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Short Report: Phylogenetic Analysis of Dengue Virus Types 1 and 4 Circulating in Puerto Rico and Key West, Florida, during 2010 Epidemics

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Abstract. We describe sequences of six strains of dengue virus (DENV): three DENV-1 isolates and two DENV-4 isolates from Puerto Rico, and a DENV-1 strain from Key West, Florida, obtained from blood donors during 2010 epidemics. Phylogenetic analysis revealed that the Puerto Rico DENV-1 strains constitute a new lineage within genotype V different from those that circulated in Puerto Rico during the past two decades. The newer Puerto Rico DENV-1 strains associated with strains from the Caribbean and South America. The DENV-1 strain from Key West, Florida clustered with a strain isolated from mosquito pools collected in that area and with a number of strains from Nicaragua and Mexico circulating during 2006–2009. The Puerto Rico DENV-4 isolates of genotype II associated with strains that have circulated on the island throughout the 1980s and 1990s and with strains from the Caribbean region and Central America. Introduction and circulation of novel DENV lineages in dengue-endemic regions have the potential to increase the severity of dengue cases.

Dengue is caused by any of the four dengue virus types (DENV-1 to DENV-4), family *Flaviviridae*, genus *Flavivirus*. Dengue virus is primarily transmitted by the urban mosquito *Aedes aegypti* and most dengue infections are asymptomatic or sub-clinical. Dengue disease spectrum ranges from a mild, influenza-like disease (dengue fever) to a potentially life-threatening condition known as severe dengue/dengue hemorrhagic fever.[1]

The four DENV types are genetically distant from each other, and phylogenetic analysis shows epidemic genotypes that vary in number and geographic distribution depending on the DENV type. Additionally, sylvatic cycles exist in Africa and Asia where sylvatic strains of DENV-2 and DENV-4 have been isolated from arboreal mosquitoes, humans, and non-human primates.[2] Four DENV-1 epidemic genotypes (I, II, IV, and V) and a sylvatic genotype have been described. However, for the sylvatic genotype recent evidence suggest a human origin, therefore being included as genotype III in newer classifications. Conversely, for DENV-4, three epidemic genotypes (I–III) and a sylvatic genotype have been described.[3] Some DENV genotypes have shown to be more virulent, with better fitness, and have been associated with increased clinical severity of dengue cases (e.g., American/Asian genotype of DENV-2).[4]

In the United States, dengue is endemic to the Commonwealth of Puerto Rico, which in 2010 experienced the largest epidemic in its history with more than 21,000 dengue suspected cases reported, of which approximately 75% were laboratory confirmed.[5] Sporadic dengue outbreaks have occurred in the Territories of Guam and American Samoa, the States of Hawaii and Texas,[6–8] and more recently in Key West, Florida during 2009–2011.[9,10] We report sequences and phylogenetic analyses of three DENV-1 and two DENV-4 strains isolated from Puerto Rico and a DENV-1 strain from Key West, Florida circulating during the 2010 epidemics.

Six plasma samples obtained from blood donors infected with DENV that were asymptomatic at the time of collection were used for further testing by using a TaqMan quantitative reverse transcription polymerase chain reaction (qRT-PCR). All specimens were subjected to RNA extraction by using the QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA), and the presence of DENV RNA was confirmed at the Food and Drug Administration by using a modified type-specific qRT-PCR in a singleplex format based on a published protocol.[11] Plasma specimens were also evaluated for infectious virus by culture in mosquito cells. In brief, 200–250 μL of plasma were added to a semi-confluent monolayer of C6/36 cells (American Type Culture Collection, Manassas, VA) propagated at 32°C in an atmosphere of 5% CO_2. Supernatants from the first and second passages in C6/36 cells were tested for viral production by qRT-PCR and infectivity was tested by focus-forming assay.

Cell culture supernatants from the second passages in C6/36 cells were used for sequencing. Extracted viral RNA was subjected to RT-PCR by using DENV-specific primers to amplify the DENV structural genes region (capsid-premembrane-envelope). In brief, fragments of approximately 3,700 and 3,500 nucleotides for DENV-1 and DENV-4, respectively, were generated by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), LA Taq polymerase (Takara, Otsu, Japan), and specific DENV-1 and DENV-4 primers (Supplemental Table 1). Amplified products were electrophoresed in agarose gels, purified from the gels and subjected to bidirectional Sanger sequencing with a minimum coverage of four-fold by using appropriate sequencing primers (Supplemental Table 1). Sequences were assembled and analyzed by using Sequencher version 5 (GeneCodes Corp., Ann Arbor, MI) and deposited in the GenBank database under accession numbers JQ045561–JQ045566.

Phylogenetic analyses were conducted with envelope protein (E) gene sequences (1,485 nucleotides) in datasets containing a total of 36 DENV-1 and 30 DENV-4 strains. Each dataset was comprised of randomly selected sequences from the Caribbean, Central America, and South America and representative sequences from diverse geographic origins encompassing all DENV genotypes retrieved from the GenBank by using the Virus Variation Tool.[12] BLAST (National Center for Biotechnology Information, Bethesda, MD) searches were conducted for each newly sequenced DENV-1 and

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Table 1
DENV-1 and DENV-4 strains used in this study*

Strain	Place of collection	Year of collection	Genotype	GenBank accession no.
DENV-1				
US-HI/Hawaii/1944	Hawaii, USA	1944	I	AB609588
MY/36000/2005	Malaysia	2005	I	FR666924
TH/TH-Sman/1954	Thailand	1954	II	D10513
TH/2543-63/1963	Thailand	1963	II	AF425629
MY/P72-1244/1972	Malaysia	1972	III	AF231721
MY/36046/2005	Malaysia	2005	III	FN825674
US-HI/HawM2540/2001	Hawaii, USA	2001	IV	DQ672562
US-HI/HawO3663/2001	Hawaii, USA	2001	IV	DQ672564
VG/BID-V2937/1985	British Virgin Islands	1985	V	GQ868601
HT/DB067/2010	Haiti	2010	V	JF969282
US-FL/KW10AG/2010	Key West, FL, USA	2010	V	JF519855
US-FL/FDA-ARC-39-10/2010†	Key West, FL, USA	2010	V	JQ045564
MQ/481013035/2008	Martinique	2008	V	JN022599
MX/BID-V3739/2007	Mexico	2007	V	GQ868527
MX/BID-V3679/2007	Mexico	2007	V	GU131966
MX/BID-V3758/2008	Mexico	2008	V	GQ868537
NI/BID-V653/2004	Nicaragua	2004	V	EU596501
NI/BID-V2343/2006	Nicaragua	2006	V	FJ562104
NI/BID-V746/2006	Nicaragua	2006	V	JN819403
NI/BID-V2646/2008	Nicaragua	2008	V	GQ199858
NI/BID-V2652/2008	Nicaragua	2008	V	GQ199859
NI/BID-V5504/2009	Nicaragua	2009	V	JF937635
NI/BID-V5067/2009	Nicaragua	2009	V	JF937644
US-PR/BID-V2136/1992	Puerto Rico	1992	V	FJ410186
US-PR/BID-V2135/1992	Puerto Rico	1992	V	FJ547087
US-PR/BID-V2133/1993	Puerto Rico	1993	V	FJ410184
US-PR/BID-V2134/1993	Puerto Rico	1993	V	FJ410185
US-PR/BID-V1743/1995	Puerto Rico	1995	V	FJ205874
US-PR/BID-V1744/1995	Puerto Rico	1995	V	FJ205875
US-PR/BID-V2139/1996	Puerto Rico	1996	V	FJ410188
US-PR/BID-V1739/1998	Puerto Rico	1998	V	FJ205872
US-PR/BID-V852/2006	Puerto Rico	2006	V	EU482591
US-PR/FDA-ARC-12-10/2010†	Puerto Rico	2010	V	JQ045561
US-PR/FDA-ARC-30-10/2010†	Puerto Rico	2010	V	JQ045562
US-PR/FDA-ARC-31-10/2010†	Puerto Rico	2010	V	JQ045563
VE/BID-V2423/2004	Venezuela	2004	V	JN819425
DENV-4				
PH/H241-P/1956	Philippines	1956	I	FJ439174
JP/D4-61NIID/1961	Japan	1961	I	AB111090
SL/No17/1978	Sri Lanka	1978	I	AY550909
CR/D4.108/1996	Costa Rica	1996	II	AY152104
DM/814669/1983	Dominica	1981	II	AF326573
DR/DB040/1997	Dominican Republic	1997	II	JF804053
SV/D4.110/1993	El Salvador	1993	II	AY152300
MQ/D4.112/1995	Martinique	1995	II	AY152100
MX/D4.111/1995	Mexico	1995	II	AY152304
US-PR/D4M.25/1982	Puerto Rico	1982	II	AY152296
US-PR/D4M.9/1982	Puerto Rico	1982	II	AY152324
US-PR/D4.115/1986	Puerto Rico	1986	II	AY152224
US-PR/D4.116/1986	Puerto Rico	1986	II	AY152272
US-PR/D4.3/1987	Puerto Rico	1987	II	AY152108
US-PR/D4.67/1987	Puerto Rico	1987	II	AY152236
US-PR/D4.85/1994	Puerto Rico	1994	II	AY152096
US-PR/D4.86/1994	Puerto Rico	1994	II	AY152116
US-PR/D4.88/1994	Puerto Rico	1994	II	AY152288
US-PR/BID-V2435/1996	Puerto Rico	1996	II	GQ199881
US-PR/D4.19/1998	Puerto Rico	1998	II	AY152040
US-PR/D4.44/1998	Puerto Rico	1998	II	AY152088
US-PR/BID-V1093/1998	Puerto Rico	1998	II	EU854296
US-PR/BID-V1082/1998	Puerto Rico	1998	II	FJ024424
US-PR/BID-V2443/1998	Puerto Rico	1998	II	FJ850059
US-PR/FDA-ARC-19-10/2010†	Puerto Rico	2010	II	JQ045565
US-PR/FDA-ARC-42-10/2010†	Puerto Rico	2010	II	JQ045566
US-VG/SC/DB045/1994	U.S. Virgin Islands	1994	II	JF804058
TH/ThD4 0439 01/2001	Thailand	2001	III	AY618940
TH/ThD4 0164 99/1999	Thailand	1999	III	AY618986
MY/P75-514/1975	Malaysia	1975	Sylvatic	AF231723

*DENV = dengue virus. DENV-2 New Guinea C and DENV-3 H87 (GenBank accession numbers AF038403 and M93130, respectively) were included as outgroup for both phylogenetic trees.
†Strains sequenced in this study.

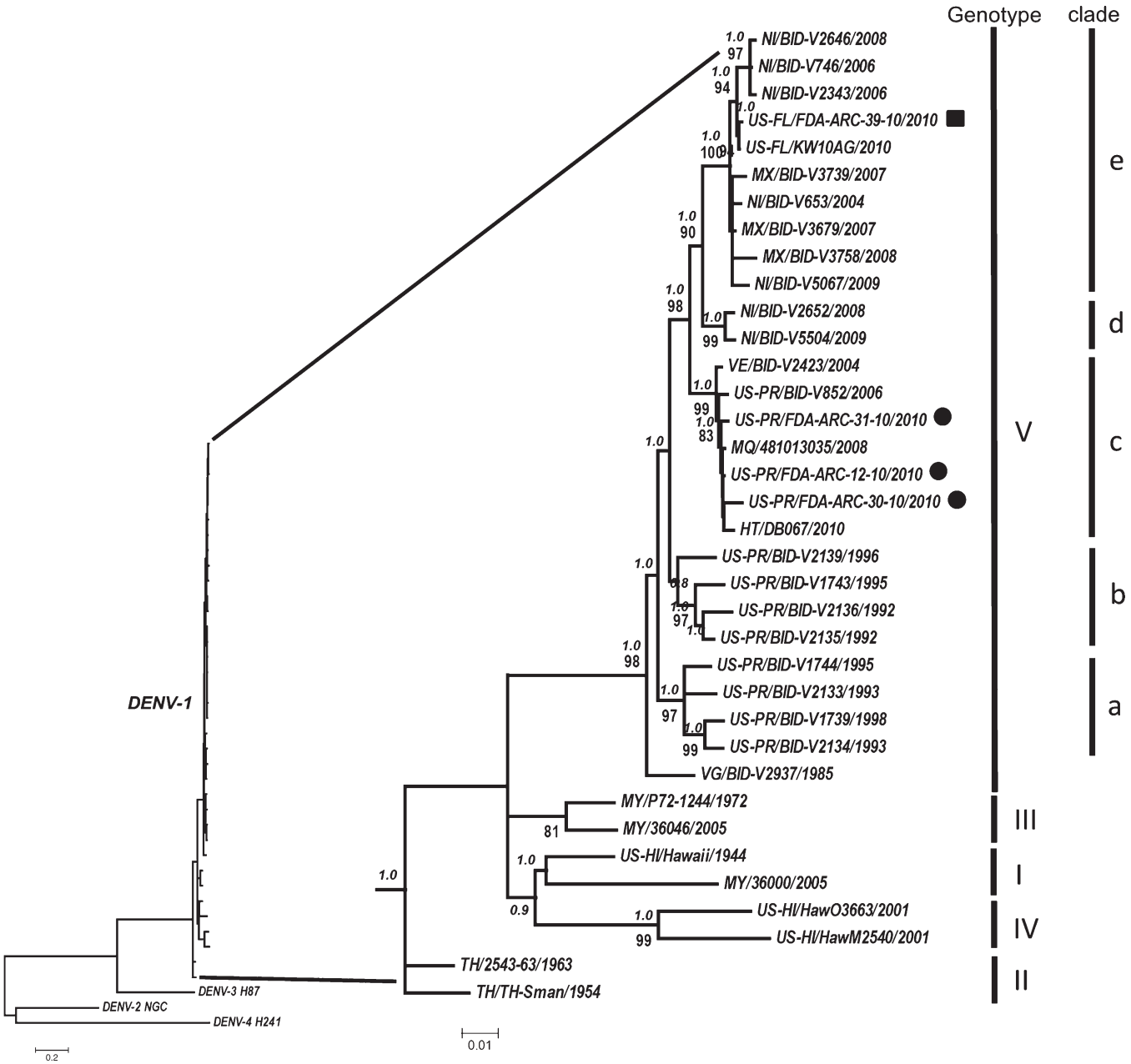

FIGURE 1. Consensus maximum-likelihood phylogenetic tree constructed with sequences of the dengue virus type 1 (DENV-1) envelope protein gene. Genotypes are identified by roman numerals. Clades of genotype V are identified by the letters a–e. Strains collected from blood donors from Puerto Rico are represented by black circles and the strain from Key West, Florida, are represented by a black square. Bayesian posterior probability (in italics) and bootstrapping values ≥ 80 are shown above and under the branch nodes, respectively. Branch lengths are proportional to the scale bar and the scale bar represents number of nucleotide substitutions between the analyzed viruses. The tree is mid-point rooted.

DENV-4 strain and the closest hits for each were included in the datasets. The E gene sequences were selected for conducting the phylogenetic analyses because a considerable number of DENV-1 and DENV-4 strains from Puerto Rico and the Caribbean region available in the database were limited to only sequences of that gene. A sylvatic sequence (for the DENV-4 analysis) and each of the DENV prototype strain sequences; i.e., DENV-2 NGC, DENV-3 H87, and DENV-4 H241 or DENV-1 Hawaii (for both DENV-1 and DENV-4 datasets, respectively) were used as outgroup to root the trees. Genotypes were classified according to Chen and Vasilakis.[3] The list of analyzed sequences is shown in Table 1.

Phylogenetic reconstruction was conducted by the maximum-likelihood method using MEGA5.[13] For the DENV-1 dataset the Tamura-Nei + Γ_4 + I model was determined to be the best fitted model for the data. For the DENV-4 dataset, the general time reversible (GTR) + Γ_4 was the best model as determined according to the Bayesian information criterion (BIC) determined by using MEGA5. A bootstrap test of 1,000 replicates was used for both datasets.

In addition, Bayesian phylogenetic (BA) and time-scale analyses were conducted by using Mr. Bayes version 3.1.2[14] and BEAST version 1.6.2,[15] respectively. For BA analysis, the GTR + Γ_4 + I model with successive branch swapping was

used as the substitution model. Four Markov Chain Monte Carlo chains were run for 10,000,000 generations, sampling every 100 generations, and the first 10,000 sampled trees were discarded as burn in. A 50% majority rule consensus tree was constructed from the posterior distribution of trees. To calculate the age for the nodes in which the newly sequenced DENV-1 and DENV-4 were located, a time-scale analysis was performed for the same datasets used for the phylogenetic analysis (excluding other DENV type sequences) by using the GTR + Γ_4 + I nucleotide substitution model, the relaxed uncorrelated lognormal clock, and constant population size tree prior as implemented in BEAST. Three independent Markov Chain Monte Carlo analyses, each run for 10,000,000 steps, were performed and combined with a burn in value set to 10% generations. A maximum clade credibility tree was generated for each DENV type, and the 95% highest posterior density intervals were obtained to determine the uncertainty in the parameters estimated.

We detected DENV RNA in all six plasma specimens using our qRT-PCR. Four of them were positive for DENV-1 and two were positive for DENV-4. All samples were infectious in cell culture as determined by positive focus-forming assay results in supernatants from infected C6/36 cells. DENV-1 and DENV-4 accounted for most of the confirmed dengue cases during the 2010 epidemic in Puerto Rico, and DENV-2 cases were also detected but at a significantly lower incidence,[5] which can explain why no DENV-2 cases were detected in the studied blood donor cohort.

Maximum-likelihood and BA analyses showed phylogenetic trees with similar topology for each DENV type and our findings are supported by high bootstrapping and/or Bayesian posterior probability values (Figures 1 and 2). Analysis of the phylogenetic trees enabled us to identify the three PR DENV-1 strains from 2010 as belonging to a different lineage within genotype V, which is distant from those that had circulated in the island during 1980s and 1990s (Figure 1). The 2010 strains represent a lineage related to those that have been detected in Central America, Mexico, and the Caribbean during the past decade,[16] which are closely associated with strains from Venezuela, Haiti, Martinique, and

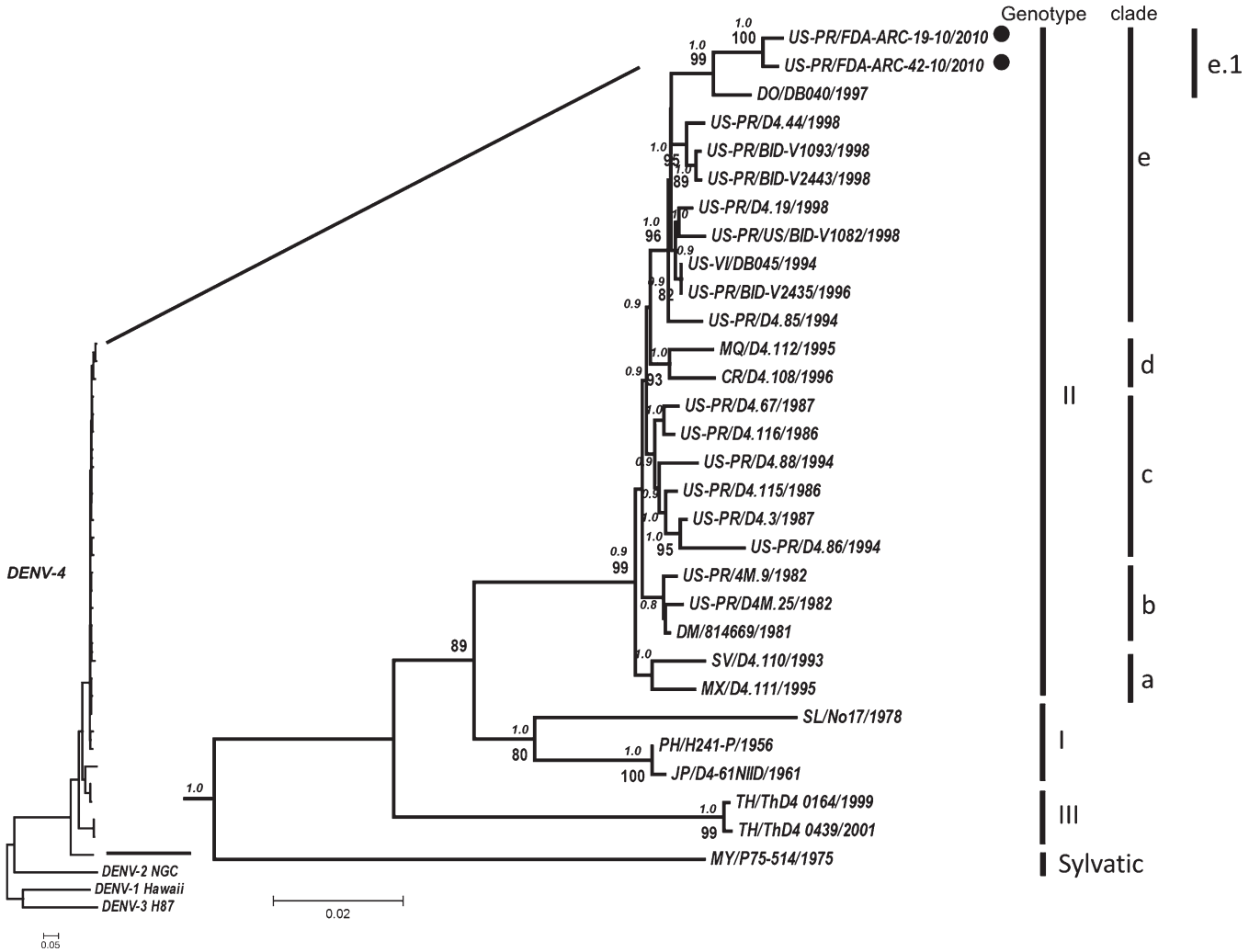

FIGURE 2. Consensus maximum-likelihood phylogenetic tree constructed with sequences of the dengue virus type 4 (DENV-4) envelope protein gene. Genotypes are identified by roman numerals. Clades of genotype II are identified by the letters a–e. Sub-clade e.1 identifies the cluster where the 2010 Puerto Rican DENV-4 strains are located, and these strains are represented by black circles. Bayesian posterior probability (in italics) and bootstrapping values ≥ 80 are shown above and under the branch nodes, respectively. Branch lengths are proportional to the scale bar and the scale bar represent the number of nucleotide substitutions between the analyzed viruses. The tree is mid-point rooted.

strain DENV-1/US/BID-V852/2006, which is the most recent strain from Puerto Rico deposited in the database that contains the entire E gene sequence (Figure 1).

As expected, the DENV-1 strain obtained from a blood donor in Key West, Florida clustered with strain KW10AG obtained from mosquito pools collected in Key West, Florida.[17] Comparison of E protein gene sequences between these two strains showed 99.9% identity among them (1,484/1,485 nucleotides). The Key West, Florida DENV-1 strains are closely related to strains from Nicaragua sampled in 2006 and 2008, and constitute a different lineage of DENV-1 within a cluster that includes a number of strains from Mexico collected during 2007–2008 (Figure 1). The age of the DENV-1 node containing the 2010 Puerto Rico strains (clade c) is 7–15 years (mean = 11 years). For clades d and e (clade e contains the 2010 DENV-1 strain from Key West, Florida), the age is 16–34 years (mean = 25 years). The age of the parent node for these three clades is 19–40 years (mean = 29 years).

Two DENV-4 strains isolated from Puerto Rico in 2010 are closely related to a strain from the Dominican Republic, which together form a separate cluster within genotype II. This cluster includes a number of strains from Puerto Rico, Central America, and the Caribbean region that have circulated throughout 1980s and 1990s (Figure 2). Although no additional Puerto Rico DENV-4 strains collected after 2000 were available for inclusion in the study to enable a better temporal resolution of the tree, our phylogenetic analysis of the DENV-4 strains circulating during the 2010 epidemic in Puerto Rico is consistent with previous studies, which indicate that the more recently sampled strains available for analysis (from the late 1990s) belong to a lineage that has been recurrently isolated in the island for a period $\geq$ 10 years. Our findings and those of previous reports[18,19] suggest that this lineage has survived numerous epidemic and inter-epidemic periods. DENV-4 has not been the dominant type for most of the past decade in Puerto Rico, and this low transmission frequency may have contributed to the lower genetic diversity observed for DENV-4 in Puerto Rico.[19,20] The calculated age for the node defining clade e is 19–24 years (mean = 21 years). For sub-clade e.1 (containing the 2010 strains), the calculated age is 14–18 years (mean = 16 years).

Introduction of new lineages of DENV in dengue-endemic regions have the potential to cause increased severity for dengue cases. This suggestion can be illustrated by a recent report from Nicaragua, which showed that introduction and replacement of the circulating lineage of DENV-2 with a new lineage displaying increased fitness correlated with an augment in disease severity.[21] A shortcoming of our study is the scarcity of Puerto Rico DENV-1 and DENV-4 sequences collected after 2000 available for analysis. Nevertheless, our results enabled us to identify circulation of a new DENV-1 lineage on the island and to speculate that its circulation can potentially lead to increased severity for cases in future epidemics. Long-term epidemiologic and *in vitro* studies are needed to determine if strains from the new DENV lineage circulating in Puerto Rico have increased fitness in comparison with older strains, and how this fitness may contribute to the already complex epidemiology of DENV on the island. In summary, our results on the molecular epidemiology of DENV-1 and DENV-4 in Puerto Rico and Florida during 2010 support the notion that an exchange of DENV strains occurrs within the Caribbean islands and between the Caribbean region and other dengue-endemic countries in Central and South America,[18–22] and that these strains and lineages circulating may have an impact on the clinical outcome of dengue cases in the region.

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Note: Supplemental table appears at www.ajtmh.org.

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