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SCIENCE DIRECT

Virology 324 (2004) 48–59

VIROLOGY

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Phylogeography and molecular evolution of dengue 2 in the Caribbean basin, 1981–2000

Jerome E. Foster,[a] Shannon N. Bennett,[b] Christine V.F. Carrington,[a,*] Helen Vaughan,[c] and W. Owen McMillan[b]

[a] *Department of Preclinical Sciences, Faculty of Medical Sciences, University of the West Indies, St. Augustine, Trinidad and Tobago*
[b] *Department of Biology, University of Puerto Rico, Rio Piedras, San Juan, Puerto Rico*
[c] *Caribbean Epidemiology Centre, Port of Spain, Trinidad and Tobago*

Received 30 October 2003; returned to author for revision 4 January 2004; accepted 9 March 2004

Abstract

We sequenced the envelope (E) genes of 59 DEN-2 isolates collected from ten Caribbean islands, six South American countries, and two Central American countries between 1981 and 2000, a period characterized by hyperendemicity and increased incidence of severe dengue. Fifty-two isolates belonged to "American/Asian" subtype IIIb, possessing a characteristic polar residue at envelope aa position 390 (N [n = 48] or S [n = 4]) common to that group. Six isolates from Trinidad (1981), Honduras (1991 [4]), and El Salvador (1987) fell into the "Native American" subtype V (D at aa 390), and one from Honduras (1986) belonged to "Asian" subtype I. The data suggest that after its first isolation in the Caribbean in 1981, genotype IIIb spread throughout the Americas and effectively replaced subtype V throughout the Caribbean basin. The strain also evolved into several distinct lineages, based on substitutions in the E glycoprotein (amino acids 91 and 131), two of which were still in circulation in 2000. Interestingly, a molecular clock did not fit the data well, suggesting that other sources of rate variation, such as differential selection or differences in effective population sizes, may exist among lineages. Our results indicate the importance of large temporal- and geographical-scale phylogenetic studies in understanding disease dynamics, particularly where replacements between regions can occur.

Keywords: Phylogeography; Molecular evolution; Caribbean basin; Dengue virus; Flavivirus

Introduction

Dengue viruses are mosquito-borne RNA viruses of the family *Flaviviridae*. There are four closely related serotypes (DEN-1–4) that are thought to represent at least three independent introductions into humans from sylvatic primates, the most recent (DEN-1) occurring within the last century (Wang et al., 2000). All four serotypes cause dengue fever (DF), a relatively mild febrile illness, and in some cases the potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The broad distribution of the mosquito vector *Aedes aegypti* in the tropics and subtropics results in the exposure of approximately 2.5 billion individuals to dengue infection (WHO, 2002). Dengue is now one of the most important re-emergent infectious diseases of the last century and one of the most serious health problems affecting tropical and subtropical regions of the Americas (McBride and Bielefeldt-Ohmann, 2000).

Before the 1980s, outbreaks in the Americas were caused by single serotypes and were geographically restricted and generally self-limiting (Gubler, 1998). However, with increases in global travel, uncontrolled population growth and urbanization within the last 30 years, and the cessation of regional mosquito control programs in the 1970s, epidemics have become steadily larger and more frequent with increased numbers of DHF/DSS cases (Gubler, 2002; WHO, 2002). In fact, few cases of hemorrhagic manifestations were reported until 1981 when the Americas experienced its first major DHF/DSS epidemic (Gubler, 1997). Before this, DEN-2 had been associated only with outbreaks of classic DF for several decades (Gubler, 1997). The 1981 DHF/DSS epidemic of DEN-2 was attributed to a strain imported from Southeast Asia, where the severe form of the disease had been endemic since the 1950s (Rico-Hesse et al., 1997).

* Corresponding author. Fax: +1-868-662-1873.
E-mail address: ccarrington@fms.uwi.tt (C.V.F. Carrington).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.03.020

This strain was related to a set of Thai and Malaysian isolates collectively classified as subtype IIIb (Lewis et al., 1993), and also referred to as the Asian–American subtype (Rico-Hesse et al., 1997).

Although the onset of DHF/DSS in the Americas in 1981 was associated with the introduction of a novel strain of DEN-2, changes in epidemic dengue severity have been associated with hyperendemic transmission patterns (the co-occurrence of multiple dengue serotypes in the same locality) in other regions (Gubler, 1998). There is strong evidence linking disease severity with secondary heterologous infection (Halstead, 1988; Kliks et al., 1989; Thein et al., 1997) and so increased hyperendemicity is likely to be one of the most significant factors contributing to increases in DHF/DSS (Gubler, 1997). By the early 1980s, all four serotypes had been reported in the Caribbean basin (CAREC, 2000) and the region experienced a rise in hyperendemicity, with two or more serotypes occasionally co-occurring in the same outbreak (Campione-Piccardo et al., 2003; Dietz et al., 1996).

The marked increase in the number of DHF cases in the Caribbean, in the wake of the appearance of DEN-2 subtype IIIb, has been largely attributed to the increased incidence of secondary infections brought about by the co-arrivals of DEN-1 and DEN-4 in 1977 and 1981, respectively (PAHO, 1979, 1981). However, the previously existing DEN-2 strain in the region, termed the "Native American" strain and classified as subtype V by Lewis et al. (1993), has never been associated with DHF/DSS epidemics, despite evidence of co-circulation with other serotypes (Gubler, 1997; Watts et al., 1999). These and other data suggest that hyperendemicity may not be the only factor contributing to the increased disease severity and that strain virulence may play an important role (Cologna and Rico-Hesse, 2003; Endy et al., 2002; Gubler et al., 1978; Leitmeyer et al., 1999; Morens et al., 1991; Rico-Hesse, 1990; Rosen, 1977; Sumarmo et al., 1983; Vaughn et al., 2000). In the case of DEN-2 in the Americas, it appears that the Asian–American strain is more virulent than its predecessor (Rico-Hesse et al., 1997).

Of the four serotypes, DEN-2 has shown the most genetic variability, reflected by its possession of the highest number of subtypes described (Lewis et al., 1993; Rico-Hesse et al., 1997). Given the epidemiological impact of the introduced Asian–American strain and other evidence associating genetic change with shifts in the epidemic potential or pathogenicity of dengue virus serotypes and other flaviviruses (Brault et al., 2002; Leitmeyer et al., 1999), it is important to understand the pattern of genetic change that has occurred in the Americas. Previous studies have shown that change in dengue virus populations is associated in part with introductions from both geographically and socioeconomically related populations (Fong et al., 1998; Foster et al., 2003; Lewis et al., 1993; Nogueira et al., 2000; Rico-Hesse et al., 1997; Singh et al., 1999; Trent et al., 1983, 1989; Uzcategui et al., 2001). For example, in parts of the Americas, the DEN-2 "Asian–American" subtype IIIb appears to have displaced the "Native American" subtype V (Rico-Hesse et al., 1997). However, the extent of this proposed displacement is not clear and subsequent phylogenetic studies on DEN-2 in the Americas have been restricted to individual countries (Dos Santos et al., 2002; Halstead et al., 2001; Nogueira et al., 2000; Ruiz et al., 2000; Sariol et al., 1999; Tolou et al., 2000; Uzcategui et al., 2001), so that we have an incomplete understanding of the subtypes' evolution since the introduction. Here we consider the phylogenetic relationships between DEN-2 isolates from throughout the Caribbean basin and neighboring territories in South and Central America (Fig. 1) collected over the 20 years following the arrival of the Asian–American strain in 1981 to assess the pattern of genetic change in DEN-2 across a region of potentially distinct populations marked by changing disease dynamics. An understanding of the links between evolution, gene flow, and selection should ultimately make it possible to identify those viral lineages that are most likely to spread and seed new epidemics, and in what way they are most likely to spread regionally.

In reporting our results, we use several terms that appear in both evolutionary biology and virology literature, but are not always used in the same sense. "Genotype" is used in its traditional genetic sense to refer to the unique sequence of bases possessed by a single isolate (we also may refer to such an individual as a "variant"). Each isolate is actually a sample of multiple and potentially variable virus particles within the host (Wang et al., 2002) presumably representing the most common genotype present. Indeed, sequence polymorphism(s), indicating the presence of multiple and equally common genotypes and virions within a single sample, was never observed in our samples. Virological nomenclature often uses the term "genotype" to refer to a class of similar (but not identical) isolates. Instead, we use the term "lineage" to describe groups of similar, and presumably related individuals that share a common ancestor (monophyletic), when that relationship is supported at least 75% of the time by bootstrap analysis. The term "gene flow" is used to refer to the movement and establishment of viral genes, and thus dengue viruses, among populations (i.e., populations of viruses in their populations of hosts). Finally, in our phylogenetic analyses, we consider genetic changes at both the base pair and amino acid level, because the former does not always result in the latter.

Results

Examination of evolutionary relationships among DEN-2 samples from around the world (Fig. 2) indicated that 46 out of 47 Caribbean island isolates (representing 27 unique country and year combinations) and all 17 of our South American isolates (representing eight unique country and year combinations) fell into a single well-defined lineage that has previously been described as subtype IIIb (Lewis

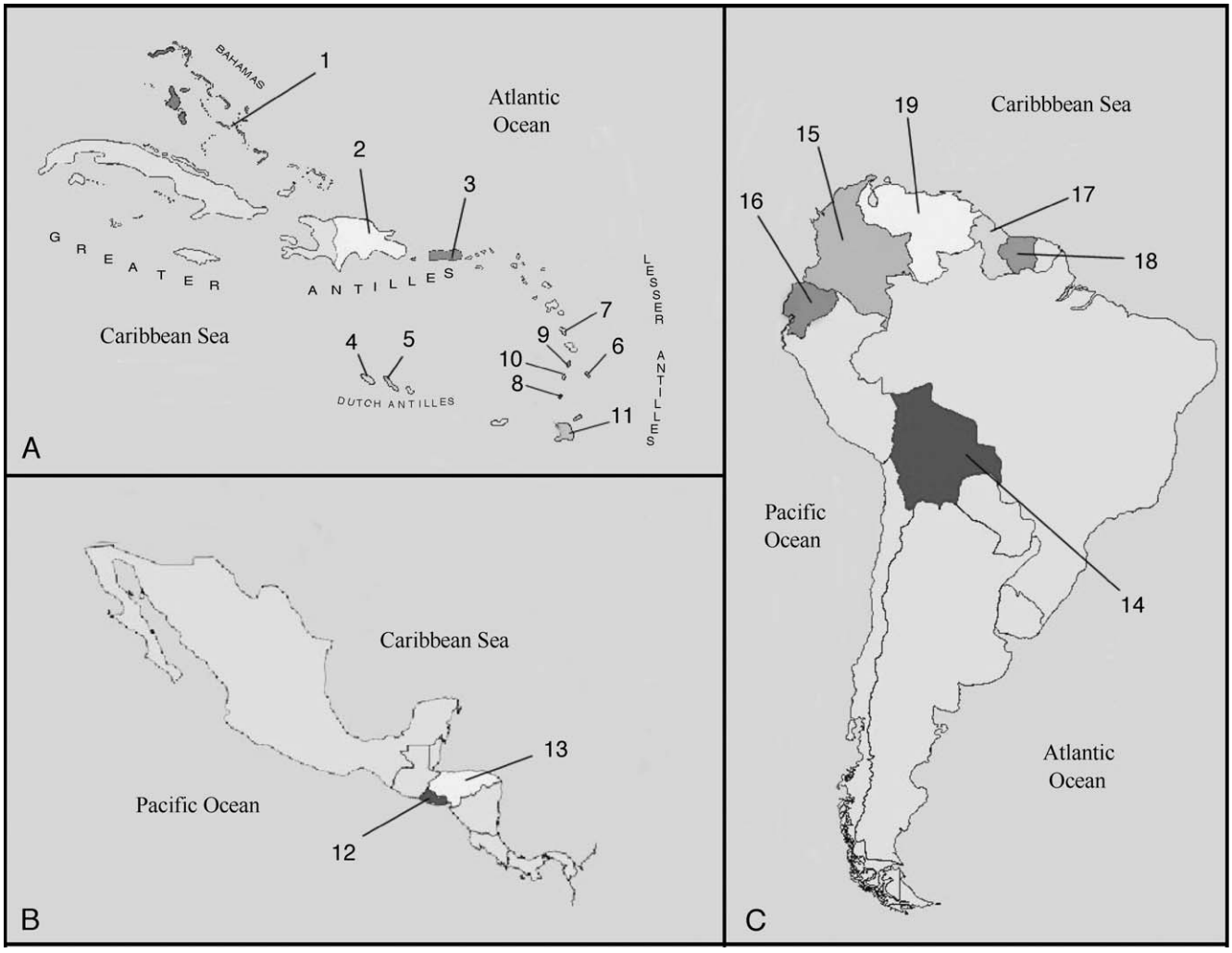

Fig. 1. Map showing countries in the Americas represented in this study. Countries represented in each region are numbered and colored to distinguish them from surrounding territories. (A) Caribbean islands—the Bahamas (labeled 1) and the Greater Antilles (Dominican Republic and Puerto Rico; labeled 2 and 3, respectively); the Dutch Antilles (Aruba, Curacao; labeled 4 and 5), and the Lesser Antilles (Barbados, Dominica, Grenada St Lucia, St. Vincent, and Trinidad and Tobago; labeled 6–11, respectively). (B) Central America—El Salvador and Honduras (labeled 12 and 13, respectively). (C) South America—Bolivia, Colombia, Ecuador, Guyana, Suriname, and Venezuela (labeled 14–19, respectively).

et al., 1993). Nucleotide sequences within this lineage differed by 0.1–3.6%. All but four of the subtype IIIb isolates examined shared an asparagine (N) at amino acid position 390, in contrast to aspartic acid (D) (the derived state with respect to the global DEN-2 phylogeny), which distinguishes the Native American subtype V (Table 1). Although both of these amino acids are polar, the former is uncharged and the latter negatively charged. Four of our subtype IIIb isolates, all Trinidadian (TRI 1987, TRI 1988, TRI 1989 and TRI 1996 A; Fig. 3, clade 1), shared an alternative substitution of serine (S) at position 390, which has been previously reported in isolates of the "Cosmopolitan" subtype IV (Twiddy et al., 2002a, 2002b). Like N, S is a polar and uncharged residue.

The sole Caribbean isolate that did not fall within subtype IIIb was a 1981 isolate from Trinidad (TRI 1981), which belonged to Native American subtype V (Fig. 2). This group also contained earlier Caribbean isolates from Puerto Rico (1969) and Trinidad (1953), along with five Central American isolates: four from Honduras (1991) and one from El Salvador (1987) (HON 1991 A–D, SAL 1987; Fig. 2, subtype V). All possessed D at amino acid position 390, characteristic of

Table 1
Amino acid changes within the E glycoprotein of DEN-2 subtypes and lineages in study

Phylogeny		Amino acid at E gene position				
Subtype	Group/lineage	91	129	131	390	485
IIIb	Clade 1	L	V	L	N	V
	Clade 2	V	V	L	N	V
	Clade 3	I	V	Q	N	V
	Clade 4	I	V	L	N	V
	Asian	V	I	Q	N	I/L
V	–	V	I	Q	D	V
I	–	V	V	Q	N	V

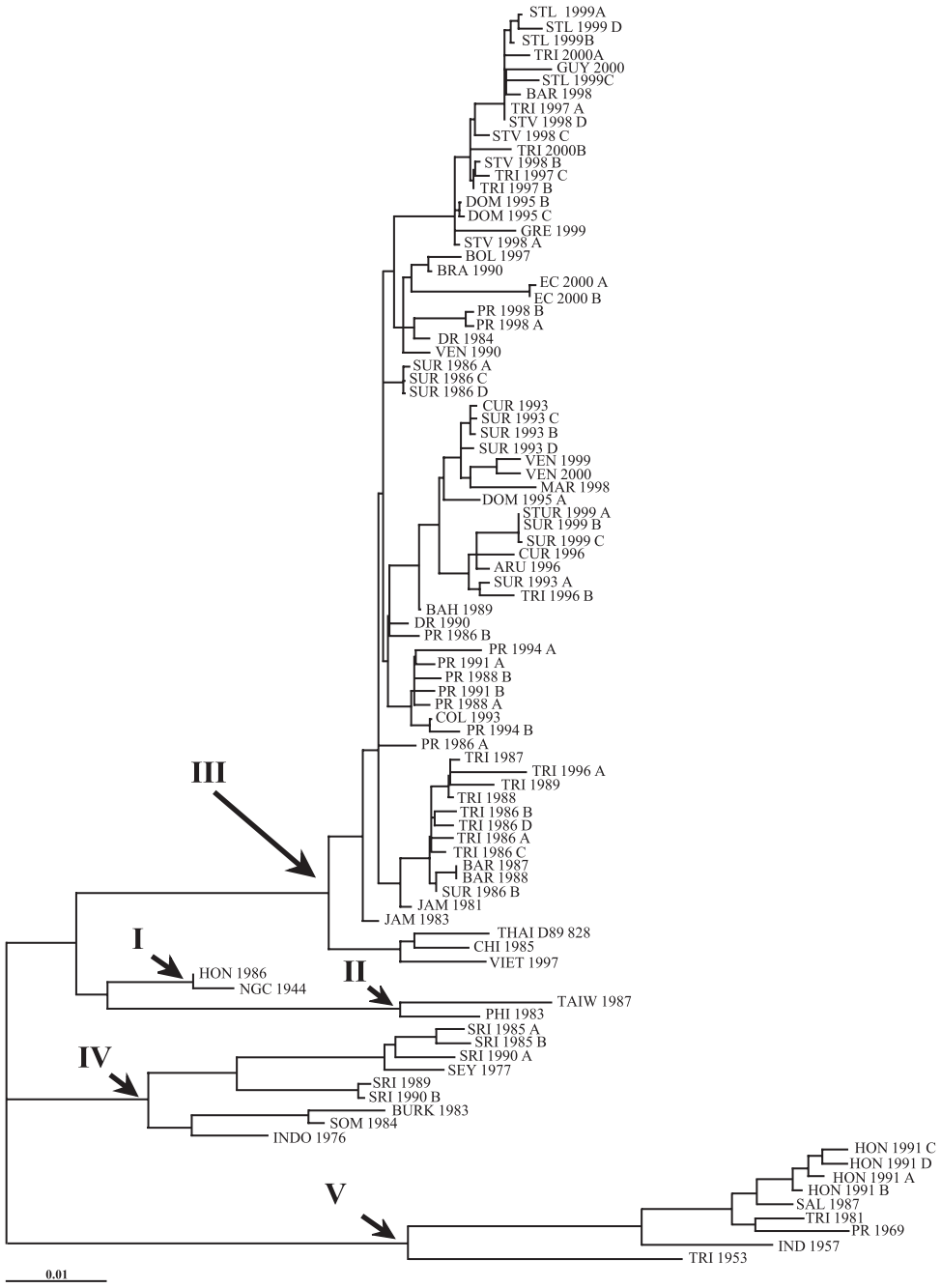

Fig. 2. Neighbor-joining tree of Global DEN-2 isolates. Neighbor-joining tree of globally distributed DEN-2 based on 1404-bp sequences of 93 isolates. DEN-2 subtypes 1–5, as described by Lewis et al. (1993), are labeled I, II, III, IV, and V, respectively. American isolates from this study falling outside subtype IIIb are labeled as follow, Honduras (HON), El Salvador (SAL), Trinidad (TRI). Remainder of global sequences in subtypes 1, 2, 4, and 5 obtained from PubMed database.

that subtype (Twiddy et al., 2002a, 2002b). The only other Central American isolate in our study, from Honduras (1986), fell into subtype I, and was closely related to the New Guinea C prototype strain (Fig. 2).

Subtype IIIb is comprised of an exclusively American clade, basal to which occur three Asian representatives of the subtype (THAI D89-828, CHI 1985, and VIE 1997; Fig. 3, labeled A). The American isolates differed from their Asian counterparts at positions 129 and 485 of the envelope protein: All American isolates have valine (V) at both positions, while Asian isolates have isoleucine (I) at position 129, and either I (CHI 1985, VIE 1997) or leucine (L, THAI D89-828) at position 485 (Table 1). All three of these amino acids have similar properties. In addition, Asian subtype IIIb isolates all bear the ancestral state at amino acid positions 91 and 131 of V and glutamine (Q), respectively, whereas American isolates, although polymorphic overall for these positions (I, L, or V at position

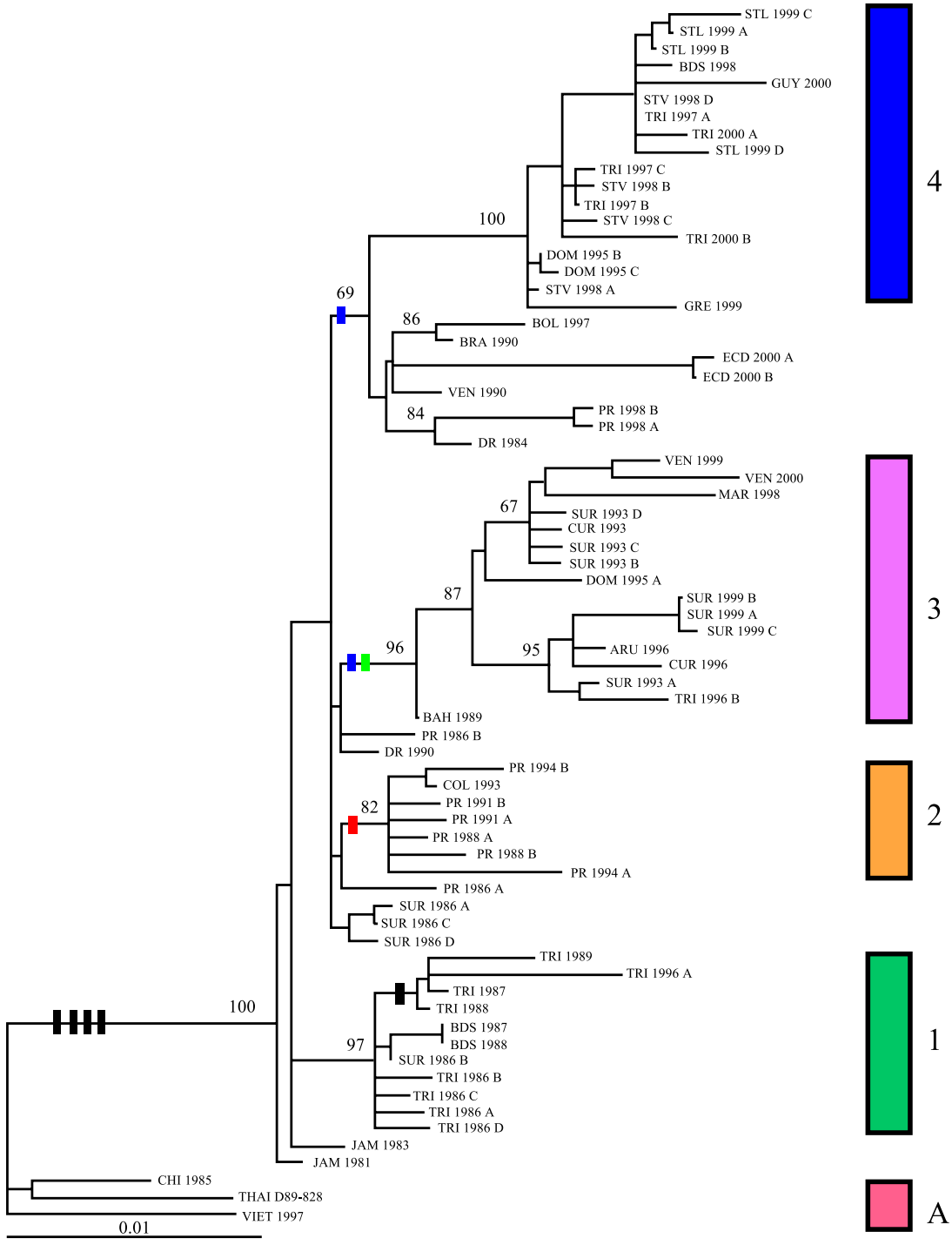

Fig. 3. Maximum likelihood tree of DEN-2 subtype IIIb. Maximum-likelihood tree of DEN-2 based on 1404-bp sequences of 71 isolates from around the Caribbean, Central and South America (1981–2000). Bootstrap support values were generated using 1000 replicate neighbor-joining trees reconstructed under the ML substitution model (Posada and Crandall, 1998). The names of isolates refer to country of origin and year of isolation. In cases where there is more than one isolate from a given country and year, a unique isolate number (and/or letter or code) is also indicated. American countries represented are Aruba (ARU), Brazil (BRA), Bahamas (BAH), Barbados (BDS), Bolivia (BOL), China (CHI), Colombia (COL), Curacao (CUR), Dominica (DOM), Dominican Republic (DR), Ecuador (EC), Grenada (GRE), Guyana (GUY), Jamaica (JAM), Puerto Rico (PR), Martinique (MAR), Mexico (MEX), St. Lucia (ATL), St. Vincent (STV), Suriname (SUR), Thailand (THAI), Trinidad and Tobago (TRI), Venezuela (VEN), Vietnam (VIE). Scale represents number of nucleotide substitutions. Sequences for BRA 1990, JAM 1981, VEN 1999, VEN 2000, MAR 1998, CHI 1985, THAI D89 828, and VIET 1997 were obtained from the PubMed database. Lineages are numbered 1–4, respectively. Black bars along the root branch represent amino acid changes between American and Asian subtype IIIb isolates at positions 91, 129, 131, and 485. Another black bar along the branch leading to Trinidadian subclade of clade 1 represents a reversion to the ancestral state at amino acid position 390. Colored bars along branches represent amino acid changes amongst American DEN-2 subtype IIIb lineages: L → V at position 91 (red), L → I at position 91(blue), and L → Q at position 131 (green).

91, and L or Q at position 131; Table 1), most likely underwent initial substitutions at both sites for L (Fig. 3).

We found no evidence for recombination within the E gene of any of the DEN-2 isolates included in this study. Split decomposition plots showed no recombinant sequences (data not shown), nor did analyses using PIST on either data set: Our estimate of the proportion of 2-state parsimony-informative changes in our ML trees (relative to all polymorphic sites), q, was 0.71 for the global data set and 0.55 for the subtype IIIb subset, neither significantly different from estimates from sequences evolved under clonality, $\hat{q}_c$, of 0.69 [$p(q = \hat{q}_c) = 0.26$] and 0.53 [$p(q = \hat{q}_c) = 0.34$], respectively.

Since the introduction of subtype IIIb into the Caribbean in 1981 (represented by JAM 1981), American isolates of DEN-2 subtype IIIb have formed four major well-supported clades (labeled 1–4 in Fig. 3). These clades are defined primarily by time period of isolation (with some overlap) and to a lesser extent by geography, with the exception of an almost exclusive Puerto Rican clade (clade 2, Fig. 3). Clade 1 consists largely of mid to late 1980s isolates from the Lesser Antilles (Barbados and Trinidad) and Suriname (Fig. 3). This clade has complete amino acid homology with the JAM 1981 isolate and is defined only by four silent mutations over the gene region examined, with the exception of the Trinidadian subclade already mentioned that nests within clade 1. Clade 2 consists of mid to late 1980s and early 1990s isolates from Puerto Rico, along with a 1993 Colombian isolate (Fig. 3). This group is distinguished from the introduction isolate by an L → V change at aa position 91 and two silent mutations. Clade 3 consists of early to late 1990s isolates from north-coastal South America (Venezuela and Suriname) associated primarily with isolates from those Caribbean islands closest to the South American mainland (i.e., Aruba, Curacao, and Trinidad) (Fig. 3). This clade is distinguished by five silent mutations and amino acid substitutions at envelope positions 91 (L to I) and 131 (L to glutamine, Q), the latter shared by two other weakly associated isolates (PR 1986 B and DR 1990; Fig. 3). At the base of clade 3 is a 1989 Bahamian isolate (BAH 1989) that may have "seeded" the lineage. Clade 4 is comprised of late 1990s to 2000 isolates from the Lesser Antilles island chain (Barbados, Dominica, Grenada, St. Lucia, St. Vincent, and Trinidad), along with a lone South American isolate from Guyana (Fig. 3). This group may have arisen as early as 1995 in Dominica (Fig. 3, clade 4A; DOM 1995 A and DOM 1995 B). Clade 4 is distinguished by eight silent changes in addition to an amino acid substitution at position 91 (L to I). It shares this amino acid change with various weakly associated Greater Antilles isolates from the same time period (PR 1998 A and PR 1998 B) or earlier (DR 1984) and South American isolates from Brazil (1990), Bolivia (1997), Ecuador (2000), and Venezuela (1990) (Fig. 3, branch with bootstrap of 69%; Table 1). In addition, there were a number of isolates from around the Caribbean basin dating from 1983 to 1990 that did not group within clear phylogenetic lineages (see Fig. 3; JAM 1983, PR 1986 B, SUR 1986 A–C, DR 1990).

Although subtype IIIb is somewhat both temporally and geographically ordered, there are several instances in which isolates from the identical time and place fall into separate clades, representing the co-circulation of multiple dissimilar genotypes. For example, two Trinidad isolates from 1996 occur in clade 1 and 3, respectively; and isolates from Dominica recovered in 1995 are associated with both clades 3 and 4, one of which appears to represent the origin of the latter.

There was considerable variation in rate of molecular change across our subtype IIIb phylogeny and the differential rates (DR) model of molecular evolution provided by far the best fit even after applying the Dunn–Sidák correction for multiple tests (data not shown). Analysis of third codon positions also did not support a molecular clock for the subtype IIIb data set according to uncorrected and corrected likelihood ratio tests, although according to the minimum Akaike information criteria (AIC; Akaike, 1974), VRDT and SRDT clock-like models outperformed DR (Table 2). Using the full codon data set, we estimated an absolute rate of

Table 2
Likelihood ratio test for different models of sequence evolution in dengue subtype IIIb

Data type	Model	−lnL	AIC	Parameters	LRT	*df*	P(χ^2)
Full codon	DR	4271.97	8821.94	139	–	–	–
	VRDT	4345.95	8835.89	72	VRDT vs. DR	67	4.83×10^{-8}
	SRDT	4349.11	8840.21	71	SRDT vs. DR	68	1.19×10^{-8}
					SRDT vs. VRDT	1	1.19×10^{-2}
	SR	4418.70	8977.4075	70	SR vs. DR	69	1.83×10^{-29}
					SR vs. VRDT	2	2.53×10^{-32}
					SR vs. SRDT	1	3.99×10^{-32}
Third codon	DR	2158.71	4595.41	139	–	–	–
	VRDT	2211.25	4566.49	72	VRDT vs. DR	67	2.04×10^{-3}
	SRDT	2213.37	4568.74	71	SRDT vs. DR	68	1.1×10^{-3}
					SRDT vs. VRDT	1	3.94×10^{-2}
	SR	2279.16	4698.32	70	SR vs. DR	69	6.79×10^{-21}
					SR vs. VRDT	2	3.21×10^{-30}
					SR vs. SRDT	1	1.85×10^{-30}

molecular evolution for subtype IIIb according to the SRDT model of evolution of 5.66×10^{-4} substitutions per site per year (confidence intervals of 4.26×10^{-4} and 7.20×10^{-4} substitutions per site per year). According to this model, the estimate for subtype IIIb's most recent common ancestor (MRCA) was 33.31 years (with confidence intervals of 29.66 and 39.41 years) before the year 2000.

Discussion

This is the first large-scale regional analysis of DEN-2 populations in the Caribbean since the introduction of the Asian–American DEN-2 strain and the onset of epidemic DHF/DSS in 1981. Our samples were drawn from countries around the Caribbean Sea, as well as greater South and Central America. The data indicate that subtype IIIb spread throughout the Americas by the early to mid 1980s, and over the past 20 years has evolved into at least four lineages, only two of which were still in circulation up to 2000. The most recent Native American (subtype V) isolate we detected in the Caribbean islands was from Trinidad (1981). The absence of this subtype beyond that year suggests that, at least within this part of the Americas, it has been displaced by the Asian–American strain, a theory first proposed by Rico-Hesse et al. (1997). However, given that we detected subtype V in El Salvador in 1987 and as recently as 1991 in Honduras, this strain may still persist in Central America. Likewise, although all of the South American isolates in our study belonged to subtype IIIb, Watts et al. (1999) confirmed the presence of subtype V in Iquitos, Peru as late as 1996 and suggested that this strain also persists in isolated pockets of South America. It is also important to consider the fact that the CAREC and CDC collections from which our isolates were derived are likely to be more representative of symptomatic than asymptomatic cases (see Materials and methods). Although both subtypes are associated with symptomatic disease, subtype V is thought to be less virulent than subtype IIIb (Rico-Hesse et al., 1997), so the apparent absence of subtype V among our isolates may also be a result of sample selection.

The presence of a New Guinea C-like strain in Honduras in 1986 (see Fig. 2; subtype I) was unexpected, because this subtype of DEN-2 has not been observed in the Americas in the last half of the century. Only two previous American studies (Halstead et al., 2001; Rico-Hesse et al., 1997) have identified isolates similar to this NGC-44 strain, from Venezuela (1994 and 1995), Mexico (1995), and more recently Haiti (2001). However, given that NGC-44 is one of the most commonly used dengue virus laboratory and reference strains, it is also plausible that isolates of this strain identified in the Caribbean may be laboratory contaminants. Based on our estimates of evolutionary rate, we would expect a 1986 isolate to differ from a 1944 strain by approximately 0.018% (conservative lower bound rate estimate 4.26×10^{-4} substitutions per site per year $\times$ 42 years = 0.018 substitutions per site). In fact, they differ by <0.005%, suggesting their origins are not 42 years apart and that the identity of the Honduran 1986 sample is erroneous.

While fluctuations in clock-like behavior increase the error in rate estimations, Jenkins et al. (2002) have shown that substitution rates estimated on large data sets are still reliable indicators of the average speed of evolution even when there is rate heterogeneity. Therefore, in spite of differential evolutionary rates among DEN-2 Caribbean subtype IIIb lineages, we estimated a single evolutionary rate across the phylogeny based on the SRDT model (5.66×10^{-4} substitutions per site per year) (confidence intervals of 4.26×10^{-4} and 7.20×10^{-4} substitutions per site per year). This estimate is comparable to rates reported in previous studies on DEN-2 subtypes (Twiddy et al., 2003; Wang et al., 2000). According to this rate, the estimate for subtype IIIb's most recent common ancestor (MRCA) was 33.31 years (with confidence intervals of 29.66 and 39.41 years) before the year 2000. Again similar similar to estimates for other DEN-2 subtypes of Asian origin (Twiddy et al., 2003) and corresponds to the post WWII period when dengue began its resurgence in Southeast Asia and the Pacific (Gubler, 1997), where the Asian–American strain is thought to have originated.

Since subtype IIIb was first introduced into the our study area in the early 1980s, its evolution has involved repeated substitutions over few amino acid positions in the E gene, suggestive of positive selection acting on these sites. This is in contrast to parallel studies of DEN-4 evolution in the Caribbean, which found little evolutionary activity in the E gene (Bennett et al., 2003; Foster et al., 2003). Three of the four major clades within subtype IIIb (clades 2, 3, and 4) are defined by amino acid mutations at a single position (91). In the case of clade 2 the L → V change at position 91 may represent reversion to the ancestral state, because this amino acid is present in sylvatic strains (Wang et al., 2000) and Asian members of the subtype (see Table 1; Blok et al., 1989). The L → I change at the same position that defines clades 3 and 4 is found only in our American isolates dated after 1988 and in Venezuelan isolates from the 1990s (Uzcategui et al., 2001). However, all substitutions we observed at position 91 involved amino acids with very similar properties. Only the additional substitution defining clade 3, a character state it shares with two immediately basal isolates and the Asian members of subtype IIIb, is likely to represent a significant phenotypic change. This substitution (which again appears to represent a reversion to the ancestral state) involved a replacement at aa residue 131 by an amino acid with different properties, where Q, a polar, hydrogen bond-forming residue, replaced L, a hydrophobic one. Position 131 resides in domain II of the flavivirus envelope glycoprotein model put forth by Rey et al. (1995). The impact of the substitution, if any, is presently unknown but since domain II is involved in virus-mediated fusion, mutations here may affect this function by disrupting the

process of conformational change at low pH (Roehrig et al., 1994; Twiddy et al., 2002a, 2002).

The temporal progression of clades within subtype IIIb suggests a complex interaction of evolutionary forces acting on DEN-2 (e.g., selection, genetic drift, and gene flow). Some subtype IIIb lineages appear to have been more successful than others. Clades 3 and 4 appear to have remained in circulation throughout the Americas up to the year 2000 while the most recent isolates in clades 1 and 2 are from the mid 1990s, suggesting that these lineages are extinct. The two persistent clades are comprised mainly of isolates dated 1993–2000. However, both lineages contain at least one isolate from the late 1980s to early 1990s (BAH 1989 in clade 3; DOM 1995 A, and B in clade 4), suggesting that they originated from isolates co-circulating with the older clades. Positive selection could account for differential success among clades: Although recent studies have provided no strong evidence for this in the dengue virus E gene (Bennett et al., 2003; Twiddy et al., 2002a, 2002b), nonstructural genes which we did not survey, are also targets for selection in dengue viruses (Bennett et al., 2003; Leitmeyer et al., 1999; Twiddy et al., 2002a, 2002b). Alternatively, local population dynamics leading to genetic bottlenecks could have randomly led to extinction and replacement of clades 1 and 2 by 3 and 4 through genetic drift. Gene flow between regions becomes an important mechanism for introducing dengue variants and establishing new lineages in a given locality, especially under such conditions.

The movement of viral variants (gene flow) between regions within the Caribbean basin and South and Central America is facilitated by geographic proximity, and cultural and economic ties. Based on its phylogeography (Fig. 3), DEN-2 subtype IIIb spread across the region after its introduction in 1981 in a manner suggesting established DEN transmission routes between the Caribbean islands and north-coastal South America. For example, although most isolates from the coast of South America bordering the Caribbean Sea, such as those from Suriname and Venezuela, grouped together into clade 3, this cluster also included isolates from nearby territories such as Trinidad, Aruba, and Curacao (Fig. 2, clade 3) rather than those from the Pacific coast of the continent such as Ecuador. Similarly, all of the 1990s isolates from the Lesser Antilles formed a close-knit group (Fig. 3, clade 4), which also included an isolate from nearby Guyana from the same time period (GUY 2000). Venezuela, Suriname, and Guyana represent obvious transit points for viruses travelling between the Caribbean islands and the mainland: Venezuela because of its close proximity (Aruba, Curacao and Trinidad, the southernmost Caribbean island, are within 56 km of Venezuela's shores); Suriname and Guyana because of their relative proximity and strong socioeconomic links from membership in the Caribbean Common Market (CARICOM). Similar geographic and socioeconomic relationships have been observed among DEN-4 populations in the Caribbean basin, which exhibit higher degrees of gene flow correlated with less divergence among Antillian and South American populations bordering the Caribbean Sea, relative to populations from Central America and Ecuador (Foster et al., 2003).

In contrast, the Greater Antilles appear to act as an evolutionarily independent DEN-2 population with gene flow restricted to member islands and only the occasional exchange of variants beyond. Puerto Rican isolates from the late 1980s to mid-1990s formed an independent clade (clade 2) that included only a single non-Puerto Rican isolate from Colombia (COL, 1993). Late 1990s, Puerto Rico isolates grouped with Dominican Republic isolates although they were separated by 14 years (84% bootstrap support, Fig. 3). Puerto Rico has a large dense urban population (in contrast to smaller islands in the Lesser Antilles) with high mosquito vector densities (Bennett et al., 2003). The island therefore has the capacity to act as a center of high viral diversity in its own right, viral lineages being less susceptible to extinction and recolonization from other populations and more likely to appear phylogenetically distinct (Bennett et al., 2003). The phylogenetic association of isolates from Puerto Rico with those from Colombia and the Dominican Republic may reflect a "Latin American" transmission route based on cultural and economic ties between these Spanish-speaking territories, presumably stronger than ties to the Lesser Antilles and other non-Spanish speaking regions. The phylogenetic association of the Bahamas isolate supports this pattern, because although derived from a Greater Antillean island, it instead shares a common phylogenetic history with clade 3, the north-coastal South American/southern Caribbean clade. This association suggests that the 1989 Bahamas isolate seeded clade 3, and reflects strong cultural (English-speaking) and economic (through CARICOM) links between the Bahamas and the Lesser Antilles.

The complexity of DEN-2 subtype IIIb evolutionary history in the Americas is further indicated by differences in evolutionary rates acting on each lineage of the phylogeny. In our analyses, the differential rates (DR) model for evolution significantly outperformed models that estimate a single or linearly changing rate for all lineages (i.e., SRDT and VRDT models, respectively), both for full codon and third codon position subset of subtype IIIb. In contrast, another recent study of molecular evolution in DEN-2 found that a molecular clock did fit (Twiddy et al., 2003). Differences in evolutionary rates across branches of a phylogeny could be due to selection (as discussed above) or lineage-specific demographic properties. Differences in viral population dynamics, particularly fluctuations in viral effective population size among regions, could create variation in both neutral and non-neutral evolutionary rates (Gillespie, 1999; Ohta, 2002). Indeed, the phylogenetic and epidemiological patterns suggest that DEN-2 populations in the Caribbean are best described as a series of loosely interconnected source and sink populations (i.e., a metapopulation; Gilpin and Hanski, 1991). Disease dynamics

among DEN populations differ depending on a number of extrinsic factors including host population sizes and immune history, vector habitat and control efforts, rainfall, and temperature (Gratz, 1999; Gubler, 1993; Kuno, 1997; WHO, 2002). The semi-independent nature of dengue viral populations is also reflected in a history of disjunct outbreaks across the region, lagging between locations by one to several years (Gubler, 1993). Furthermore, it is unlikely that all DEN-2 populations within the Caribbean basin are at evolutionary equilibrium (easily disrupted by bottlenecks), and this could also lead to extensive rate variation (Ohta and Gillespie, 1996). Markedly unstable, dengue populations fluctuate between very large sizes during epidemics and periods of extremely small population sizes leading in some instances to local extinction (Kuno, 1997; Sittisombut et al., 1997). Even on large islands like Puerto Rico, which sustain continuous dengue transmission, effective population size (sensitive to the lowest extreme) is on the order of 500 viruses during epidemic periods where up to an estimated 850,000 people could have been infected (Bennett et al., 2003).

In conclusion, our large regional and temporal scale phylogenetic study suggests that DEN-2 in the Caribbean basin including parts of South America is primarily subtype IIIb, displacing the former subtype over a wide geographic area since its introduction in 1981. The phylogeographic pattern of subtype IIIb evolution underscores the absence of physical barriers to DEN transmission among territories within the Caribbean basin and emphasizes the need for dengue control efforts that focus on a regional rather than local scale (Foster et al., 2003). The temporal progression of clade evolution within subtype IIIb and observed variation in rates of molecular evolution suggest the presence of complex evolutionary dynamics throughout its history. Further work is needed to determine what factors have dictated the success or failure of individual lineages, and to determine whether the phylogenetic diversity observed translates into differences in virulence and transmittability.

Materials and methods

Viral archive, RNA extraction, and RT-PCR

Virus isolates were obtained from the Caribbean Epidemiology Centre (CAREC), Trinidad, and the Centers for Disease Control and Prevention (CDC), Dengue Branch, San Juan, Puerto Rico. At both institutions, viruses were isolated from sera and, if necessary, cultured in C6/36 mosquito cells. These institutions are principal sources of dengue identification and serotyping in the Caribbean basin and regularly receive sera from medical institutions throughout the Americas. The samples received therefore usually represent symptomatic infections. We randomly selected viruses from the collections, stratified to represent the multiple localities and years in which DEN-2 has been recorded between 1981 and 2000. In total, we sequenced 59 isolates from 10 Caribbean islands (n = 36), 6 South American countries (n = 17), and 2 Central American countries (n = 6). Table 3 shows the year, country of origin, and number of isolates included in this study.

Viral RNA was extracted from sera or cell culture supernatant using QIAamp Viral RNA Mini kits (Qiagen GmbH, Germany) according to the manufacturer's instruc-

Table 3
Country, year of isolation, and number of DEN-2 isolates sequenced in this study

Country of isolation	Year of isolation																
	1981	1983	1984	1986	1987	1988	1989	1990	1991	1993	1994	1995	1996	1997	1998	1999	2000
Aruba													1				
Bahamas							1										
Barbados					1	1									1		
Bolivia														1			
Colombia										1							
Curacao										1			1				
Dominica												3					
Dominican Republic			1					1									
Ecuador																	2
El Salvador					1												
Grenada																1	
Guyana																	1
Honduras				1					4								
Jamaica[a]		1															
Puerto Rico[a]				2		2			2		2				2		
St. Lucia																4	
St. Vincent															4		
Suriname				4						4						3	
Trinidad	1			4	1	1	1						2	3			2
Venezuela								1									

[a] Bennett et al., unpublished data.

tions. For each isolate, we amplified a 1404-bp region of the genome encoding almost the entire 1485-bp envelope (E) gene in reverse transcriptase-polymerase chain reactions (RT-PCRs). RT-PCR amplification primer sequences are provided in Table 4. Where necessary, RT-PCR products were purified before sequencing using Qiagen PCR purification kits (Qiagen), as outlined by the manufacturer.

RT-PCR fragment sequencing

Cycle-sequencing reactions were performed using Taq DyeDeoxy Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, with sequencing primers described in Table 4. Products were cleaned by standard precipitation before sequencing on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems). Sequences were then aligned against a DEN-2 reference sequence (Irie et al., 1989) (GenBank number M29095) and fragments combined for a given isolate using Sequencher 3.1.1 software (Gene Codes, Michigan) to obtain a continuous nucleotide sequence for each sample. All sequences generated in this study can be accessed in GenBank (accession numbers AY484598–AY484667).

Phylogenetic analysis

Sequences were aligned using MEGALIGN v 3.1.7 (Lasergene), employing the Clustal algorithm (Higgins and Sharp, 1988; Higgins et al., 1996) and the alignments manually checked for errors. Two phylogenetic trees were then constructed using PAUP 4.0b10 (Swofford, 1998). In the first, all 59 sequences were compared with 11 unpublished sequences (2 Jamaican, 9 Puerto Rican; Bennett et al., manuscript in preparation) and 21 previously published sequences (representing all five DEN-2 subtypes) in a neighbor-joining tree, as a means of subtyping our samples. For all sequences falling into the DEN-2 subtype IIIb clade (n = 71 including eight reference sequences (L10041, M15075, AF119661, AF195041, AF208496, AF398108, AF398114, AF410358), we then constructed a second tree derived by maximum likelihood for a more rigorous assessment of the subtype's microevolution. Tree calculations were based on models of sequence evolution whose parameters were estimated by maximum likelihood, using Modeltest 3.06 PPC (Posada and Crandall, 1998). The resultant phylogenetic tree was rooted with a subgroup of Asian isolates belonging to subtype III that served as an outgroup against which to compare our Caribbean isolates. We assessed the reliability of the tree topology by bootstrapping the data set 1000 times to generate neighbor-joining trees. Internal nodes of particular interest are labeled with percent times obtained, if greater than 50. We denote groups as "well-supported" if their bootstrap values are at least 75% (this value was arbitrarily selected because it is midway between 50 and 100). Initial recombination analysis was performed on the data by comparing neighbor-joining trees based on 500 continuous bases at a time for topological incongruities. SplitsTree v2.4 (Huson, 1998) was also used at this stage to examine sequences for recombination by way of the split decomposition method, which produces network-like trees when recombination is present in data sets. Finally, we assessed the overall degree of recombination among our DEN-2 isolates' E gene sequences using PIST 1.0 (Worobey, 2001) on both data sets (i.e., the global DEN-2 data set and the DEN-2 subtype IIIb subset). This method assesses the maximum-likelihood tree for elevated proportions of two-state parsimony-informative polymorphic sites, by comparing this index to the number of such sites observed over 1000 simulations of clonal evolution (constrained to the same tree and model of evolution; Worobey, 2001).

Finally, to estimate the rate of evolution within subtype IIIb and/or detect differences in these rates over time and/or across lineages, we compared the fit of different models of evolutionary rates, using hierarchical likelihood ratio tests (Rambaut, 2000) and Akaike Information Criteria (AIC) (Posada and Crandall, 1998). Models of substitution rate include a non-clock-like rate of evolution indicated by different rates for each branch (DR model), and three clock-like models of evolution given by a rate for all branches that varies linearly over time (VRDT), a single rate for all branches, with noncontemporaneous tips (SRDT), and a single rate for all branches where tips are constrained to be contemporaneous (SR; estimated by

Table 4
List of primers used to amplify and sequence envelope of DEN-2

Primer[a]	Sequence	Function	Genome position
U1019	5′-AACATGGAAGTTGTGTGACGACGAT	amplification	1019
L2526	5′-TTTGAAGGGGATTCTGGTTGGAAC	amplification	2526
U1027	5′-AGTTGTGTGACGACGATGGCAAAAA	sequencing	1027
L1405	5′-TGATTTCCTTGCCATGTTTTCCTGT	sequencing	1405
U1417	5′-GGCAAGGAAATCAAAATAACACCAC	sequencing	1417
U1736	5′-CAGGGGCCACAGAAATCCAGATGTC	sequencing	1736
L1776	5′-CCTGCACTTGAGATGTCCTGTGAAC	sequencing	1776
L2131	5′-TTGTCTCAAACATTTGGCCGATGGA	sequencing	2131
U2149	5′-GAGACAACAATGAGAGGAGCGAAGA	sequencing	2149
L2476	5′-CGTTATCTGTGATGAAGATCCCGCT	sequencing	2476

[a] Designed by Bennett et al.

enforcing a molecular clock in PAUP* with no consideration for noncontemporaneous samples). Likelihood scores for each model were generated in TipDate version 1.2 (Rambaut, 2000), along with a maximum-likelihood estimate of substitution rates and 95% confidence intervals for the rooted ML tree (Rambaut, 2000). Confidence intervals were estimated on a reduced, representative data set of 20 isolates, due to computational constraints. All calculations were performed using the REV six-parameter nucleotide substitution model (Lanave et al., 1984; Rodriguez et al., 1990), the best-fitting model for our data set according to Modeltest 3.06 PPC (Posada and Crandall, 1998).

Acknowledgments

The authors thank the Caribbean Epidemiology Centre (CAREC) and CDC Dengue Branch (Puerto Rico) for providing isolates for our study. In particular we are grateful to V. Vorndam of the CDC, and V. Morris-Glasgow and A. George of CAREC. We are also grateful to laboratory personnel at the Dept. of Biology, University of Puerto Rico and CDC Dengue Branch for their technical assistance and use of their sequence database. In particular, we thank D. Rodriguez, M. Beltran and M. Chirivella. We would also like to thank D. Gubler for helpful comments on the manuscript. Sequence data presented in this study was collected at the UPR Sequencing and Genotyping Facility, established in part by grants from NIH (RCMI and BRIN), NSF, and from Puerto Rico's EPSCoR program. This work was funded by a UNDP/World Bank/ WHO TDR training grant (CVFC, JEF, HV) and research grants from the University of the West Indies, St. Augustine Campus Research and Publication Fund (CVFC), the Caribbean Health Research Council (CVFC), and the United States National Institute of Health (WOM).

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