Virus Research 213 (2016) 23–31

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Insights into the molecular evolution of *Dengue virus* type 4 in Puerto Rico over two decades of emergence

Estelle Martin[a], Maritza Chirivella[b], Juliene K.G. Co[c], Gilberto A. Santiago[b], Duane J. Gubler[d], Jorge L. Muñoz-Jordán[b], Shannon N. Bennett[a,e,*]

[a] *Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawai'i at Manoa, Honolulu, HI, USA*
[b] *Dengue Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, San Juan, PR, USA*
[c] *Department of Biology, De La Salle University, Manila, Philippines*
[d] *Program in Emerging Infectious Diseases, Duke-National University of Singapore Graduate Medical School, Singapore*
[e] *Department of Microbiology, California Academy of Sciences, San Francisco, CA, USA*

ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form 5 November 2015
Accepted 6 November 2015
Available online 10 November 2015

Keywords:
Dengue virus
Evolution
Lineage turnover
Emergence
Natural selection

ABSTRACT

Dengue has emerged globally as a major human health problem since the 1950s and is now the most important arboviral disease of humans, infecting nearly 400 million people annually. While some cases are asymptomatic, others can develop a febrile illness (dengue fever) or even progress to severe and fatal dengue. Dengue is caused by any of 4 closely related but distinct viruses, known as *Dengue virus* serotype 1 to 4 (DENV-1 to DENV-4) which are maintained in endemic transmission to humans in large urban centers of the tropics by *Aedes* mosquitoes. Since the early 1960s, Puerto Rico, a major metropolitan center in the Caribbean, has experienced increasingly larger and clinically more severe epidemics following the introduction of all four dengue serotypes. The first dengue hemorrhagic fever epidemic in 1986, and a particularly severe outbreak in 1998 were dominated by novel DENV-4 strains that evolved in Puerto Rico, replacing earlier strains and spreading throughout the region. Sequence characterization of 54 complete DENV-4 genomes and their comparative evolution against 74 previously published viral sequences from the region over several decades shows that DENV-4 strains from these periods were genetically distinct based on unique changes in the envelope and non-structural genes. Their replacement of earlier strains in Puerto Rico progressed rapidly, suggesting that strong natural selection played a role in their fixation. This study confirms that DENVs evolve through rapid lineage turnover driven in part by natural selection and genetic drift.

1. Introduction

Dengue virus (DENV), a mosquito-borne pathogen of humans, causes an estimated 390 million infections annually throughout the tropics (Bhatt et al., 2013). Two vector species, principally *Aedes aegypti* and to a lesser extent *Ae. albopictus*, are responsible for most of the transmissions. DENV is a fast-evolving single-stranded, positive sense RNA virus of the genus *Flavivirus* in the family Flaviviridae. Evidence suggests that the four distinct serotypes of DENV (DENV-1 to DENV-4) originally diverged in canopy-dwelling mosquitoes and nonhuman primates in the rainforests of Asia, before colonizing humans several hundred years ago, each serotype undergoing a burst of evolutionary change associated with adaptations to new vectors and/or humans, and their demographic expansions (Wang et al., 2000; Twiddy et al., 2002a,b; Vasilakis et al., 2008; Gubler, 2014).

DENV have continued to evolve in humans, diversifying into genotypes or subtypes within each serotype, accompanied by lineage extinctions and replacements within a country, or sometimes due to exchange from other regions, that have been correlated with epidemic activity and/or disease severity (Gubler, 1988, 1998; Gubler et al., 1978, 1981; Rico-Hesse et al., 1997; Messer et al., 2002, 2003; Bennett et al., 2003, 2006; Steel et al., 2010).

Infection with any DENV serotype can result in a range of disease syndromes from asymptomatic to fever with rash. A small proportion of cases can progress to more severe disease characterized by

* Corresponding author at: Department of Microbiology, California Academy of Sciences, 55 Music Concourse Drive, San Francisco, CA 94118, USA. Fax: +1 415 379 5715.
E-mail addresses: sbennett@calacademy.org, sbennett@hawaii.edu (S.N. Bennett).

http://dx.doi.org/10.1016/j.virusres.2015.11.009
0168-1702/

a variety of syndromes ranging from hemorrhagic to neurologic disease; a vascular leak syndrome, with or without hemorrhage, is the most common form of severe dengue. Case fatality rates vary across populations and average about 5%. Many factors contribute to the risk for severe disease, including whether the patient has been previously infected by another serotype (Halstead et al., 1970; Kurane et al., 1991; Green and Rothman 2006; Midgley et al., 2011; Rothman 2011), the genetic background of the individual (Sierra et al., 2007; García et al., 2011Khor et al., 2011; Alagarasu et al., 2013), and the genetic makeup of the virus (Rosen 1977; Gubler et al., 1978, 1981; Gubler, 1988, 1998; Rico-Hesse et al., 1997; Leitmeyer et al., 1999). Control of dengue remains problematic, relying on vector control since there are as yet no vaccines available.

Dengue has emerged as the most important arboviral disease of humans. Historically, epidemic DENV transmission involved a single serotype in specific geographic regions (Gubler, 1998). However, beginning in the 1940s with World War II, DENV began to expand geographically with the movement of mosquito vectors and people. During the past few decades in particular, with increasing globalization and unprecedented urban growth, all DENV serotypes have spread throughout the tropics and now co-circulate in more than 100 countries with increasing frequency and magnitude of epidemics (Gubler, 2002, 2011; Kroeger and Nathan, 2006; Ranjit and Kissoon, 2011).

Puerto Rico exemplifies this expansion, beginning with early circulation of only one serotype at a time, followed by the establishment and co-circulation of multiple serotypes and mounting disease burden. DENV became sequentially re-established in Puerto Rico and the rest of the new world in the 1970s, following the cessation of the hemispheric *Ae. aegypti* eradication program. Early dengue activity in Puerto Rico was characterized by periodic outbreaks of one or at most two serotypes: 1963 (DENV-3), 1969 (DENV-2), 1972/73 and 1975/76 (DENV-2), 1977 (DENV-2 and DENV-3), 1978 (DENV-1), 1981/82 (DENV-1 and DENV-4) (Gubler and Trent, 1993; Dietz et al., 1996). The 1981/1982 outbreak was the first involving DENV-4, after which co-circulation of multiple serotypes became the norm, along with epidemics of increasing size and disease severity (Gubler and Trent, 1993; Dietz et al., 1996). DENV-1 was introduced in late 1977 and caused a major epidemic of classical dengue fever in 1978, remaining the predominant serotype during 1984/85 and 1991–93; DENV-2 (Jamaican/Asian-American genotype) was introduced in 1984, but did not cause an epidemic until 1994, dominating from 1988 to 1990 and 1994 to 1996, while DENV-4 was the predominant serotype during three large epidemics in 1982, 1986/87 and 1998. The emergence of dengue hemorrhagic fever in Puerto Rico occurred during the 1986 epidemic (Gubler and Trent, 1993; Dietz et al., 1996), and the 1998 outbreak was the most clinically severe outbreak up to that time (Bennett et al., 2003, 2010). In the following years, DENV-4, DENV-1 and DENV-2 declined dramatically and DENV-3 became the dominant serotype from 1999 to 2003, supplanted briefly by DENV-2, and then re-emerged in 2007 (McElroy et al., 2011). From 2007 to 2010, all four DENV serotypes were reported in Puerto Rico (Santiago et al., 2012).

Because DENV-4 has played a significant role in the epidemiology of dengue in Puerto Rico for 20 years, and was responsible for causing three of the four epidemics during that period, it has served as a model for understanding how virus evolution correlates with epidemic potential and severity. DENV-4 sequence characterization over two decades based on partial genomes (<40%) indicated that strong adaptive evolution in the nonstructural gene 2A (NS2A) correlated with the 1998 epidemic (Bennett et al., 2003). However, the impact of changes in other important genes, such as the polymerase (NS5), remains uncharacterized. Whole-genome characterization will complete our understanding of the genetic basis of lineage formation across epidemic and non-epidemic periods. The aim of this work was to provide a comprehensive phylogenetic analysis based on full genome sequences of DENV-4 in Puerto Rico from its first appearance in 1981 through the large outbreak in 1998. Our study revealed additional amino acid substitutions in nonstructural genes NS1 and NS5, as well as nucleic acid changes in non-translated regions (NTR), associated with the changing epidemic dynamics and lineage turnover in DENV evolution in Puerto Rico.

2. Materials and methods

2.1. Virus collection

Viruses were originally isolated from human serum samples of dengue cases collected since 1981, by inoculation of *Ae. aegypti* or *Toxorynchities amboinensis* mosquitoes or C6/36 *Ae. albopictus* cells, and archived, by the Dengue Branch of the U.S. Centers for Disease Control and Prevention (CDC), in San Juan, Puerto Rico, as part of its surveillance program (Gubler et al., 1984). We randomly subsampled, stratified by year group, 21 archived strains of DENV-4 for whole-genome sequencing guided by the previously partially characterized 82 specimens (Bennett et al., 2003) spanning the period of dengue emergence in Puerto Rico from 1986 to 1998. In addition, full genome sequencing of five randomly sampled DENV-4 viruses stratified by year group from five Puerto Rico municipalities was conducted by the CDC in collaboration with the BROAD Institute. Table 1 documents all of the viruses sampled and included in this study by year and municipality. Viral stocks were produced in *A. albopictus* C6/36 cells and the total number of passages was kept to below three to minimize *in vitro* evolution. This work has been performed under Institutional Review Board approvals at the Centers for Disease Control and Prevention, the University of Hawaii, and the BROAD Institute.

2.2. RNA extraction, RT-PCR and nucleotide sequencing

Viral RNA was extracted using the QIAamp viral RNA mini kit and reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen). For each strain, the entire genome was amplified by polymerase chain reaction (PCR) using Pfu Ultra II Fusion HS DNA polymerase (Stratagene), a high-fidelity enzyme, and primer pairs of our own design. Amplicons represented overlapping fragments of approximately 400 bp covering the entire 10,700 nucleotide genome, including the viral structural genes capsid (C), precursor membrane (prM) and envelope (E), the seven non-structural genes (NS1, NS2A/B, NS3, NS4A/B and NS5), and the NTR at the 5′ and 3′ ends of the open reading frame (ORF). Each amplicon was confirmed by gel electrophoresis and purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare). Purified DNA products were sequenced with primers of our own design targeted for both strands, to generate 2x to 6x high-quality coverage in both directions. Sequencing services were performed by the UH Manoa Advanced Studies in Genomics, Proteomics and Bioinformatics Facility using an Applied Biosystem 3730XL DNA Analyzer. Primer sequences are provided as Supplementary material.

2.3. Phylogenetic analysis

Sequences were trimmed, cleaned and assembled using Sequencher 5.0 (Gene Code Corp). Complete assembled genomes were aligned in TranslatorX (http://translatorx.co.uk/) and verified in Se-Al 2.0 (http://tree.bio.ed.ac.uk/software/seal/), along with publicly available sequences, both whole and partial genomes, covering DENV-4 circulation in Puerto Rico. Alignments were analyzed using RAxML to generate a maximum-likelihood (ML) tree with

Table 1
DENV-4 used in this study with virus label, country, year of isolation, municipality, GenBank accession number and references. Complete genomes were used unless otherwise noted[a].

Virus (Label)	Country	Year	PR municipality, health region	Accession number (s)	Reference
D4/DM/M44/1981	Dominica	1981	NA	AY152360 - AY152363	Bennett et al. (2003)[a]
D4/CO/750/1982	Colombia	1982	NA	TBA	This study
D4/US/PR M10/1982	US	1982	Villalba, Ponce	AY152316 - AY152319	Bennett et al. (2003)[a]
D4/US/PR M12/1982	US	1982	Guanica, Ponce	AY152328 - AY152331	Bennett et al. (2003)[a]
D4/US/PR M13/1982	US	1982	San Juan	AY152312 - AY152315	Bennett et al. (2003)[a]
D4/US/PR M15/1982	US	1982	Fajardo	AY152340 - AY152343	Bennett et al. (2003)[a]
D4/US/PR M16/1982	US	1982	Fajardo	AY152348 - AY152351	Bennett et al. (2003)[a]
D4/US/PR M20/1982	US	1982	San Juan	AY152356 - AY152359	Bennett et al. (2003)[a]
D4/US/PR M21/1982	US	1982	Arroyo, Ponce	AY152332 - AY152335	Bennett et al. (2003)[a]
D4/US/PR M24/1982	US	1982	Fajardo	AY152308 - AY152311	Bennett et al. (2003)[a]
D4/US/PR M25/1982	US	1982	Carolina, San Juan	AY152296 - AY152299	Bennett et al. (2003)[a]
D4/US/PR M3/1982	US	1982	San Juan	AY152344 - AY152347	Bennett et al. (2003)[a]
D4/US/PR M4/1982	US	1982	Guaynabo, San Juan	AY152320 - AY152323	Bennett et al. (2003)[a]
D4/US/PR M5/1982	US	1982	Carolina, San Juan	AY152336 - AY152339	Bennett et al. (2003)[a]
D4/US/PR M7/1982	US	1982	Trujillo Alto, San Juan	AY152352 - AY152355	Bennett et al. (2003)[a]
D4/US/PR M9/1982	US	1982	Guanica, Ponce	AY152324 - AY152327	Bennett et al. (2003)[a]
D4/US/PR 114/1985	US	1985	UK (Puerto Rico)	GU318311	Bennett et al. (2010)[a]
D4/US/PR M31/1985	US	1985	Cataño, Bayamón	GU318312	Bennett et al. (2010)[a]
D4/US/PR M32/1985	US	1985	Guaynabo, San Juan	GU318313	Bennett et al. (2010)[a]
D4/US/PR M34/1985	US	1985	San Juan	GU318315	Bennett et al. (2010)[a]
D4/US/PR M35/1985	US	1985	Guaynabo, San Juan	GU318316	Bennett et al. (2010)[a]
D4/US/PR M36/1985	US	1985	San Juan	GU318317	Bennett et al. (2010)[a]
D4/US/PR M37/1985	US	1985	San Juan	GU318318	Bennett et al. (2010)[a]
D4/US/PR 115/1986	US	1986	San Juan	AY152224 - AY152227, TBA	Bennett et al. (2003) this study
D4/US/PR 116/1986	US	1986	San Juan	AY152272 - AY152275	Bennett et al. (2003)[a]
D4/US/PR 117/1986	US	1986	San Juan	AY152276 - AY152279, TBA	Bennett et al. (2003) this study
D4/US/PR M42/1986	US	1986	San Juan	AY152280 - AY152283	Bennett et al. (2003)[a]
D4/US/PR 1D/1987	US	1987	Vega Baja, Arecibo	AY152228 - AY152231, TBA	Bennett et al. (2003) this study
D4/US/PR 3/1987	US	1987	Lares, Arecibo	AY152108 - AY152111	Bennett et al. (2003)[a]
D4/US/PR 5D/1987	US	1987	Caguas	AY152232 - AY152235, TBA	Bennett et al. (2003) this study
D4/US/PR 8D/1987	US	1987	Guayama, Ponce	AY152240 - AY152243, TBA	Bennett et al. (2003) this study
D4/US/PR 9/1987	US	1987	Aguada, Aguadilla	AY152284 - AY152287	Bennett et al. (2003)[a]
D4/US/PR 60/1987	US	1987	Ponce	AY152244 - AY152247	Bennett et al. (2003)[a]
D4/US/PR 62/1987	US	1987	San Juan	AY152220 - AY152223	Bennett et al. (2003)[a]
D4/US/PR 63/1987	US	1987	San Juan	AY152256 - AY152259	Bennett et al. (2003)[a]
D4/US/PR 64/1987	US	1987	Mayaguez	AY152260 - AY152263, TBA	Bennett et al. (2003) this study
D4/US/PR 65/1987	US	1987	Mayaguez	AY152264 - AY152267, TBA	Bennett et al. (2003) this study
D4/US/PR 66/1987	US	1987	Ponce	AY152248 - AY152251	Bennett et al. (2003)[a]
D4/US/PR 69/1987	US	1987	Toa Alta, Bayamón	AY152252 - AY152255	Bennett et al. (2003)[a]
D4/US/PR 72/1987	US	1987	San Juan	AY152216 - AY152219	Bennett et al. (2003)[a]
D4/US/PR 73/1987	US	1987	Cabo Rojo, Mayaguez	AY152268 - AY152271	Bennett et al. (2003)[a]
D4/US/PR 100/1990	US	1990	San Juan	TBA	This study
D4/US/PR 107/1990	US	1990	Jayuya, Ponce	GU318310, TBA	Bennett et al. (2010) this study
D4/US/PR 24/1992	US	1992	San Germán, Mayaguez	AY152188 - AY152191	Bennett et al. (2003)[a]
D4/US/PR 25/1992	US	1992	Las Marías, Mayaguez	AY152164 - AY152167	Bennett et al. (2003)[a]
D4/US/PR 26/1992	US	1992	San Germán, Mayaguez	AY152192 - AY152195	Bennett et al. (2003)[a]
D4/US/PR 27/1992	US	1992	San Sebastián, Aguadilla	AY152172 - AY152175	Bennett et al. (2003)[a]
D4/US/PR 28/1992	US	1992	Mayaguez	AY152196 - AY152199, TBA	Bennett et al. (2003) this study
D4/US/PR 29/1992	US	1992	Las Piedras, Caguas	AY152200 - AY152203, TBA	Bennett et al. (2003) this study
D4/US/PR 30/1992	US	1992	San Sebastián, Aguadilla	AY152168 - AY152171	Bennett et al. (2003)[a]
D4/US/PR 31/1992	US	1992	Moca, Aguadilla	AY152176 - AY152179	Bennett et al. (2003)[a]
D4/US/PR 32/1992	US	1992	San Sebastián, Aguadilla	AY152180 - AY152183	Bennett et al. (2003)[a]
D4/US/PR 34/1992	US	1992	Yabucoa, Caguas	AY152204 - AY152207	Bennett et al. (2003)[a]
D4/US/PR 35/1992	US	1992	San Juan	AY152208 - AY152211	Bennett et al. (2003)[a]
D4/US/PR 36/1992	US	1992	Cidra, Caguas	AY152112 - AY152115	Bennett et al. (2003)[a]
D4/US/PR 37/1992	US	1992	Quebradillas, Arecibo	AY152160 - AY152163	Bennett et al. (2003)[a]
D4/US/PR 41/1992	US	1992	Morovis, Arecibo	AY152212 - AY152215	Bennett et al. (2003)[a]
D4/US/PR 42/1992	US	1992	Cabo Rojo, Mayaguez	AY152184 - AY152187	Bennett et al. (2003)[a]
D4/US/PR 76/1994	US	1994	Aguada, Aguadilla	AY152136 - AY152139	Bennett et al. (2003)[a]
D4/US/PR 77/1994	US	1994	Aguadilla, Aguadilla	AY152132 - AY152135	Bennett et al. (2003)[a]
D4/US/PR 78/1994	US	1994	Añasco, Mayaguez	AY152128 - AY152131	Bennett et al. (2003)[a]
D4/US/PR 79/1994	US	1994	Barceloneta, Arecibo	AY152156 - AY152159	Bennett et al. (2003)[a]
D4/US/PR 80/1994	US	1994	Bayamón	AY152124 - AY152127	Bennett et al. (2003)[a]
D4/US/PR 82/1994	US	1994	Caguas	AY152140 - AY152143	Bennett et al. (2003)[a]
D4/US/PR 83/1994	US	1994	Camuy, Arecibo	AY152144 - AY152147	Bennett et al. (2003)[a]
D4/US/PR 84/1994	US	1994	Ciales, Arecibo	AY152084 - AY152087	Bennett et al. (2003)[a]
D4/US/PR 85/1994	US	1994	Florida, Arecibo	AY152096 - AY152099	Bennett et al. (2003)[a]
D4/US/PR 86/1994	US	1994	Guaynabo, San Juan	AY152116 - AY152119	Bennett et al. (2003)[a]
D4/US/PR 87/1994	US	1994	Hormigueros, Mayaguez	AY152152 - AY152155	Bennett et al. (2003)[a]
D4/US/PR 88/1994	US	1994	Lajas, Mayaguez	AY152288 - AY152291	Bennett et al. (2003)[a]
D4/US/PR 89/1994	US	1994	Las Marías, Mayaguez	AY152120 - AY152123	Bennett et al. (2003)[a]
D4/US/V2429/1994	US	1994	Arecibo	GQ199878	BROAD direct submission
D4/US/V860/1994	US	1994	San Juan	FJ226067	BROAD direct submission
D4/US/V2430/1994	US	1994	San Juan	GQ199879	BROAD direct submission
D4/US/PR 118/1995	US	1995	Cidra, Caguas	TBA	This study
D4/US/PR 119/1995	US	1995	Loíza, San Juan	TBA	This study

Table 1 (*Continued*)

Virus (Label)	Country	Year	PR municipality, health region	Accession number (s)	Reference
D4/US/PR 120/1995	US	1995	Humacao, Caguas	TBA	This study
D4/US/V2434/1995	US	1995	San Juan	FJ850057	BROAD direct submission
D4/US/V2431/1995	US	1995	Guayama, Ponce	GQ199880	BROAD direct submission
D4/US/V2432/1995	US	1995	Caguas	GQ252675	BROAD direct submission
D4/US/V2433/1995	US	1995	Salinas, Ponce	FJ810417	BROAD direct submission
D4/US/PR 182/1996	US	1996	Bayamón	TBA	This study
D4/US/PR 183/1996	US	1996	Caguas	TBA	This study
D4/US/PR 184/1996	US	1996	Caguas	TBA	This study
D4/US/PR 185/1996	US	1996	Caguas	TBA	This study
D4/US/V2435/1996	US	1996	Caguas	GQ199881	BROAD direct submission
D4/US/V2436/1996	US	1996	Ponce	GQ199882	BROAD direct submission
D4/US/V2439/1996	US	1996	Ponce	GQ199885	BROAD direct submission
D4/US/V2440/1996	US	1996	Ponce	FJ850058	BROAD direct submission
D4/US/V2437/1996	US	1996	San Lorenzo, Caguas	GQ199883	BROAD direct submission
D4/US/V2438/1996	US	1996	Humacao, Caguas	GQ199884	BROAD direct submission
D4/US/PR 162/1997	US	1997	Bayamón	TBA	This study
D4/US/PR 188/1997	US	1997	Ponce	TBA	This study
D4/US/PR 222/1997	US	1997	UK (Puerto Rico)	TBA	This study
D4/US/PR 163/1997	US	1997	Ponce	TBA	This study
D4/US/PR 12/1998	US	1998	San Juan	AY152052 - AY152055	Bennett et al. (2003)[a]
D4/US/PR 13/1998	US	1998	Aguadilla, Aguadilla	AY152068 - AY152071	Bennett et al. (2003)[a]
D4/US/PR 14/1998	US	1998	Lajas, Mayaguez	AY152048 - AY152051	Bennett et al. (2003)[a]
D4/US/PR 15/1998	US	1998	San Juan	AY152080 - AY152083	Bennett et al. (2003)[a]
D4/US/PR 17/1998	US	1998	Arroyo, Ponce	AY152056 - AY152059	Bennett et al. (2003)[a]
D4/US/PR 18/1998	US	1998	Aibonito, Caguas	AY152044 - AY152047	Bennett et al. (2003)[a]
D4/US/PR 19/1998	US	1998	Coamo, Ponce	AY152040 - AY152043	Bennett et al. (2003)[a]
D4/US/PR 20/1998	US	1998	Dorado, Bayamón	AY152036 - AY152039	Bennett et al. (2003)[a]
D4/US/PR 44/1998	US	1998	Barceloneta, Arecibo	AY152088 - AY152091	Bennett et al. (2003)[a]
D4/US/PR 45/1998	US	1998	Aguas Buenas, Caguas	AY152060 - AY152063	Bennett et al. (2003)[a]
D4/US/PR 46/1998	US	1998	Bayamón	AY152072 - AY152075	Bennett et al. (2003)[a]
D4/US/PR 47/1998	US	1998	Arecibo	AY152064 - AY152067	Bennett et al. (2003)[a]
D4/US/PR 48/1998	US	1998	Caguas	AY152076 - AY152079	Bennett et al. (2003)[a]
D4/US/PR 218/1998	US	1998	San Juan	TBA	This study
D4/US/PR 219/1998	US	1998	San Juan	TBA	This study
D4/US/PR 220/1998	US	1998	Arecibo	TBA	This study
D4/US/PR 221/1998	US	1998	Bayamón	TBA	This study
D4/US/V2444/1998	US	1998	San Juan	FJ882597	BROAD direct submission
D4/US/V2445/1998	US	1998	Arecibo	FJ882598	BROAD direct submission
D4/US/V1093/1998	US	1998	San Juan	EU854296	BROAD direct submission
D4/US/V1094/1998	US	1998	Vega Baja, Arecibo	EU854297	BROAD direct submission
D4/US/V2441/1998	US	1998	Bayamón	FJ882595	BROAD direct submission
D4/US/V2442/1998	US	1998	Guaynabo, San Juan	FJ882596	BROAD direct submission
D4/US/V2443/1998	US	1998	Guaynabo, San Juan	FJ850059	BROAD direct submission
D4/VE/V2164/1998	Venezuela	1998	NA	FJ639737	BROAD direct submission
D4/US/V2446/1999	US	1999	Bayamón	FJ882599	BROAD direct submission
D4/US/V2447/1999	US	1999	Bayamón	FJ882600	BROAD direct submission
D4/US/V2448/1999	US	1999	Caguas	FJ88260	BROAD direct submission
D4/CO/V3408/2001	Colombia	2001	NA	GQ868581	BROAD direct submission
D4/CO/V3412/2005	Colombia	2005	NA	GQ868585	BROAD direct submission
D4/VE/V2501/2008	Venezuela	2008	NA	FJ882592	BROAD direct submission
D4/US/ARC_19/2010	US	2010	UK (Puerto Rico)	JQ045565	Añez et al. (2012)
D4/US/ARC_42/2010	US	2010	UK (Puerto Rico)	JQ045566	Añez et al. (2012)

[a] Incomplete genomes: 4014 nucleotides from capsid, pre-membrane, membrane, envelope, NS1 (partial), NS2A, NS4B genes.

Table 2
Positive and negative selection by gene in DENV-4 from Puerto Rico.

Gene	Length	Number of variable sites	Prop. of variable sites	Mean proportion of synonymous substitutions (S)	Mean proportion of nonsynonymous substitutions (N)	Omega (dN/dS)	Selected codon (s)
Capsid	339	48	0.14	0.0117	0.0022	0.26	111[a]
Pre+membrane	498	77	0.15	0.0206	0.0010	0.08	
Membrane	225	33	0.15	0.0258	0.0014	0.10	
Envelope	1485	253	0.17	0.0334	0.0019	0.11	
NS1	1056	866	0.82	0.0355	0.0035	0.18	
NS2a	654	245	0.37	0.0167	0.0074	0.17	
NS2b	390	39	0.10	0.0441	0.0006	0.03	
NS3	1854	161	0.09	0.0349	0.0005	0.03	
NS4a	381	46	0.12	0.0371	0.0025	0.07	
2K protein	69	7	0.10	0.0436	0.0059	0.04	
NS4b	735	369	0.50	0.0240	0.0009	0.05	
NS5	2700	2581	0.96	0.0354	0.0035	0.17	3345[b]

[a] According to 3 of 5 codon-based methods.
[b] According to 4 of 5 codon-based methods.

ML bootstrap support based on the optimal number of ML bootstrap replicates (Stamatakis et al., 2008). The tree was rooted on the closest DENV-4 isolate from the region available since its re-establishment in Puerto Rico, D4/DM/M44/1981 from Dominica.

To identify nucleotide substitutions that have occurred on the internal branches of lineages formed during the evolution of DENV-4 in Puerto Rico, particularly in correlation with the high epidemic activity observed in 1986/87 and 1998, we mapped changes onto the ML phylogenetic tree using parsimony-based methods (MacClade) (Maddison and Maddison, 1989). Amino acid substitutions for the coding region, and nucleotide changes in the 5′ and 3′ NTR were mapped onto the ML topology.

To identify selection pressures on DENV-4 in Puerto Rico, we estimated the relative rates of nonsynonymous (dN) to synonymous (dS) nucleotide substitution across the coding portion of the genome. Estimates of dN/dS ratios (w) greater than 1 suggested positive selection, whereas $w < 1$ indicated negative or purifying selection and $w = 1$ neutral selection, in other words, the absence of selection, when random genetic drift became more important in evolution. Analyses were implemented in HyPhy v.2.0 (Kosakovsky Pond et al., 2005), the online version Data Monkey (http://www.datamonkey.org/) (Kosakovsky Pond and Frost, 2005; Delport et al., 2010) and PAML v.4.8 (http://abacus.gene.ucl.ac.uk/software/paml.html). We applied five different models that use alternative algorithms, from empirical counts to maximum-likelihood estimation, to determine dN/dS across codon sites: the Single Likelihood AnCestor (SLAC), Fixed Effect Likelihood (FEL), Random Effect Likelihood (REL), Fast Unbiased Bayesian AppRoximation (FUBAR) and the Mixed Effect Model of Evolution (MEME) (Kosakovsky Pond and Frost, 2005; Kosakovsky Pond et al., 2011). In addition, to account for different ways in which selection may be acting, that is, on entire lineages rather than individual codons, we implemented the Branch-Site REL method (Kosakovsky Pond et al., 2011). For SLAC, FEL, REL and MEME, significance levels were set to $p = 0.1$ and for FUBAR, the Posterior Probability was set to 0.9, as recommended by program authors. The REV nucleotide substitution model was used as the best substitution model for the data set based on previous findings and confirmed in HyPhy v. 2.0 (Bennett et al., 2003).

Alignments were scanned for recombinants (GARD, implemented in HYPHY v.2.0) before analyses. None were detected. Similar to previous studies we estimated expected fixation rates under neutral evolution using a coalescent approach (Bennett et al., 2003). In brief, the average time to fixation of neutral mutations in a haploid population is related to population size (2Ne) and generation time. Mutations fixed much faster than this are assumed to be non-neutral and therefore may have been fixed by positive selection rather than drift. We estimated effective population sizes from theta = 2Ne × mu, (Kuhner, 2006) for the 1994 and 1998 lineages (theta = 0.012137 and 0.017732 respectively) using the viral generation time for DENV as 14 days comprising intrinsic (within human) and extrinsic (within mosquito) replication (Holmes et al., 1998) and the DENV-4 synonymous substitution rate of 1.768×10^{-3} substitutions/site/year, which we estimated from complete coding genomes recovered in this study using BEAST v2.0 (8 runs of 500 million generations each, sampling every 10,000 generations with a 25% burnin, under a strict clock model and HKY model of substitution) to derive the neutral mutation rate per site per generation (mu) as 6.78×10^{-5}. Putatively positively selected amino acid changes were identified as those that fall on the internal branches of the tree that separate sampling times (for example, on the branch leading to the viral isolates sampled in 1998); at the population genetic level, mutations that are absent from an early time-point yet present in all sequences from a later time-point can be assumed to have gone to fixation over the course of the sampling period.

3. Results

We conducted a comprehensive evolutionary analysis of DENV-4 emergence in Puerto Rico over several decades that included 54 additional complete genomes along with 74 previously published partial genomes from our group (38% coverage) from isolates collected between 1981 and 1999 from Puerto Rico, Dominica, Venezuela and Colombia, as well as two publicly available virus genomes from Puerto Rico collected in 2010 (Table 1). A ML phylogeny rooted on an isolate from Dominica (1981), associated with the establishment of DENV-4 in Puerto Rico revealed three different major and well-supported monophyletic lineages evolving following the 1981 introduction that reflect a pattern of multiple lineage replacements during DENV-4's history in Puerto Rico (Fig. 1). Distinct from the original strains circulating in Puerto Rico in 1982, and marking the next period of epidemic DENV-4 activity, group I comprised the majority of isolates from 1985 to 1987 (99% bootstrap support) and was distinguished by three amino acid changes: E gene position 163, Met-to-Thr, E351, Iso-to-Val, and NS1 gene position 11, Iso-to-Val.

Following the epidemic of 1986/87, a period of relatively low DENV-4 activity occurred between 1990 and 1996, from which viruses formed a second group, group II (76% bootstrap support). Group II DENV-4 were distinguished by two additional changes in the 3′ NTR region at nucleotide positions 14 and 112. Subsequently, most isolates collected between 1996 and 1998, which included the large 1998 epidemic in Puerto Rico and the Caribbean, formed group III (100% bootstrap support). Group III was distinguished by two substitutions, in the 5′ NTR (position 77) and 3′ NTR (position 325), and a subset of viruses, including all of the 1998 viruses, formed a cluster (100% bootstrap support) that was further distinguished by five substitutions: three in the NS2A (Val-to-Ala at pos. 35, Iso-to-Thr at pos. 72, and Pro-to-Ser at pos. 158), one in NS5 (Thr-to-Iso at pos. 826), and one in 3′ NTR (position 112). Notably, we observe a pattern throughout DENV-4 emergence in PR where lineages descend from a few variants from previous periods: in addition to the introduced viruses spreading and ultimately leading to the group I lineage, group II (1992–1994) includes three 1986/87 isolates at the root, and group III (1998) includes a few variants from 1994/1995 at the root. All lineages were distributed island-wide (no significant geographic structure was found), although group III appears to be the most widespread (Fig. 1).

The mechanisms behind lineage turnover and DENV-4 ongoing activity in Puerto Rico were further investigated by examining the role of selection in lineage formation. Under codon site-based models for positive or negative selection (SLAC/FEL/REL and FUBAR, HYPHY v.2.0), as well as the MEME method, which further detects phylogeny-wide patterns of episodic selection, two sites were identified with a significantly high probability of being under positive selection: codon site 111, in the capsid gene, and 3345, in the NS5 gene. In contrast, up to 699 codon sites (or 21%) were estimated as being under pervasive purifying selection (Table 2, FUBAR posterior probability >0.9). Positively selected substitutions in codon 111 (Fig. 1, black circles) or 3345 (triangles) were distributed in terminal taxa in multiple lineages. Substitutions at codon 111 involved either Val-to-Iso (virus variants in group II) or Val-to-Ala (variants in group III), both conservative substitutions. All substitutions in 3345 involved Ala-to-Pro, a non-conservative substitution. A branch-based model of positive selection was not supported by the data (branch-site REL, HYPHY v.2.0). The mean dN/dS for internal branches was 0.03.

Lineage turnover in DENV-4 history in Puerto Rico can occur rapidly. To determine the speed of lineage turnover associated with the 1998 epidemic, we randomly screened an additional 106 DENV-4 from Puerto Rico collected between 1994 and 1998 to determine their association with either the 1992/1994 lineage (group II) or the

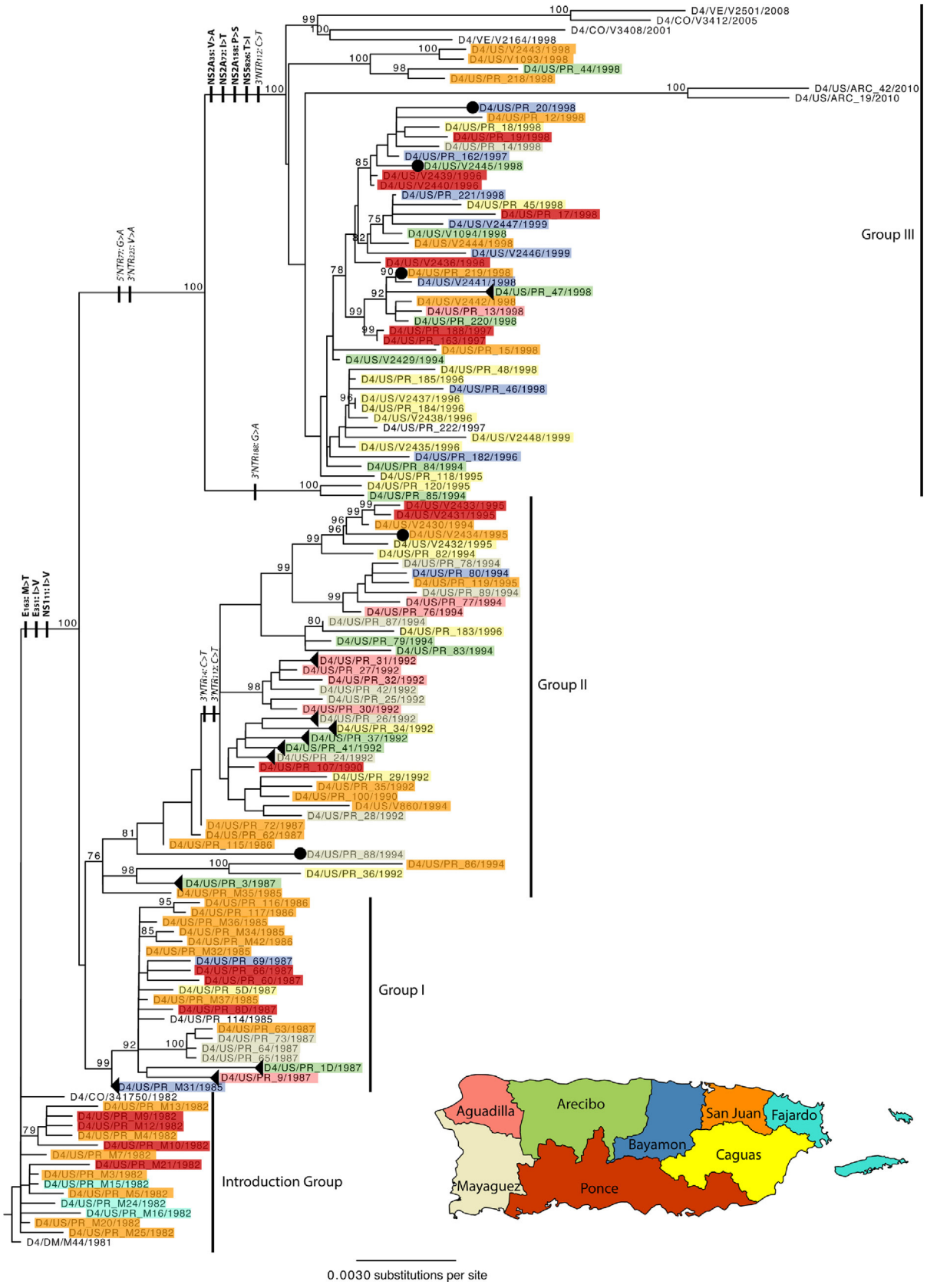

Fig. 1. Maximum likelihood (ML) phylogeny of Puerto Rico DENV-4 from 1981 to 1998. The phylogenetic tree was rooted with a 1981 isolate from Dominica and includes 54 whole genome sequences of DENV-4 from Puerto Rico generated in this study, as well as 74 previously published sequences from Puerto Rico and the region. Four clades are evident and associated with distinct circulation periods: the introduction group, 1981–1982 (Introduction Group), 1985–1987 (Group I), 1992–1994 (Group II), and the 1998 outbreak (Group III). Bootstrap values are shown for each clade based on 100 ML replicates generated in RAxML (Stamatakis et al., 2008). Amino acid changes between the different major clades are identified along branches and positively selected sites identified on terminal nodes are represented by either a black circle (codon 111) or triangle (codon 3345). Samples are colored-coded by the PR Health Region in which they were collected, as indicated in the map legend.

Table 3
Additional DENV-4 viruses from Puerto Rico screened for association with Group II or Group III lineages based on envelope sequence homology[a]. Percentages reported with actual isolate numbers associating with a given clade in parentheses.

Year	Clade	
	Group II (1992/94)	Group III (1998)
1994	90% (27)	10%[b] (3)
1995	39% (9)	61% (14)
1996	35% (6)	65% (11)
1997	5% (1)	95%[b] (21)
1998	0% (0)	100%[b] (14)

[a] ML bootstrap support > 95%.
[b] Significantly nonrandom, $p < 0.05$ after sequential Bonferroni correction for multiple comparisons.

1998 lineage (group III) according to their E gene sequences. Table 3 demonstrates that group II dominated in 1994 but was virtually replaced by group III as early as 1997 (96% of samples assigned), a turnover, defined as a switch in dominance, of three years. Group III became fixed (100% of isolates) by 1998. The time to most recent common ancestor of the group III lineage under the assumption of no selection was estimated as 1992 (BEAST v.2.0, ESS > 11,000, Bouckaert et al., 2014). The estimated rate of fixation for changes along this branch under neutrality using the coalescent was previously reported as 37 years based on gene fragments (Bennett et al., 2003). Re-estimated time to fixation under neutrality based on whole genome data was 8.44 years, almost three times as slow as the three years observed. The emergence of the group I lineage from the introduced strain may have been similarly rapid: the introduced virus caused an island-wide epidemic in 1982 (Waterman et al., 1985) then virtually disappeared for two years before reemerging in 1985 to cause an epidemic in 1986 (Dietz et al., 1996).

4. Discussion

The evolutionary history of DENV since emerging into humans several hundred years ago has been shaped by natural selection and genetic drift acting on variations introduced by *in situ* mutation or migration from elsewhere. Here, we present a comprehensive analysis of the evolutionary trajectory of an important serotype in Puerto Rico (DENV-4) over two decades during which hyperendemicity set in.

Phylogenetic inference on whole-genome sequenced DENV-4 isolated over time since 1981 indicated frequent lineage turnover in some instances associated with a change in epidemic activity. Distinct post-introduction lineages were noted in the 1986/87 epidemic (group I), 1992 (group II), a period of non-epidemic sporadic transmission, and 1998 (group III), the year of a particularly large epidemic in Puerto Rico. Lineage turnover associated with the 1998 epidemic was rapid, with a switch in the dominant lineage within three years, and involved several amino acid substitutions in NS2A and NS5, as well as changes in both NTR. A multi-national region-wide analysis by Foster et al. (2003) suggested that these three major DENV-4 lineages evolved in Puerto Rico rather than by importation, and group III persisted as the dominant DENV-4 strain until at least 2010 (Fig. 1). Interestingly, frequency-dependent processes of evolution, such as negative frequency-dependent selection, may be operating since two of the three major lineages were preceded by small numbers of variants from earlier time periods. One possible mechanism for this may be immune escape, a phenomenon observed in other pathogens, such as influenza, in which newly arisen variants are poorly recognized by the host's immune system and have a selective advantage over common strains. In the case of DENV, opportunities for immune escape could arise if serotype-specific immunity were incomplete or if selection for rare variants was operating at cross-reactive sites. Lineage turnover has been well documented in DENV, for example, in DENV-2 evolution in the Pacific (Steel et al., 2010), Puerto Rico (McElroy et al., 2011) and Thailand (Sittisombut et al., 1997), and DENV-3 evolution in Thailand (Wittke et al., 2002) and Sri Lanka (Messer et al., 2003).

The rapidity of lineage turnover *in situ* involving more amino acid substitutions than on any other internal branch against a background rate evolving three times slower suggests a pattern of non-neutral evolution. Our results indicate that the substitutions shared by the group III viruses (associated with the 1998 epidemic and beyond) would have taken 8.44 years to fix under neutral evolution, when in fact they were fixed after arising within the Puerto Rico virus population within three years. This rapid rate of evolution is strongly suggestive of positive selection for the group III strains. Some amino acid changes, particularly in NS2A, occurred in a region of few synonymous substitutions, also consistent with positive selection. Based on the large number of cases and presumable transmission events in the 1998 outbreak and the high rates of disease severity, where disease severity has been correlated with levels of virus (Gubler et al., 1978, 1981,b; Libraty et al., 2002a,b; Steel et al., 2010; Srikiatkhachorn et al., 2012), it may be that group III viruses replicate to a greater degree and/or transmit more efficiently at the population level, factors that contribute to greater viral fitness (defined as the number of "offspring" contributed to the next generation), relative to earlier strains. Fitness differences among DENV have previously been suggested or observed directly (Gubler et al., 1981; Gubler and Kuno, 1997; Rico-Hesse et al., 1997; Leitmeyer et al., 1999; Steel et al., 2010; Ohainle et al., 2011). However, DENV evolution has generally been dominated by purifying selection (Sittisombut et al., 1997; Wittke et al., 2002; McElroy et al., 2011; Santiago et al., 2012): the amino acid sites under positive selection are few across a genome dominated by purifying selection, making them statistically difficult to detect (Twiddy et al., 2002a,b), but nonetheless, not precluding them from being potentially important.

Targets of positive selection potentially shaping DENV evolution could be operating in both the vertebrate host and mosquito vector. NS2A, which underwent a cluster of three substitutions defining the group III viruses, forms a small, hydrophobic protein containing a number of predicted transmembrane domains involved in virus replication. Substitutions have been shown to block the production of virus particles (Liu et al., 2003; Leung et al., 2008), cleavage and maturation (Kümmerer and Rice, 2002). NS5, which also experienced a substitution in the emergence of the group III, 1998 clade, is a bi-functional enzyme with an N-terminal methyltransferase (Egloff et al., 2002) and C-terminal RNA dependent RNA polymerase (Yap et al., 2007). Substitutions have resulted in lower viral titer for flavivirus WNV *in vitro* and reduced replicative fitness in chickens but not *Culex pipiens* mosquitoes (Van et al., 2012). The 3′NTR, in which different substitutions were observed defining the 1992/94 viruses and group III viruses, interacts with viral and/or cellular proteins in viral RNA synthesis, replication and genome cyclization (Alvarez et al., 2006; Friebe et al., 2011). Other studies implicate different genetic alterations in dengue viruses associated with changes in epidemic intensity: Steel et al. (2010) report changes in prM, NS2A and NS4A genes associated with attenuation, as measured by number of cases and virus isolation rates, of DENV-2 viruses from the 1970s causing a series of outbreaks in the south Pacific (Gubler and Kuno, 1997; Gubler, 2014); changes in the envelope gene and the untranslated regions of DENV-2 have been associated with increased epidemic intensity within human populations (Leitmeyer et al., 1999; Bennett et al., 2006). A change

in the circulating strains of DENV-3 have been associated with the onset of clinical severity in Sri Lanka (Lanciotti et al., 1994; Messer et al., 2003), and attenuation in Indonesia (Gubler et al., 1981). In light of these and other studies that have experimentally demonstrated links between lineage turnover, genetic changes and epidemic dynamics (Rico-Hesse et al., 1997; Armstrong and Rico-Hesse, 2003; Anderson and Rico-Hesse, 2006; Hanley et al., 2008; Mota and Rico-Hesse, 2009), the potential for altered phenotypes and their fitness consequences for dengue viruses should be addressed in future experimental studies.

Genetic drift remains an important factor in DENV evolution. Stochastic events during transmission and within hosts lead to population bottlenecks, which exacerbate evolution by drift (Bennett et al., 2010; McElroy et al., 2011). Two significant reductions in virus population sizes as reflected in reduced genetic diversity, also known as population genetic bottlenecks, occurred in DENV-4 just prior to the turnover events, in 1983–5 and 1995 (Bennett et al., 2010), and presumably reduced the standing variation for selection to act upon. Similar reports have noted the importance of genetic drift on DENV evolution in Puerto Rico: DENV-2 (McElroy et al., 2011) and DENV-3 (Santiago et al., 2012) as well as elsewhere (Jarman et al., 2008).

5. Conclusion

DENV emergence in Puerto Rico has been marked by increasingly severe epidemics since the 1960s, exemplifying similar trends throughout the region. Based on a comprehensive analysis of DENV-4 virus sequence evolution over this decades-long period of emergence, we find that viruses have been evolving rapidly, with major turnovers in the dominant strains forming novel lineages that in two cases were associated with major outbreaks, in 1986 and 1998. After 1981 these novel lineages do not appear to have been seeded by the importation of new strains but rather arose *in situ* through evolutionary processes such as positive selection and genetic drift. In the case of the group III viruses, associated with the 1998 epidemic and beyond, substitutions across multiple gene regions were fixed three times more rapidly than expected under neutral evolution, suggesting that these viruses were under selection for greater fitness. Indeed, 1998 was marked by severe epidemic activity in Puerto Rico and the surrounding region, and the same DENV lineage persisted to mark another burst of epidemic activity starting in 2010. This study demonstrates the importance of DENV genetic variation and evolution in the emergence of dengue as the most significant mosquito-borne viral pathogen of humans.

Acknowledgements

We gratefully acknowledge the work of Vance Vorndam and other members of the CDC San Juan Dengue Branch for their contributions to this study and the curation of an extensive archival DENV collection; Ric Yanagihara for editorial comments; and financial support from NIH/NIAID Pacific Southwest Regional Center of Excellence (PSWRCE U54AI065359), NIH/NCRR COBRE (P20RR018727) and RCMI (G12RR003061) programs, the US Department of Defense (DOD06187000) and the Hawaii Community Foundation (13ADVC-60318).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2015.11.009.

References

Alagarasu, K., Mulay, A.P., Singh, R., Gavade, V.B., Shah, P.S., Cecilia, D., 2013. Association of HLA-DRB1 and TNF genotypes with dengue hemorrhagic fever. Hum. Immunol.

Alvarez, D.E., Lodeiro, M.F., Filomatori, C.V., Fucito, S., Mondotte, J.A., Gamarnik, A.V., 2006. Structural and functional analysis of dengue virus RNA. Novartis Found. Symp. 277, 120–132, discussion 132–5–251–3 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17319158&retmode=ref&cmd=prlinks.

Anderson, J.R., Rico-Hesse, R., 2006. *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. Am. J. Trop. Med. Hyg. 75, 886–892.

Armstrong, P.M., Rico-Hesse, R., 2003. Efficiency of dengue serotype 2 virus strains to infect and disseminate in *Aedes aegypti*. Am. J. Trop. Med. Hyg. 68, 539–544.

Bennett, S.N., Holmes, E.C., Chirivella, M., Rodriguez, D.M., Beltran, M., Vorndam, V., Gubler, D.J., McMillan, W.O., 2003. Selection-driven evolution of emergent dengue virus. Mol. Biol. Evol. 20, 1650–1658.

Bennett, S.N., Holmes, E.C., Chirivella, M., Rodriguez, D.M., Beltran, M., Vorndam, V., Gubler, D.J., McMillan, W.O., 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.

Bennett, S.N., Drummond, A.J., Kapan, D.D., Suchard, M.A., Muñoz-Jordán, J.L., Pybus, O.G., Holmes, E.C., Gubler, D.J., 2010. Epidemic dynamics revealed in dengue evolution. Mol. Biol. Evol. 27, 811–818.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., et al., 2013. The global distribution and burden of dengue. Nature 496, 504–507.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Comput. Biol. 10, e1003537.

Delport, W., Poon, A.F.Y., Frost, S.D.W., Kosakovsky Pond, S.L., 2010. Datamonkey a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 26, 2455–2457 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=20671151&retmode=ref&cmd=prlinks.

Dietz, V., Gubler, D.J., Ortiz, S., Kuno, G., Casta-Vélez, A., Sather, G.E., Gómez, I., Vergne, E., 1996. The 1986 dengue and dengue hemorrhagic fever epidemic in Puerto Rico: epidemiologic and clinical observations. P. R. Health Sci. J. 15, 201–210.

Egloff, M.-P., Benarroch, D., Selisko, B., Romette, J.-L., Canard, B., 2002. An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J. 21, 2757–2768 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=12032088&retmode=ref&cmd=prlinks.

Foster, J.E., Bennett, S.N., Vaughan, H., Vorndam, V., McMillan, W.O., Carrington, C.V.F., 2003. Molecular evolution and phylogeny of dengue type 4 virus in the Caribbean. Virology 306, 126–134.

Friebe, P., Shi, P.-Y., Harris, E., 2011. The 5 and 3 downstream AUG region elements are required for mosquito-borne flavivirus RNA replication. J. Virol. 85, 1900–1905.

García, G., del, P., uerto, F., Pérez, A.B., Sierra, B., Aguirre, E., Kikuchi, M., Sánchez, L., Hirayama, K., Guzmán, M.G., 2011. Association of MICA and MICB alleles with symptomatic dengue infection. Hum. Immunol. 72, 904–907.

Green, S., Rothman, A., 2006. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr. Opin. Infect. Dis. 19, 429–436.

Gubler, D.J., Kuno, G., 1997. Dengue and Dengue Hemorrhagic Fever. CAB International.

Gubler, D.J., Reed, D., Rosen, L., Hitchcock, J.R., 1978. Epidemiologic, clinical, and virologic observations on dengue in the Kingdom of Tonga. Am. J. Trop. Med. Hyg. 27, 581–589.

Gubler, D.J., Suharyono, W., Lubis, I., Eram, S., Gunarso, S., 1981. Epidemic dengue 3 in central Java:associated with low viremia in man. Am. J. Trop. Med. Hyg. 30, 1094–1099.

Gubler, D.J., Kuno, G., Sather, G.E., Velez, M., Oliver, A., 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am. J. Trop. Med. Hyg. 33, 158–165.

Gubler, D.J., Trent, D.W., 1993. Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. Infect. Agents Dis. 2, 383–393.

Gubler, D.J., 1988. Dengue. In: Monath, T.P. (Ed.), The Arboviruses: Epidemiology and Ecology, vol. II. Boca Raton, FL, CRC Press, pp. 223–260.

Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11, 480–496.

Gubler, D.J., 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10, 100–103.

Gubler, D.J., 2011. Dengue, urbanization and globalization: the unholy trinity of the 21(st) century. Trop. Med. Health 39, 3–11.

Gubler, D.J., 2014. Dengue viruses: their evolution, history and emergence as a global public health problem. In: Gubler, D.J., Ooi, E.-E., Vasudevan, S., Farrar, J.F. (Eds.), Dengue and Dengue Hemorrhagic Fever. , 2nd ed. CAB International, Wallingford, UK, pp. 1–29.

Halstead, S.B., Nimmannitya, S., Cohen, S.N., 1970. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J. Biol. Med. 42, 311–328.

Hanley, K.A., Nelson, J.T., Schirtzinger, E.E., Whitehead, S.S., Hanson, C.T., 2008. Superior infectivity for mosquito vectors contributes to competitive displacement among strains of dengue virus. BMC Ecol. 8, 1.
Holmes, E.C., Bartley, L.M., Garnett, G.P., 1998. 10 the emergence of dengue: past present and future. Biomed. Res. Rep. http://www.sciencedirect.com/science/article/pii/S1874532607800342.
Jarman, R.G., Holmes, E.C., Rodpradit, P., Klungthong, C., Gibbons, R.V., Nisalak, A., Rothman, A.L., Libraty, D.H., Ennis, F.A., Mammen, M.P., Endy, T.P., 2008. Microevolution of dengue viruses circulating among primary school children in Kamphaeng Phet, Thailand. J. Virol. 82, 5494–5500.
Khor, C.C., Chau, T.N.B., Pang, J., Davila, S., Long, H.T., Ong, R.T.H., Dunstan, S.J., Wills, B., Farrar, J., Van Tram, T., et al., 2011. Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1. Nat. Genet. 43, 1139–1141.
Kosakovsky Pond, S.L., Frost, S.D.W., 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 21, 2531–2533 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=15713735&retmode=ref&cmd=prlinks.
Kosakovsky Pond, S.L., Frost, S.D.W., Muse, S.V., 2005. HyPhy: hypothesis testing using phylogenies. Bioinformatics 21, 676–679.
Kosakovsky Pond, S.L., Murrell, B., Fourment, M., Frost, S.D.W., Delport, W., Scheffler, K., 2011. A random effects branch-site model for detecting episodic diversifying selection. Mol. Biol. Evol. 28, 3033–3043.
Kroeger, A., Nathan, M.B., 2006. Dengue: setting the global research agenda. Lancet 368, 2193–2195.
Kuhner, M.K., 2006. LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. Bioinformatics, 768 http://bioinformatics.oxfordjournals.org/content/22/6.
Kurane, I., Mady, B.J., Ennis, F.A., 1991. Antibody-dependent enhancement of dengue virus infection. Rev. Med. Virol. 1, 211–221.
Kümmerer, B.M., Rice, C.M., 2002. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. J. Virol. 76, 4773–4784 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11967294&retmode=ref&cmd=prlinks.
Lanciotti, R.S., Lewis, J.G., Gubler, D.J., Trent, D.W., 1994. Molecular evolution and epidemiology of dengue-3 viruses. J. Gen. Virol. 75 (Pt 1), 65–75.
Leitmeyer, K.C., Vaughn, D.W., Watts, D.M., Salas, R., Villalobos, I., de Chacon Ramos, C., Rico-Hesse, R., 1999. Dengue virus structural differences that correlate with pathogenesis. J. Virol. 73, 4738–4747.
Leung, J.Y., Pijlman, G.P., Kondratieva, N., Hyde, J., Mackenzie, J.M., Khromykh, A.A., 2008. Role of nonstructural protein NS2A in *Flavivirus* assembly. J. Virol. 82, 4731–4741 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=18337583&retmode=ref&cmd=prlinks.
Libraty, D.H., Endy, T.P., Houng, H.-S.H., Green, S., Kalayanarooj, S., Suntayakorn, S., Chansiriwongs, W., Vaughn, D.W., Nisalak, A., Ennis, F.A., Rothman, A.L., 2002a. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J. Infect. Dis. 185, 1213–1221.
Libraty, D.H., Young, P.R., Pickering, D., Endy, T.P., Kalayanarooj, S., Green, S., Vaughn, D.W., Nisalak, A., Ennis, F.A., Rothman, A.L., 2002b. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis. 186, 1165–1168.
Liu, W.J., Chen, H.B., Khromykh, A.A., 2003. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. J. Virol. 77, 7804–7813.
Maddison, W.P., Maddison, D.R., 1989. Interactive analysis of phylogeny and character evolution using the computer program MacClade. Folia Primatol. 53, 190–202 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=2606395&retmode=ref&cmd=prlinks.
McElroy, K.L., Santiago, G.A., Lennon, N.J., Birren, B.W., Henn, M.R., Muñoz-Jordán, J.L., 2011. Endurance, refuge, and reemergence of dengue virus type 2, Puerto Rico, 1986–2007. Emerg. Infect. Dis. 17, 64–71.
Messer, W.B., Gubler, D.J., Harris, E., Sivananthan, K., De Silva, A.M., 2003. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg. Infect. Dis. 9, 800–809.
Messer, W.B., Vitarana, U.T., Sivananthan, K., Elvtigala, J., Preethimala, L.D., Ramesh, R., Withana, N., Gubler, D.J., De Silva, A.M., 2002. Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. 66, 765–773.
Midgley, C.M., Bajwa-Joseph, M., Vasanawathana, S., Limpitikul, W., Wills, B., Flanagan, A., Waiyaiya, E., Tran, H.B., Cowper, A.E., Chotiyarnwong, P., et al., 2011. An in-depth analysis of original antigenic sin in dengue virus infection. J. Virol. 85, 410–421.
Mota, J., Rico-Hesse, R., 2009. Humanized mice show clinical signs of dengue fever according to infecting virus genotype. J. Virol. 83, 8638–8645.
Ohainle, M., Balmaseda, A., Macalalad, A.R., Tellez, Y., Zody, M.C., Saborío, S., Nuñez, A., Lennon, N.J., Birren, B.W., Gordon, A., et al., 2011. Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. Sci. Transl. Med. 3, 114ra128.
Ranjit, S., Kissoon, N., 2011. Dengue hemorrhagic fever and shock syndromes. Pediatr. Crit. Care Med. 12, 90–100.
Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., de Mesa, M.T., Nogueira, R.M., da Rosa, A.T., 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230, 244–251.
Rosen, L., 1977. The Emperor's New Clothes revisited: or reflections on the pathogenesis of dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. 26, 337–343.
Rothman, A.L., 2011. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat. Rev. Immunol. 11, 532–543.
Santiago, G.A., McElroy-Horne, K., Lennon, N.J., Santiago, L.M., Birren, B.W., Henn, M.R., Muñoz-Jordan, J.L., 2012. Reemergence and decline of dengue virus serotype 3 in Puerto Rico. J. Infect. Dis. 206, 893–901.
Sierra, B., Alegre, R., Pérez, A.B., García, G., Sturn-Ramirez, K., Obasanjo, O., Aguirre, E., Alvarez, M., Rodriguez-Roche, R., Valdés, L., et al., 2007. HLA-A, -B, -C, and -DRB1 allele frequencies in Cuban individuals with antecedents of dengue 2 disease: advantages of the Cuban population for HLA studies of dengue virus infection. Hum. Immunol. 68, 531–540.
Sittisombut, N., Sistayanarain, A., Cardosa, M.J., 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. Med. Hyg. 57, 100–108 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=9242328&retmode=ref&cmd=prlinks.
Srikiatkhachorn, A., Wichit, S., Gibbons, R.V., Green, S., Libraty, D.H., Endy, T.P., Ennis, F.A., Kalayanarooj, S., Rothman, A.L., 2012. Dengue viral RNA levels in peripheral blood mononuclear cells are associated with disease severity and preexisting dengue immune status. PLoS One 7, e51335.
Stamatakis, A., Hoover, P., Rougemont, J., 2008. A rapid bootstrap algorithm for the RAxML web servers. Syst. Biol. 57, 758–771.
Steel, A., Gubler, D.J., Bennett, S.N., 2010. Natural attenuation of dengue virus type-2 after a series of island outbreaks: a retrospective phylogenetic study of events in the South Pacific three decades ago. Virology 405, 505–512.
Twiddy, S.S., Farrar, J.J., Chau, N.V., Wills, B., Gould, E.A., Gritsun, T., Lloyd, G., Holmes, E.C., 2002a. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology 298, 63–72.
Twiddy, S.S., Woelk, C.H., Holmes, E.C., 2002b. Phylogenetic evidence for adaptive evolution of dengue viruses in nature. J. Gen. Virol. 83, 1679–1689.
Van, S., lyke, G.A., Ciota, A.T., Willsey, G.G., Jaeger, J., Shi, P.-Y., Kramer, L.D., 2012. Point mutations in the West Nile virus (Flaviviridae; *Flavivirus*) RNA-dependent RNA polymerase alter viral fitness in a host-dependent manner in vitro and in vivo. Virology 427, 18–24.
Vasilakis, N., Fokam, E.B., Hanson, C.T., Weinberg, E., Sall, A.A., Whitehead, S.S., Hanley, K.A., Weaver, S.C., 2008. Genetic and phenotypic characterization of sylvatic dengue virus type 2 strains. Virology 377, 296–307.
Wang, E., Ni, H., Xu, R., Barrett, A.D., Watowich, S.J., Gubler, D.J., Weaver, S.C., 2000. Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J. Virol. 74, 3227–3234.
Waterman, S.H., Novak, R.J., Sather, G.E., Bailey, R.E., Rios, I., Gubler, D.J., 1985. Dengue transmission in two Puerto Rican communities in 1982. Am. J. Trop. Med. Hyg. 34, 625–632.
Wittke, V., Robb, T.E., Thu, H.M., Nisalak, A., Nimmannitya, S., Kalayanrooj, S., Vaughn, D.W., Endy, T.P., Holmes, E.C., Aaskov, J.G., 2002. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. Virology 301, 148–156.
Yap, T.L., Xu, T., Chen, Y.L., Malet, H., Egloff, M.-P., Canard, B., Vasudevan, S.G., Lescar, J., 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J. Virol. 81, 4753–4765 http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17301146&retmode=ref&cmd=prlinks.