Arch Virol (2010) 155:1401–1412
DOI 10.1007/s00705-010-0721-1

ORIGINAL ARTICLE

Evolution of dengue virus in Mexico is characterized by frequent lineage replacement

Erik Carrillo-Valenzo · Rogelio Danis-Lozano · Jorge X. Velasco-Hernández · Gilma Sánchez-Burgos · Celia Alpuche · Irma López · Claudia Rosales · Cécile Baronti · Xavier de Lamballerie · Edward C. Holmes · José Ramos-Castañeda

Received: 12 February 2010 / Accepted: 1 June 2010 / Published online: 15 June 2010

Abstract Both dengue fever and its more serious clinical manifestation, dengue hemorrhagic fever, represent major public health concerns in the Americas. To understand the patterns and dynamics of virus transmission in Mexico, a country characterized by a marked increase in dengue incidence in recent years, we undertook a molecular evolutionary analysis of the largest sample of Mexican strains of dengue virus compiled to date. Our E gene data set comprises sequences sampled over a period of 27 years and representing all of the Mexican states that are endemic for dengue. Our phylogenetic analysis reveals that, for each of the four dengue viruses (DENV-1 to DENV-4), there have been multiple introductions of viral lineages in Mexico, with viruses similar to those observed throughout the Americas, but there has been strikingly little co-circulation. Rather, dengue virus evolution in Mexico is typified by frequent lineage replacement, such that only a single viral lineage dominates in a specific serotype at a specific time point. Most lineage replacement events involve members of the same viral genotype, although a replacement event involving different genotypes was observed with DENV-2, and viral lineages that are new to Mexico are described for DENV-1, DENV-3 and DENV-4.

Introduction

Dengue is one of the most important re-emerging infections in the Americas. The rise of dengue in this region is particularly striking given that many countries were declared free of the principal mosquito vector, *Aedes aegypti*, by the mid-1950s, which greatly reduced the levels of transmission of dengue virus [33]. However, the breakdown of mosquito-control measures precipitated the reappearance of both *A. aegypti* and dengue. An important case in point concerns Mexico, where re-emergent dengue

Electronic supplementary material The online version of this article (doi:10.1007/s00705-010-0721-1) contains supplementary material, which is available to authorized users.

E. Carrillo-Valenzo · J. Ramos-Castañeda (✉)
Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Av. Universidad 655, Santa María Ahuacatitlan, 621000 Cuernavaca, Morelos, México
e-mail: jcastane@correo.insp.mx

E. Carrillo-Valenzo
Departamento de Epidemiología, Servicios de Salud de Morelos, Cuernavaca, Morelos, México

R. Danis-Lozano
Centro Regional de Investigaciones en Salud Pública, Instituto Nacional de Salud Pública, Tapachula, Chiapas, México

J. X. Velasco-Hernández
Programa en Matemáticas Aplicadas y Computación, Instituto Mexicano del Petroleo, México D.F., México

G. Sánchez-Burgos
Unidad de Investigación Médica, CMN Ignacio García Téllez, Instituto Mexicano del Seguro Social, Mérida, Yucatán, México

C. Alpuche · I. López · C. Rosales
Instituto de Diagnóstico y Referencia Epidemiológicos, Secretaria de Salud, México D.F., México

C. Baronti · X. de Lamballerie
Unité des Virus Emergents, Université de la Méditerranée, Marseille, France

E. C. Holmes
Center for Infectious Disease Dynamics, Department of Biology, The Pennsylvania State University, University Park, PA 16802, USA

fever (DF) was first described in 1978. DENV-1 was the first virus detected, and cases occurred throughout the country until 1981, when DENV-2 was introduced, followed by DENV-4 in 1982 and DENV-3 in 1995. The first cases of dengue hemorrhagic fever (DHF) were reported in 1984 [20]. All four DEN viruses now cause regular dengue epidemics in Mexico, sometimes with relatively high levels of mortality due to DHF, and with a marked increase in incidence at locations close to the border with the United States [4, 26]. In total, some 26,500 cases of DHF were reported in Mexico between 1995 and 2007, and it now represents a major public health problem, with increases in dengue incidence noted in a number of states, such as Oaxaca [14].

Dengue is caused by a group of four viruses (DENV-1 to DENV-4; also referred to as serotypes) that are classified within the family *Flaviviridae* of single-stand positive-sense RNA viruses. Notably, each of the four viruses is comprised of a number of phylogenetically distinct lineages, termed 'genotypes' or 'subtypes', which differ in both geographical and sometimes clinical association [16, 27]. Although phylogenetic trees of DENV are typically drawn comprising all available genotypes, it is clear that DENV lineages, including whole genotypes, experience regular birth and death, such that some lineages proliferate while others perish (reviewed in ref. [15]). It is just such a process that has seemingly led to replacement of the American by 'Asian' genotype viruses in the Americas [28], while more fine-scale lineage replacement events have been observed in several Asian countries [32, 35, 36].

Although the renewed presence of the *A. aegypti* mosquito, coupled with a largely susceptible host population, is undoubtedly a major reason for the reappearance of dengue in the Americas, the factors that drive the irregular appearance of dengue epidemics in countries such as Mexico are unclear. In principle, the reasons underlying these distinctive epidemiological patterns could be either ecological or virological, involving such factors as climate change, the increasing global connectedness of human populations, rapid urbanization, and the presence of viral genotypes (or component lineages) that have elevated transmissibility in either the human or mosquito populations in this geographical region. Although it is tempting to invoke climate as a driving force of dengue epidemics, a recent analysis revealed that multiyear incidence patterns of dengue in Mexico could not be explained by either changes in precipitation, temperature, or the El Niño southern oscillation [17]. However, it is clear that other ecological factors are important in Mexico, as competence of the *A. aegypti* mosquito vector differs across the country in a manner that is explained by major geographic features, particularly the Neovolcanic axis [23].

Despite the public health importance of dengue in Mexico, there have been relatively few studies of the molecular epidemiology of DENV in this country. Most of the studies undertaken to date have tended to focus on specific geographic regions within Mexico, such as the Yucatan [8] or Oaxaca [5, 21, 25], have considered very small genetic regions [24], or have focused on the prM gene, even though most studies of DENV genetic variability on a global scale have considered the envelope (E) protein. The most comprehensive study to date was that of Díaz et al. [8], who surveyed the phylogenetic diversity in 40 E gene sequences representing all four DEN viruses sampled up to the year 2002. As is true of other geographical locations, clear cases of lineage replacement were noted within the Mexican population.

Our aim in this paper was to update the analysis of Díaz et al. [8] by examining patterns of sequence evolution in a large number (83) of E gene sequences sampled up to the year 2007 and from a variety of locations within Mexico. In particular, we wished to reveal the number of independent introductions of DENV into Mexico, the time-scale of these events, and the occurrence of all lineage replacement events. Given that the introduction of distinct viral lineages may trigger dengue epidemics, such a phylogenetic survey is of clear importance.

Materials and methods

Samples and E gene sequencing

All samples included in this study were obtained from the dengue virus collection of the Instituto de Diagnóstico y Referencia Epidemiológica, Secretaría de Salud, México. This produced a total 14 DENV-1, 16 DENV-2, 3 DENV-3 and 1 DENV-4 new samples (Table 1; Fig. 1). Samples consisted of acute serum or first-passage C6/36-infected cells from DF cases. Serotyping was performed either by immunofluorescence or RT-PCR [18, 19] according to the standard procedure recommended by PAHO/WHO. E gene sequencing was performed as described previously [29]. Specifically, RNA was extracted from 200 µl of the sample using a QIAmp viral RNA kit (Qiagen) according to the manufacturer's recommendations. cDNA was produced using random hexamers (Promega) and MuLV reverse transcriptase (Promega). Amplicons for direct sequencing were generated by PCR for the entire E gene using the following specific primers: DENV-1: EIF (AACAA GARCYGARACRTGGATGTC) and E4R (YARTTCAT TTGATATTTGYTTCCACAT); DENV-2: DEN2-env-S3 (ACACCATAGGRACGACRYATTT) and DEN2-env-R3 (CCRCTGCCACAYTTYAGTTCT); DENV-3: DEN3-E2-S (GARAARGTAGARACATGGGC) and DEN3-E2-R

(TCNGCYTGRAAATTTGTATTGCTC); DENV-4: DEN4-env-S2 (TGGATACTYAGAAAYCCAGGART) and DEN4-env-R2 (ACTCTGGTTGYAATTMGTACTG). All sequences generated here have been submitted to GenBank and assigned accession numbers HM171538–HM171571 (Table 1).

Phylogenetic analysis

All complete E gene sequences of the four DEN viruses from which the country and year of isolation were available were downloaded from GenBank and combined with these sequences generated here. Viruses sequenced by The Broad

Table 1 Isolates of DENV from Mexico newly sequenced as part of this study

Isolate	Year of sampling	Place of sampling	State	GenBank accession number
DENV-1				
D1/Mexico/Cuernavaca/2/2006	2006	Cuernavaca	Morelos	HM171557
D1/Mexico/Veracruz/23/2006	2006	Veracruz	Veracruz	HM171558
D1/Mexico/Mocorito/10/2006	2006	Mocorito	Sinaloa	HM171559
D1/Mexico/Jalpan de Serra/9/2006	2006	Jalpan de Serra	Queretaro	HM171560
D1/Mexico/Othon P Blanco/14/2007	2007	Othon P Blanco	Quintana Roo	HM171561
D1/Mexico/Ocosingo/22/2006	2007	Ocosingo	Chiapas	HM171562
D1/Mexico/Mujica/16/2007	2007	Mujica	Tabasco	HM171563
D1/Mexico/Ixtaczoquitlan/17/2007	2007	Ixtaczoquitlan	Veracruz	HM171564
D1/Mexico/Leon/18/2007	2007	Leon	Guanajuato	HM171565
D1/Mexico/Huimanguillo/2006	2006	Huimanguillo	Tabasco	HM171566
D1/Mexico/La Colorada/13/2006	2006	La Colorada	Sonora	HM171567
D1/Mexico/Puerto Vallarta/6/2006	2006	Puerto Vallarta	Jalisco	HM171568
D1/Mexico/Cd Victoria/5/2007	2007	Cd Victoria	Tamaulipas	HM171569
D1/Mexico/Nejapa de Morelos/3/2006	2006	Nejapa de Morelos	Oaxaca	HM171570
DENV-2				
D2/Mexico/Amatepec/2/2002	2002	Amatepec	Estado de Mexico	HM171541
D2/Mexico/Jiutepec/3/2002	2002	Jiutepec	Morelos	HM171542
D2/Mexico/Apodaca/4/2002	2002	Apodaca	Nuevo León	HM171543
D2/Mexico/Desconocido/5/2002	2002	Unknown	Unknown	HM171544
D2/Mexico/La Paz/6/2003	2003	La Paz	Baja California Sur	HM171545
D2/Mexico/Tlayacapan/10/2003	2003	Tlayacapan	Morelos	HM171546
D2/Mexico/Cosala/12/2003	2003	Cosala	Sinaloa	HM171547
D2/Mexico/Huatabampo/13/2003	2003	Huatabampo	Sonora	HM171548
D2/Mexico/Playa Vicente/15/2003	2003	Playa Vicente	Veracruz	HM171549
D2/Mexico/Frontera/16/2005	2005	Frontera	Tabasco	HM171550
D2/Mexico/Huautla/17/2005	2005	Huautla	Oaxaca	HM171551
D2/Mexico/Doctor Arroyo/18/2005	2005	Doctor Arroyo	Nuevo León	HM171552
D2/Mexico/Mazatlan/19/2005	2005	Mazatlan	Sinaloa	HM171553
D2/Mexico/Ocosingo/20/2007	2007	Ocosingo	Chiapas	HM171554
D2/Mexico/Tapachula/21/2002	2002	Tapachula	Chiapas	HM171555
D2/Mexico/Ciudad Madero/22/2004	2004	Ciudad Madero	Tamaulipas	HM171556
DENV-3				
D3/Mexico/Alto Lucero/5/2006	2006	Alto Lucero	Veracruz	HM171538
D3/Mexico/La Paz/1/2003	2003	La Paz	Baja California Sur	HM171539
D3/Mexico/Tlaltizapan/3/2006	2006	Tlaltizapan	Morelos	HM171540
DENV-4				
D4/Mexico/Cardenas/2/2006	2006	Cardenas	Tabasco	HM171571

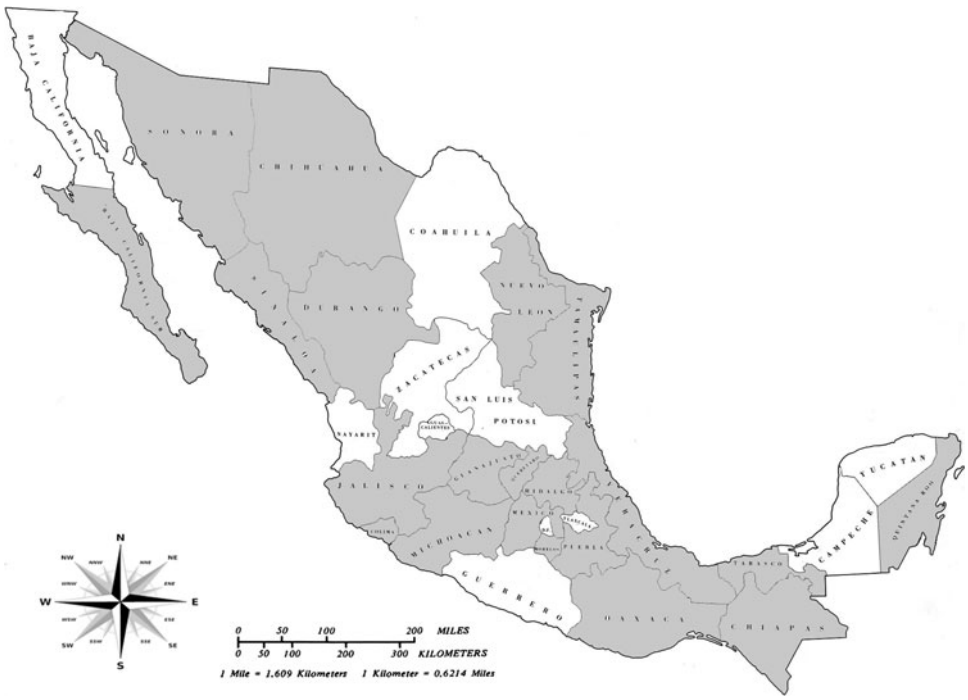

Fig. 1 Map of Mexico showing the division into individual states. Those states from which sequences included in this study were available are *shaded gray*

Institute were excluded, as these will be described in other publications. This resulted in data sets of the following sizes: DENV-1 = 700 sequences, 1,485 nt; DENV-2 = 860 sequences, 1,485 nt; DENV-3 = 525 sequences, 1,479 nt; DENV-4 = 247 sequences, 1,485 nt. The sequences were aligned manually (available from the authors on request). These data contained the following numbers of Mexican sequences, either generated here or taken from GenBank: DENV-1 = 23 sequences; DENV-2 = 37 sequences; DENV-3 = 10 sequences; DENV-4 = 13 sequences. These sequences were sampled over a period of 27 years (1980–2007) and from 20 different states within Mexico (Fig. 1). Our analysis excluded two isolates previously assigned to the Asian 2 genotype of DENV-2—C932/Guerrero-Mx/97 and C1077/Guerrero-Mx/97 [8]. Because the E gene sequences of these viruses are extremely closely related to those of the New Guinea C strain isolated in 1944, it cannot be conclusively excluded that they are laboratory contaminants. Similarly, we excluded a DENV-2 isolate sampled in Mexico in 1983 (GenBank accession L04561), as it is connected to members of the American genotype by an anomalously long branch [30]. Finally, DENV-2 isolates Oaxaca/1656/2005 and Oaxaca/1038/2005 were excluded because they are claimed to represent inter-genotypic recombinants [25].

To obtain a provisional understanding of the phylogenetic relationships of these sequences, we inferred maximum-likelihood trees using the PHYML package [13], employing SPR branch swapping. In all cases, the GTR + Γ_4 model of nucleotide substitution was utilized (exact parameter values available from the authors on request). From these initial phylogenies, we selected 100 'background' isolates of each of the four DEN viruses, along with those sampled from Mexico, that could be subjected to more in-depth phylogenetic analysis. Background sequences were selected so as (1) to represent all of the circulating genotypes of each of the four DEN viruses, (2) to include sequences from as wide a sample of years as possible in order to maximize the sensitivity of analysis of temporal dynamics (see below), and (3) to include all sequences that are closely related to those sampled in Mexico and which therefore give information on the introduction of these viruses into this country. This selection process resulted in data sets of the following sizes: DENV-1 = 123 sequences; DENV-2 = 137 sequences; DENV-3 = 110 sequences; DENV-4 = 113 sequences.

Bayesian MCMC analysis

We estimated both the rate of nucleotide substitution per site and the time to the most recent common ancestor (TMRCA) for each DENV data set using the Bayesian Markov chain Monte Carlo (MCMC) approach available in the BEAST package (http://beast.bio.ed.ac.uk/; ref. [10]). In each case, we used both strict and relaxed (uncorrelated lognormal) molecular clocks and a substitution model employing the GTR substitution matrix with a different rate assigned to each codon position (as this is often an appropriate descriptor of evolution of RNA viruses including DENV; ref. [31]). As expected, very similar

results were obtained under both strict and relaxed molecular clocks. We also employed the Bayesian skyline population coalescent prior in all cases, as this is clearly the best descriptor of the complex population dynamics of DENV, and inferring demographic processes was not the aim of this study. In each case, MCMC chains were run for a sufficient time to achieve convergence (assessed using the TRACER program; http://tree.bio.ed.ac.uk/software/tracer/), with uncertainty in parameter estimates reflected in values of the 95% highest probability density (HPD). Finally, for the relaxed clock analysis, we also used BEAST to compute the maximum clade credibility (MCC) tree using the TreeAnnotator program, with the first 10% trees removed as burn-in. The level of support for each node on the tree is given as the Bayesian posterior probability (BPP) value. With these trees in hand we were able to (1) determine the number of independent introductions of each DEN virus in Mexico, defined as the presence of phylogenetically distinct clusters of sequences supported by BPP values >0.90, and (2) compute the 95% HPD values on the age of each Mexican lineage, as this provides information on the timing of each introduction event into the Mexican population (although it is important to note that age of a lineage is not necessarily the same as time of introduction).

Results

Our provisional phylogenetic analysis of 2,332 complete E genes of all four DEN viruses revealed that the 83 sequences of Mexican origin fell into a number of discrete clusters indicative of independent introductions into this population (results not shown; available from the authors on request). To analyze these evolutionary patterns in more detail, we selected 100 representative 'background' sequences from each DEN virus, combined these with those sampled from Mexico, and undertook more rigorous Bayesian phylogenetic analysis.

The Bayesian relaxed molecular clock MCC trees for each of the four DEN viruses (human isolates only) are shown in Figs. 2, 3, 4 and 5. Those sequences sampled from Mexico are shaded in each figure, and the number of discrete Mexican clusters, indicative of separate introductions, is marked. In this context it is important to note that because we defined introductions as phylogenetically distinct clusters of viruses with high posterior probability values, our count of the number separate introduction events is inherently conservative. Indeed, in a number of cases, clusters of Mexican viruses also contain isolates sampled in other localities, principally from Central America, that might be indicative of multiple entries into Mexico. This is considered in more detail below. Rates of nucleotide substitution were similar to those estimated previously [34]: 95% HPD values across all DEN viruses of 5.7–9.5 $\times$ 10^{-4} nucleotide substitutions per site, per year (Supplementary Table 1).

In the case of DENV-1, all 23 Mexican E gene sequences analyzed here, sampled from the states of Sonora, Sinaloa, Tamaulipas, Jalisco, Querétaro, Michoacán, Morelos, Veracruz, Tabasco, Oaxaca, Chiapas and Quintana Roo (from north to south of the country; Fig. 1), fall into genotype III of this virus that is widespread throughout the Americas (Fig. 2). However, even though only a single DENV-1 genotype is observed in Mexico, our phylogenetic analysis reveals that there have been at least three introductions of genotype III, as signified by the presence of three phylogenetically distinct lineages. In addition, our analysis of times to common ancestry (and sampling) indicates that there was little, if any, co-circulation of the viral lineages associated with these three introductions. The cluster of viruses associated with introduction #1 has an estimated age dating to 1973–1978, compatible with an appearance in Mexico shortly before the major epidemic of 1979–1981, and circulates until at least 1986 (the date of the last sequence sampled). In contrast, the TMRCA of the viruses associated with introduction #2 is between 1985 and 1992, suggesting a more recent introduction into Mexico, and viruses of this cluster circulate until 1995. Finally, the largest phylogenetically distinct group of viruses, denoted here as introduction #3, has an inferred date of common ancestry of 1997–2001 and circulates until 2007. As 2007 is also the date of the most recent sample, this viral lineage is likely to be circulating today. Introduction #3 is also of note because it is described here for the first time, it circulates in nearly all Mexican states, and it seems to have dispersed more widely around Central America, as isolates from El Salvador and Nicaragua are very closely related to those from Mexico.

In the case of DENV-2, our phylogenetic analysis suggests that there have been at least three separate introductions of this virus into Mexico (Fig. 3). As with DENV-1, these introductions are staggered in time and hence are indicative of limited co-circulation and persistence but frequent lineage replacement. However, unlike the case of DENV-1, all of these introductions involve a different genotype of DENV-2. The first introduction of DENV-2 sampled here involves viruses of the American genotype, which evidently spread to a variety of localities throughout the Americas. Our Bayesian coalescent analysis suggests that the age of this lineage dates to the mid to late 1970s (95% HPD = 1974–1980), although entry into Mexico may not have occurred until the early 1980s, corresponding to a major epidemic of DENV-2 in the Americas [8]. As this lineage has not been sampled since 1995, it is likely


D1_AB074760_Japan_1943
D1_EU848545_Hawaii_1944
D1_AF425619_Hawaii_1945
D1_AY732481_Thailand_1982
D1_AY732420_Thailand_1986
D1_AF425630_Thailand_1980
D1_AF350498_China_1980
D1_AB003090_Lao_1996
D1_AY732463_Thailand_1993
D1_AM746212_SaudiArabia_2006
D1_AY732381_Thailand_1988
D1_AY732382_Thailand_1991
D1_AY732415_Thailand_1993
D1_EU448387_Vietnam_2004
D1_AY732452_Thailand_2000
D1_EU448391_Indonesia_2006
D1_AY732469_Thailand_2000
D1_AY732417_Thailand_1997
D1_AB111067_Singapore_2001
D1_EF113153_China_2006
D1_AY871812_China_2004
D1_EU448393_Thailand_2003
D1_AB111064_Thailand_1998
D1_AF309641_Cambodia_1998
D1_AY708047_Myanmar_2001
D1_AY726554_Myanmar_1998
D1_EU448397_Vietnam_2007
D1_DQ016650_Thailand_2004
D1_FJ158611_China_2007
D1_DQ193572_China_2004
D1_EF508202_China_2004
D1_EU081281_Singapore_2006
D1_D00503_Philippines_1984
D1_U88535_NauruIsland_1974
D1_D00501_Caribbean_1977
D1_D00502_Thailand_1980
D1_EU448404_Indonesia_2003
D1_AY858983_Indonesia_2004
D1_AB111068_Samoa_2001
D1_EU448405_Philippines_2007
D1_EF440432_EastTimor_2000
D1_EU448409_Indonesia_2007
D1_AB232666_Indonesia_2002
D1_DQ285561_Seychelles_2004
D1_EU448411_Indonesia_2005
D1_AB189120_Indonesia_1998
D1_EU863647_Chile_2002
D1_DQ016651_FrenchPolynesia_2001
D1_DQ672560_FrenchPolynesia_2001
D1_DQ672561_Hawaii_2001
D1_D10513_Thailand_1958
D1_AF425629_Thailand_1963
D1_EF457905_Malaysia_1972
D1_AF425622_Malaysia_1972
D1_AF231721_Malaysia_1972
D1_AF298807_IvoryCoast_1998
D1_AF425620_IvoryCoast_1985
D1_AY713473_Myanmar_1971
D1_AF425615_Myanmar_1976
D1_AY762084_Singapore_1993
D1_DQ016652_India_1996
D1_DQ285562_Comoros_1993
D1_EU448413_India_2006
D1_AF425613_Brazil_1982
D1_AF425617_Colombia_1996
D1_Mexico_1378_1983
D1_Mexico_1379_1982
D1_Mexico_Puebla_1462_1984
D1_Mexico_Yucatan_1298_1980
D1_Mexico_1756_1986
D1_Mexico_Sonora_1463_1984
1. Mexico
(1973-1978)
D1_AF425616_Colombia_1985
D1_AF425639_Trinidad_1986
D1_AF425626_Peru_1991
D1_FJ410183_PuertoRico_1993
D1_EU482567_PuertoRico_1998
D1_FJ410181_PuertoRico_1995
D1_FJ410175_PuertoRico_1994
D1_FJ390374_PuertoRico_1995
D1_FJ390379_PuertoRico_1998
D1_AB111065_Paraguay_1999
D1_FJ390378_PuertoRico_1998
D1_AF425610_Angola_1988
D1_AF425621_Jamaica_1977
D1_AF425618_Grenada_1977
D1_EF122232_FrenchGuiana_1989
D1_AF425609_Aruba_1985
D1_AY153755_CostaRica_1993
D1_DQ016656_Nicaragua_1996
D1_FJ410188_PuertoRico_1996
D1_Mexico_Campeche_4642_1995
D1_Mexico_Yucatan_3425_1994
D1_Mexico_Quintana Roo_4942_1995
2. Mexico
(1985-1992)
D1_AF425631_Trinidad_1978
D1_S64849_Brazil_1990
D1_AF425614_Brazil_1997
D1_AF311956_Brazil_1997
D1_AF514883_Paraguay_2000
D1_AF513110_Brazil_2001
Genotype III
D1_AF425632_Venezuela_1995
D1_AF425638_Venezuela_1995
D1_EU482609_Venezuela_2007
D1_AF425634_Venezuela_1997
D1_AF425635_Venezuela_1995
D1_FJ024485_Nicaragua_2005
D1_Mexico_La_Colorada_13_2006
D1_Mexico_Huimanguillo_2006
D1_Mexico_Mocorito_10_2006
D1_Mexico_Mujica_16_2007
D1_Mexico_Ocosingo_22_2006
D1_Mexico_Nejapa_de_Morelos_3_2006
D1_Mexico_Cd_Victoria_5_2007
D1_Mexico_Puerto_Vallarta_6_2006
D1_EU448814_ElSalvador_2006
D1_FJ024423_Nicaragua_2005
D1_EU596504_Nicaragua_2005
D1_Mexico_Veracruz_23_2006
D1_FJ024479_Nicaragua_2006
D1_Mexico_Cuernavaca_2_2006
D1_Mexico_Othon_P_Blanco_14_2007
D1_Mexico_Ixtaczoquitlan_17_2007
D1_Mexico_Jalpan_de_Serra_9_2006
D1_Mexico_Leon_18_2007
3. Mexico
(1997-2001)
Genotype I
Genotype II
1.0
1.0
1.0
1.0
1.0
DENV-1
1900
1925
1950
1975
2000
Year

◀ **Fig. 2** MCC tree of 123 isolates of human DENV-1 circulating globally. The 23 viruses sampled from Mexico are *shaded gray*, with the number of separate introductions into Mexico noted as well as the 95% credible intervals for the age of each introduced lineage. Isolates sampled outside of Mexico that fall in Mexican clusters are shown in *bold italic*. As this phylogeny was estimated using a relaxed molecular clock, the position of the root is assigned automatically, and the tip position of each isolate corresponds to the time of its sampling (with time shown on the *x*-axis). BPP values are shown for relevant nodes

that it is now extinct in Mexico [8]. Indeed, the demise of this genotype reflects part of the continent-wide replacement of the American genotype by viruses of Asian origin in the Americas [28]. The second introduction of DENV-2 into Mexico concerns the presence of a single virus of the geographically widespread Cosmopolitan genotype that was sampled in 1996, with a credible time of origin ranging from 1966 to 1996 (as in all cases where single viral isolates are involved, the credible intervals on estimates of TMRCA are necessarily large). Although the identification of a single Cosmopolitan virus makes its countrywide prevalence difficult to judge, phylogenetic analyses of prM sequences have noted its presence in regions such as the Yucatan [9], so there has clearly been some onward transmission of this genotype within Mexico. However, the fact that viruses of the Cosmopolitan genotype have not been reported since 2002 suggests that this lineage may also have suffered extinction.

The final introduction of DENV-2 recorded here involves viruses of the Asian/American genotype, which is clearly now the dominant genotype in Mexico. The appearance of Asian/American genotype viruses was initially described in the Yucatan during a DENV-2 epidemic in 2002 [22]. Subsequent phylogenetic analysis showed that this genotype had spread to the state of Oaxaca, and our study shows that viruses from the states of Sonora, Coahuila, Nuevo León, Tamaulipas, Baja California Sur, Sinaloa, Durango, Hidalgo, Estado de México, Morelos, Veracruz and Chiapas can also be assigned to the Asian/American genotype. In addition, that the Mexican viruses assigned to introduction #3 are closely related to those sampled from Costa Rica and Nicaragua indicates that this genotype has spread to multiple countries in Central America. Our analysis of the TMRCA suggests that Asian/American genotype viruses in Mexico have a credible time of origin of between 1993 and 1997, although it is unclear exactly when these viruses first entered the country. Finally, as viruses of the Asian/American genotype are associated with DHF and have relatively high fitness in both humans and mosquitoes [1, 6, 7], such that they are able to out-compete co-circulating viruses, their apparent establishment in the Mexican population is a major cause for concern.

In a similar manner to DENV-1, all introductions of DENV-3 into Mexico involve viruses of a single genotype—genotype III—that is relatively widespread in the Americas (Fig. 4). Like DENV-1, there have also been at least three independent introductions of DENV-3 into Mexico, although the very small sample size cautions against strong conclusions. The first introduction sampled here is represented by a single isolate sampled in 1995. Although our estimate of the TMRCA of this lineage goes back to 1991, that DENV-3 was not detected in Mexico until 1995 suggests that it most likely appeared close to this latter time [8]. Similarly, we estimate that viruses of the second lineage of genotype III viruses in Mexico last shared a common ancestor during the period 1991–1995, which is again compatible with their first appearance in Mexico at a time concomitant with the DENV-3 epidemic of 1995. This lineage then appears to have circulated until at least 2000. Finally, introduction #3, identified here for the first time, clearly took place more recently, with an inferred common ancestry dating to 2002–2003. As viruses of this lineage circulate until at least 2006, it can also be regarded as the current lineage of DENV-3 in Mexico, although this will clearly need to be confirmed on a larger sample of viruses. Hence, despite the small sample size, it is clear that DENV-3 also experiences a clear turnover of viral lineages through time.

The small sample of sequences available for DENV-4 again provides strong evidence for an episode of lineage replacement (Fig. 5). Specifically, our phylogenetic analysis reveals at least two introductions of genotype II viruses into Mexico. The first of these is likely to have occurred at the time of a DENV-4 outbreak in 1981, although our molecular clock analysis suggests that these group of viruses in fact share a common ancestry that dates back to 1975–1979. This is the most common lineage of DENV-4 in the Mexican population, and one that was clearly successful in Latin America and the Caribbean region more broadly [3, 11]. However, as this lineage has not been sampled since 1997, it is likely to have gone extinct. The second introduction, newly detected here, comprises a single viral isolate that was sampled in 2006, with an inferred ancestry that may date back to 1989 (although it is obviously likely to have entered Mexico at a time far closer to 2006). However, as this is a single virus lineage, it is difficult to assess its potential for future spread in the Mexican population.

Discussion

Our phylogenetic analysis reveals that DENV evolution in Mexico is characterized by the independent entry of multiple viral lineages, with at least 11 separate introductions over the last 30 years, relatively limited co-circulation of lineages of individual DEN viruses (even though the 4

Fig. 3 MCC tree of 137 isolates of human DENV-2 circulating globally. The 37 viruses sampled from Mexico are *shaded gray*, with the number of separate introductions into Mexico noted as well as the 95% credible intervals for the age of each introduced lineage. Isolates sampled outside of Mexico that fall in Mexican clusters are shown in *bold italic*. BPP values are shown for relevant nodes. See Fig. 2 for other details

DEN viruses themselves often co-circulate), and frequent lineage replacement. This pattern of lineage turnover becomes most apparent when the phylogenies produced here are compared to those generated by Díaz et al. [8] and which cover viruses sampled up to 2002: in DENV-1, DENV-3, and DENV-4, the currently dominant lineage identified by Díaz et al. [8] has seemingly been replaced by a new viral lineage identified here for the first time. The

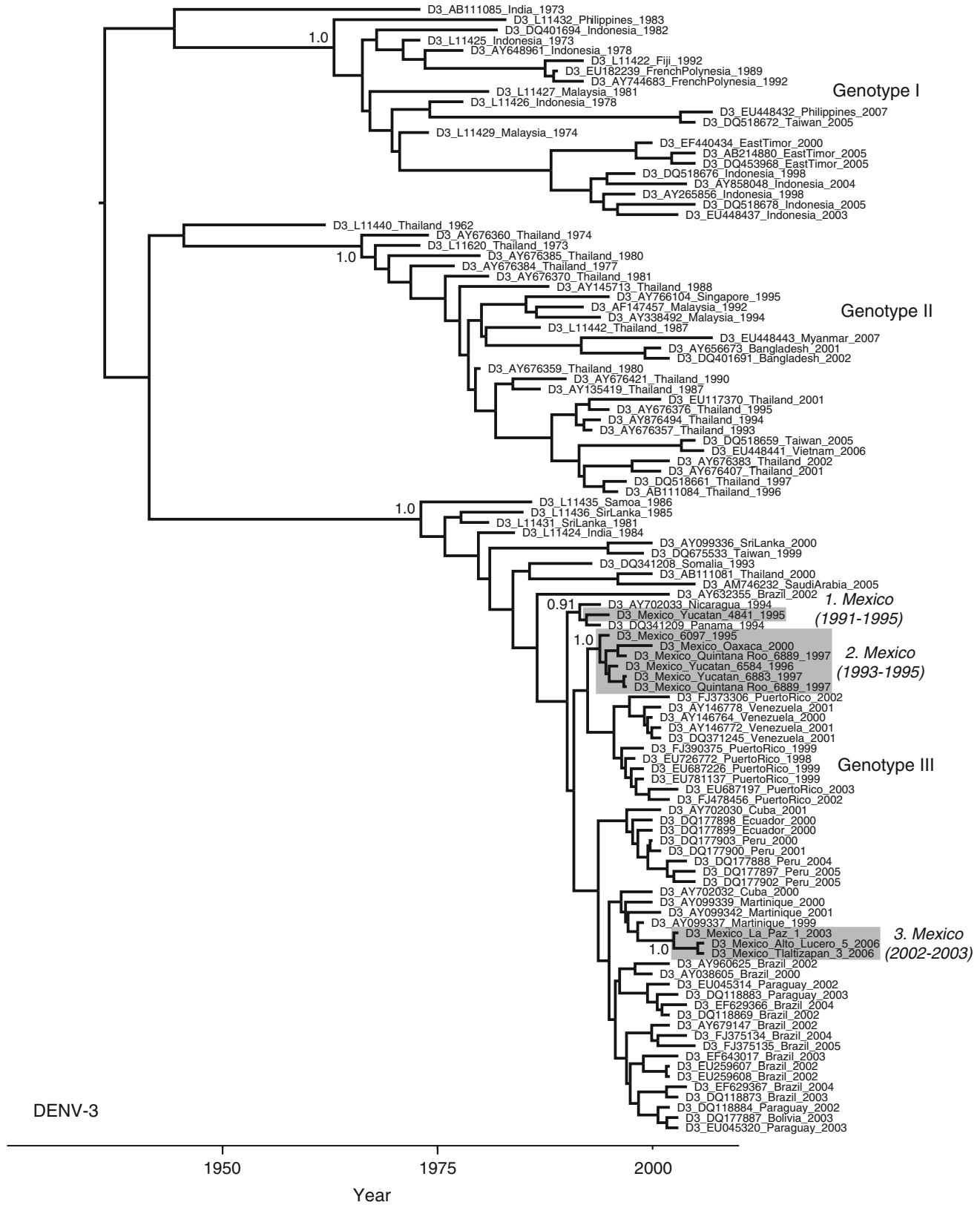

Fig. 4 MCC tree of 110 isolates of human DENV-3 circulating globally. The ten viruses sampled from Mexico are *shaded gray*, with the number of separate introductions into Mexico noted as well as the 95% credible intervals for the age of each introduced lineage. BPP values are shown for relevant nodes. See Fig. 2 for other details


D4_U18440_Thailand_1963
D4_FJ439174_Philippines_1956
D4_U18434_Philippines_1964
D4_U18435_Philippines_1984
D4_EU448449_Philippines_2003
D4_EU448448_Philippines_2005
D4_AB111086_India_1996
D4_U18437_SriLanka_1978
D4_AF231722_Malaysia_1969
D4_AY618952_Thailand_1980
D4_U18441_Thailand_1978
D4_AY618950_Thailand_1977
D4_AY618949_Thailand_1976
D4_AY618953_Thailand_1981
D4_AY780644_Thailand_1985
D4_AY618962_Thailand_1986
D4_AY618957_Thailand_1983
D4_AY618958_Thailand_1984
D4_AY550909_SriLanka_1978
D4_AY618972_Thailand_1994
D4_EU448453_Cambodia_2003
D4_AB111089_Thailand_2002
D4_EU448452_Vietnam_2004
D4_EU448450_Vietnam_2006
D4_AY618990_Thailand_1991
D4_AY618976_Thailand_1996
D4_AB111087_Thailand_1999
D4_AY618974_Thailand_1995
D4_AY618992_Thailand_2001
D4_AY618935_Thailand_2000
D4_AY618987_Thailand_1999
D4_EU448456_Thailand_2005
D4_EU448454_Thailand_2007
D4_AY618988_Thailand_1997
D4_EF436279_China_1978
D4_EF440435_EastTimor_2000
D4_EU448459_Indonesia_2007
D4_EU448458_Philippines_2004
D4_AJ428556_Malaysia_2001
D4_EU448461_Indonesia_2004
D4_EU448462_Solomon_2007
D4_EU448464_Singapore_2001
D4_AY858049_Indonesia_2004
D4_EU448463_Indonesia_2007
D4_AY762085_Singapore_1995
D4_U18430_Indonesia_1977
D4_U18429_Indonesia_1976
D4_U18438_Tahiti_1979
D4_AY152857_PuertoRico_1985
D4_AY152328_PuertoRico_1982
D4_AY152312_PuertoRico_1982
D4_AY152336_PuertoRico_1982
D4_AF326573_DominicanRepublic_1981
D4_AY152389_Jamaica_1981
D4_AY152296_PuertoRico_1982
D4_AY152383_Trinidad_1982
D4_AY152380_Trinidad_1984
D4_AF231726_Thailand_1994
D4_Mexico_1414_1983
D4_Mexico_1420_1983
D4_U18432_NewCaledonia_1984
D4_AY152384_Jamaica_1983
D4_U18431_Mexico_1984
D4_Mexico_Yucatan_1503_1984
D4_AY152379_Honduras_1991
D4_DQ341219_Colombia_1996
D4_AY152292_Ecuador_1994
D4_Mexico_1554_1985
D4_Mexico_1551_1985
D4_U18427_ElSalvador_1994
D4_AY152300_ElSalvador_1993
D4_Mexico_D4.111_1995
D4_AY152378_Mexico_1991
D4_Mexico_Yucatan_4915_1995
D4_Mexico_Quintana Roo_4959_1995
D4_Mexico_Yucatan_6637_1997
D4_Mexico_Yucatan_5962_1996
D4_U18439_Tahiti_1985
D4_AY152288_PuertoRico_1994
D4_AY152284_PuertoRico_1987
D4_AY152276_PuertoRico_1986
D4_AY152252_PuertoRico_1987
D4_AY152108_PuertoRico_1987
D4_AY152112_PuertoRico_1992
D4_AY152855_PuertoRico_1996
D4_U18436_PuertoRico_1986
D4_AY152160_PuertoRico_1992
D4_AY152156_PuertoRico_1994
D4_AY152376_Barbados_1993
D4_AY152377_Trinidad_1994
D4_AY152100_Martinique_1995
D4_AY152375_Barbados_1993
D4_AY152104_CostaRica_1996
D4_AY152372_Surinam_1994
D4_DQ390322_FrenchGuiana_1993
D4_DQ390329_FrenchGuyana_1994
D4_DQ390324_FrenchGuyana_1995
D4_AY152096_PuertoRico_1994
D4_AY152365_Bahamas_1998
D4_AY934757_CostaRica_1993
D4_DQ390321_Martinique_2004
D4_DQ390320_Guadalupe_2004
D4_DQ390319_Martinique_2004
D4_DQ390328_FrenchGuyana_2004
D4_AY152092_Venezuela_1995
D4_AY152369_Montserrat_1994
D4_AY152371_Montserrat_1994
D4_AY152368_Barbados_1999
D4_Mexico_Cardenas_2_2006
D4_AY152088_PuertoRico_1998
D4_AY152084_PuertoRico_1994
D4_AY152052_PuertoRico_1998
D4_AY152044_PuertoRico_1998
0.94
1.0
1.0
1.0
Genotype I
Genotype II
1. Mexico
(1975-1979)
2. Mexico
(1989-2006)
DENV-4
1900
1925
1950
1975
2000
Year

◀ **Fig. 5** MCC tree of 113 isolates of human DENV-4 circulating globally. The 13 viruses sampled from Mexico are *shaded gray*, with the number of separate introductions into Mexico noted as well as the 95% credible intervals for the age of each introduced lineage. Isolates sampled outside of Mexico that fall in Mexican clusters are in *bold*. BPP values are shown for relevant nodes. See Fig. 2 for other details

exception is DENV-2, where the emergent Asian/American genotype has seemingly established itself as the major lineage of DENV-2 in Mexico.

As is now increasingly frequently observed in studies of DENV evolution, lineage replacement appears to be a more common occurrence than long-term lineage persistence [2, 12]. Unfortunately, determining the evolutionary basis of individual replacement events is inherently problematic, particularly whether they are selectively mediated because lineages differ sufficiently in fitness that natural selection is able to favor one over another, they reflect larger-scale oscillations in the prevalence of each DEN virus, or they are largely due to stochastic events such as periodic bottlenecks in vector population size [15]. Growing experimental evidence for the relative inferiority of the American genotype of DENV-2 [27], coupled with the non-overlapping nature of lineage distributions in Mexico, suggests that more attention should be paid to the possible role of natural selection in determining patterns of lineage turnover.

More generally, the viral lineages that have circulated in Mexico appear to be of the same evolutionary origin as those detected more widely in the Americas, and particularly in countries that are geographically close to Mexico, indicating that there has been considerable local diffusion across relatively fluid borders. A simple prediction for the future is therefore that any newly emerging lineages that are successful in Mexico are likely to be equally able to spread across a far wider geographic area. Such a dynamic evolutionary process further illustrates the ability of DENV to exploit the movement patterns of the human host populations and argues that Mexico could eventually be exposed to viral strains with very different phenotypic features, such as increased virulence. The continued surveillance of dengue in Mexico, using the modern methods of molecular epidemiology, is therefore of utmost importance.

Acknowledgments The authors wish to thank Ms. Silvia Tenorio-Salgado for technical assistance. This work was supported by a grant FOSSIS-CONACYT SALUD-2005-01-13852 to JRC.

References

1. Armstrong PM, Rico-Hesse R (2001) Differential susceptibility of *Aedes aegypti* to infection by the American and Southeast Asian genotypes of dengue type 2 virus. Vector Borne Zoonotic Dis 1:159–168
2. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, Vorndam V, Gubler DJ, McMillan WO (2003) Selection-driven evolution of emergent dengue virus. Mol Biol Evol 20:1650–1658
3. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, Vorndam V, Gubler DJ, McMillan WO (2006) Molecular evolution of dengue 2 virus in Puerto Rico: positive selection in the viral envelope accompanies clade reintroduction. J Gen Virol 87:885–893
4. Brunkard JM, Robles López JL, Ramirez J, Cifuentes E, Rothenberg SJ, Hunsperger EA, Moore CG, Brussolo RM, Villarreal NA, Haddad BM (2007) Dengue fever seroprevalence and risk factors, Texas-Mexico border, 2004. Emerg Infect Dis 13:1477–1483
5. Cisneros A, Díaz-Badillo A, Cruz-Martínez G, Tovar R, Ramírez-Palacios LR, Jiménez-Rojas F, Beaty B, Black WC 4th, de Lourdes Muñoz M (2006) Dengue 2 genotypes in the state of Oaxaca, Mexico. Arch Virol 151:113–125
6. Cologna R, Armstrong PM, Rico-Hesse R (2005) Selection for virulent dengue viruses occurs in humans and mosquitoes. J Virol 79:853–859
7. Cologna R, Rico-Hesse R (2003) American genotype structures decrease dengue virus output from human monocytes and dendritic cells. J Virol 77:3929–3938
8. Díaz FJ, Black WC 4th, Farfán-Ale JA, Loroño-Pino MA, Olson KE, Beaty BJ (2006) Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 37:760–773
9. Díaz FJ, Farfán-Ale JA, Olson KE, Loroño-Pino MA, Gubler DJ, Blair CD, Black WC, Beaty BJ 4th (2002) Genetic variation within the premembrane coding region of dengue viruses from the Yucatan peninsula of Mexico. Am J Trop Med Hyg 67:93–101
10. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214
11. Foster JE, Bennett SN, Vaughan H, Vorndam V, McMillan WO, Carrington CVF (2003) Molecular evolution and phylogeny of dengue type 4 virus in the Caribbean. Virology 306:126–134
12. Goncalvez AP, Escalante AA, Pujol FH, Ludert JE, Tovar D, Salas RA, Liprandi F (2002) Diversity and evolution of the envelope gene of dengue virus type 1. Virology 303:110–119
13. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Mol Biol Evol 52:696–704
14. Günther J, Ramírez-Palacio LR, Pérez-Ishiwara DG, Salas-Benito JS (2009) Distribution of dengue cases in the state of Oaxaca, Mexico, during the period 2004–2006. J Clin Virol 45:218–222
15. Holmes EC (2009) The evolution and emergence of RNA viruses. In: Harvey PH, May RM (eds) Oxford series in ecology and evolution (OSEE). Oxford University Press, Oxford
16. Holmes EC, Twiddy SS (2003) The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3:19–28
17. Johansson MA, Cummings DA, Glass GE (2009) Multiyear climate variability and dengue: El Niño southern oscillation, weather, and dengue incidence in Puerto Rico, Mexico, and Thailand: a longitudinal data analysis. PLoS Med 6:e1000168
18. Lanciotti RS, Gubler DJ, Trent DW (1997) Molecular evolution and phylogeny of dengue-4 viruses. J Gen Virol 78:2279–2286
19. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30:545–551
20. Loroño-Pino MA, Cropp CB, Farfán JA, Vorndam AV, Rodríguez-Angulo EM, Rosado-Paredes EP, Flores-Flores LF, Beaty BJ, Gubler DJ (1999) Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg 61:725–730

21. Loroño-Pino MA, Farfán-Ale JA, Rosado-Paredes EP, Kuno G, Gubler DJ (1993) Epidemic dengue 4 in the Yucatán, Mexico, 1984. Rev Inst Med Trop Sao Paulo 35:449–455
22. Loroño-Pino MA, Farfán-Ale JA, Zapata-Peraza AL, Rosado-Paredes EP, Flores-Flores LF, García-Rejón JE, Díaz FJ, Blitvich BJ, Andrade-Narváez M, Jiménez-Ríos E, Blair CD, Olson KE, Black WC, Beaty BJ 4th (2004) Introduction of the American/Asian genotype of dengue 2 virus into the Yucatan State of Mexico. Am J Trop Med Hyg 71:485–492
23. Lozano-Fuentes S, Fernandez-Salas I, de Lourdes Muñoz M, Garcia-Rejon J, Olson KE, Beaty BJ, Black WC 4th (2009) The Neovolcanic axis is a barrier to gene flow among *Aedes aegypti* populations in Mexico that differ in vector competence for dengue 2 virus. PLoS Negl Trop Dis 3:e468
24. Mota J, Ramos-Castañeda J, Rico-Hesse R, Ramos C (2002) Phylogenetic analysis of the envelope protein (domain III) of dengue 4 viruses. Salud Publica Mex 44:228–236
25. Perez-Ramirez G, Diaz-Badillo A, Camacho-Nuez M, Cisneros A, de Lourdes Muñoz M (2009) Multiple recombinants in two dengue virus, serotype-2 isolates from patients from Oaxaca, Mexico. BMC Microbiol 9:260
26. Ramos MM, Mohammed H, Zielinski-Gutierrez E, Hayden MH, Lopez JL, Fournier M, Trujillo AR, Burton R, Brunkard JM, Anaya-Lopez L, Banicki AA, Morales PK, Smith B, Muñoz JL, Waterman SH, Dengue Serosurvey Working Group (2008) Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico border: results of a household-based seroepidemiologic survey, December 2005. Am J Trop Med Hyg 78:364–369
27. Rico-Hesse R (2003) Microevolution and virulence of dengue viruses. Adv Virus Res 59:315–341
28. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MTR, Nogueira RMR, da Rosa AT (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230:244–251
29. Roca Y, Baronti C, Revollo RJ, Cook S, Loayza R, Ninove L, Fernandez RT, Flores JV, Herve JP, de Lamballerie X (2009) Molecular epidemiological analysis of dengue fever in Bolivia from 1998 to 2008. Vector Borne Zoonotic Dis 9:337–344
30. Ruiz BH, Sánchez I, Ortega G, López I, Rosales L, Medina G (2000) Phylogenetic comparison of the DEN-2 Mexican isolate with other flaviviruses. Intervirology 43:48–54
31. Shapiro B, Rambaut A, Drummond AJ (2006) Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. Mol Biol Evol 23:7–9
32. Sittisombut N, Sistayanarain A, Cardosa MJ, Salminen M, Damrongdachakul S, Kalayanarooj S, Rojanasuphot S, Supawadee J, Maneekarn N (1997) Possible occurrence of a genetic bottleneck in dengue serotype 2 viruses between the 1980 and 1987 epidemic seasons in Bangkok, Thailand. Am J Trop Hyg Med 57:100–108
33. Tapia-Conyer R, Méndez-Galván JF, Gallardo-Rincón H (2009) The growing burden of dengue in Latin America. J Clin Virol 46(Suppl 2):S3–S6
34. Twiddy SS, Holmes EC, Rambaut A (2003) Inferring the rate and time-scale of dengue virus evolution. Mol Biol Evol 20:122–129
35. Wittke V, Robb TE, Thu HM, Nimmannitya S, Kalayanrooj S, Vaughn DW, Endy TP, Holmes EC, Aaskov JG (2002) Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. Virology 301:148–156
36. Zhang C, Mammen MP Jr, Chinnawirotpisan P, Klungthong C, Rodpradit P, Monkongdee P, Nimmannitya S, Kalayanarooj S, Holmes EC (2005) Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. J Virol 79:15123–15130