 and DENV-3 and lowest in DENV-4 (Table 4). These differences could be partially due to sampling. In this study more isolates were included from the Americas than from other continents. American strains segregated into four genotypes of DENV-2, but only into two, one and one genotypes of DENV-3, -1 and -4, respectively. Thus, it is understandable that the average nucleotide diversity would be greater for DENV-2 (Table 4). Some serotypes have circulated in the Americas longer than others: since at least 1954 for DENV-2, 1966 for DENV-3, 1977 for DENV-1 and 1981 for DENV-4. Persistence for long times allows a larger number of substitutions to accumulate. These factors may also account for the greater level of differentiation in DENV-2 and the lower in DENV-4. Thus, differences in strains trafficking into the Americas and differences in amounts of time for virus evolution probably account for the different levels of variation observed among serotypes.

Sequence data collected in this study also provide insight into the forces acting in DENV evolution. The ratios of diversity at non-synonymous and synonymous sites ($\pi ns/\pi sy$) in the aligned sequences were between 0.042 and 0.056, which indicates that mutations leading to amino acid substitutions were strongly constrained. Since codon bias does not appear to play a significant role in DENV evolution (67), synonymous substitutions accounting for most

of the variations observed are presumed to be neutral and more likely fixed by genetic drift. However, we cannot rule out that some punctual non-synonymous changes or that a few of the synonymous substitutions altering the secondary structure of genomic RNA were positively selected. We can say that positive selection accounts for a minimal proportion, if any, of the observed changes in the E gene. This assertion is supported by the negative value obtained for all serotypes in Tajima' D and Fu and Li's F* tests of neutrality (Table 4) and is in agreement with results reported by others (45,68). Thus, genetic drift and gene flow, represented by multiple introductions of foreign strains, are the predominant forces acting in DENV evolution in Mexico.

This is the most comprehensive study of the molecular epidemiology of dengue in Mexico. However, there are several issues that need more study. More work needs to be done to determine the association of virus genotypes with disease severity. Analysis of more isolates from the endemic zones like the states of the Gulf Coast and from interepidemic periods like 1987–1993 and 1998–1999 would strengthen the inferences about the dispersion and persistence of DENV strains inside the country. Future studies should address these issues by including more isolates, sequencing longer genomic sequences and using new analytical methods to study the molecular evolution of dengue viruses.

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