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Epidemiological Implications of the Genetic Diversification of Dengue Virus (DENV) Serotypes and Genotypes in Mexico

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Abstract

Variation and clade shifts in dengue virus (DENV) genotypes are responsible for numerous dengue fever outbreaks throughout Latin America in the past decade. Molecular analyses of dengue serotypes have revealed extensive genetic diversification and the emergence of new genotypes in Brazil (DENV-4 genotype I) and elsewhere in tropical and subtropical America. The goal of the present study is to assess the extent to which the adventitious introduction of DENV genotypes and their increasing genetic diversity affects dengue epidemiology in Mexico. A nuanced sequence inspection and phylogenetic analysis of the C-prM nucleotide region of DENV was performed for specimens collecting in 2009 from the Veracruz State, Mexico. Findings were contrasted with specimens collected in adjacent years and analysed based on the epidemiological patterns reported between 1990 and 2019. Additionally, the identification process of various DENV genotypes was assessed, including: (1) DENV-1, genotype V, (2) the DENV-2 Asian/American and Asian II genotypes (3) DENV-3, genotype III, and (4) DENV-4, genotype I. This resulted in the discovery of a distinct genetic cladistic pattern for serotype DENV-2. Lastly, study findings suggest that a correlation exists between the emergence of novel genotypes and genetic diversification, with the increasing incidence of DENV infections in Mexico in 2009.

Keywords: emergence of new genotypes; geographic distribution of DENV serotypes; phylogenetic analysis of dengue; dengue epidemiology

1. Introduction

In tropical and subtropical America today, the co-circulation of dengue (DENV), zika (ZIKV) and chikungunya (CHIKV) viruses is a major public health concern. The transmission of these diseases is dependent on mosquito vectors, specifically, the "bite" of infected *Aedes aegypti* or *Aedes albopictus* mosquitoes. Mass human population movement, climate change, and the introduction of novel technologies (related to agriculture, construction, damming, etc.) have contributed to the geographic redistribution and expansion of the range of mosquito vectors and the diseases that they transmit (Ryan et al., 2018).

Aedes aegypti is the principal dengue vector due to its enhanced viral replication capacity, therefore causing an increased likelihood of DENV transmission (Clyde et al., 2006). Since the vector was introduced from Africa in the 17th century, various DENV genotypes have been recorded (Moore et al., 2013; Gubler, 2004). Symptoms of these infections are similarly, making accurate diagnoses difficult. Nonetheless, dengue transmission in the Americas has been carefully monitored for approximately 50 years. Notably, its incidence has increased dramatically over the last quarter-century.

Most dengue infections are asymptomatic, although mild to moderate symptoms may occur, including: fever, malaise, headache, retro-orbital pain, myalgia, arthralgia, nausea/vomiting, and rash (Murray et al., 2013). In cases of "severe dengue", or dengue haemorrhagic fever (DHF)—the primary symptom are plasma leakage (vascular permeability) that may result in pleural effusion and dyspnoea, leading to dengue shock syndrome (DSS), possibly causing the death of the patient (Huy et al., 2013). More than 50 million dengue fever cases occur each year worldwide, resulting in ~24,000 deaths annually (Huy et al., 2013). In Mexico, ~548,000 cases of dengue fever and ~153,682cases of severe dengue were reported between 1990 and 2019 (Dirección

General de Epidemiología, 2020). Although dengue is the most common vector-borne viral disease worldwide, few studies have investigated the underlying relationship between disease epidemiology and evolutionary virology, specifically the influx of genetic diversity and novel genotypes. This relationship becomes increasingly convoluted with the presence of concomitant chikungunya and zika virus infections (Mercado-Reyes et al., 2019).

Globally, DENV-2 was the predominant dengue serotype reported between 1990 and 2004. Between 2005 and 2009, coinfections among all four serotypes (DENV-1 – Denv-4) largely characterized outbreaks. After 2009, various serotypes predominated in different regions of the world. DENV-1 and DENV-2 were the most common serotypes in Africa and the Americas; DENV-1 was most prevalent in Europe; coinfection among all four serotypes predominated in the eastern Mediterranean (Levant); and DENV-1 was the most common serotype in the western Pacific (Oceania) (Cisneros-Solano et al., 2004; Guo et al., 2017). These results, although valuable as a global distribution overview, do not inform the geographic fine-mapping of dengue serotypes and genotypes which are etiologically dependent on climate and distinct viral evolution trajectories. Such microevolutionary patterns are more readily apparent in phylogeographic analyses (Cisneros et al., 2006; Gardella-Garcia et al., 2008; Pinheiro and Corber, 1997; Rico-Hesse et al., 1997).

DENV serotypes emerged in the Americas beginning with DENV-1 in 1977. By 1981, DENV-2 and DENV-4 appeared, and in 1994, a novel DENV-3 genotype emerged. These developments resulted in a shift from nonendemicity or hypoendemicity, to hyperendemicity, and ultimately, the arrival of severe dengue, reported between 1981 and 1997 in 24 American countries (Gubler, 1998; Pinheiro and Corber, 1997). Severe dengue was first reported in Mexico among eight patients in

1985. By 1995–1996, 539 cases of severe dengue, including 30 deaths were reported. During this same period, Mexican dengue endemicity was established, and later confirmed in Oaxaca State in 2010 (Istúriz et al., 2000; Torres-Galicia et al., 2014). Significant epidemics of serotype DENV-1 occurred throughout numerous Mexican States between 1979 and 1983, including: Chiapas, Mexico State, Guerrero, Hidalgo, Jalisco, Michoacán, Oaxaca, Puebla, Querétaro, Quintana Roo, San Luis Potosí, Tabasco, Tamaulipas, Veracruz, and Yucatán. Serotype DENV-2 first appeared in Oaxaca and Tamaulipas in 1982. DENV-3 was introduced in Chiapas, Puebla, San Luis Potosi, Tamaulipas and Veracruz in 1995. Lastly, cases of DEN-4, the least common dengue serotype in Mexico, was reported in Yucatán in 1984 (Loroño-Pino et al., 1993). By 1996, DEN-4 was reported in Oaxaca, Veracruz and other Mexican States (Cisneros-Solano et al., 2004; Cisneros et al., 2006; Danis et al., 2014; Falcón-Lezama et al., 2009; Gardella-Garcia et al., 2008). The variable geographic distribution of dengue serotypes corroborates the complex and dynamic nature of DENV introduction across Mexico.

In 2009, Hurricane Ida flooded the States of Veracruz, Tabasco, and Jalisco creating an ideal breeding ground for mosquito vectors, leaving many people susceptible to infection. During the subsequent dengue outbreak, the Mexican Epidemiological Surveillance Single Information System—SINAVE (DREF, 2009) reported 41,687 confirmed cases of dengue fever in Veracruz, representing a 30% increase from the previous year. A total of 7,898 of these cases were classified as severe dengue. There were an additional 11,222 unconfirmed cases reported as well (DREF, 2009).

Studies have shown that DENV strains possess variable levels of virulence (Rodriguez-Roche and Gould, 2013). For example, the occurrence of particularly severe

DHF in Cuba in 1981 was linked to the introduction of a Southeast Asian strain of DENV-2 (Rico-Hesse et al., 1997; Gardella-Garcia et al., 2008). Subsequently, an extended outbreak of DHF/DSS arose in Venezuela, Mexico, Colombia, and Brazil, linked to the same Southeast Asian strain of DENV-2 (Rico-Hesse et al., 1997). In 1995, following a 5-year DENV-1 epidemic, a novel DENV-2 epidemic (American genotype) was reported in Peru (Kochel et al., 2002). This indicated that the American DENV-2 genotype strain lacked the properties necessary to cause the development of severe disease. This episode corroborates earlier findings that associate the DENV-2 American genotype with mild disease and the DENV-2 Southeast Asian genotype with the appearance of DHF in the Americas (Rico-Hesse et al., 1997). Viral virulence and immune response are hence considered two key determinants in the pathogenesis of DHF. In agreement with other researchers, we hypothesize that the displacement of one genotype by another more pernicious type will precipitate severe dengue. This system operates within the context of vector density, in turn influenced by climate and ecological factors. To investigate this hypothesis, it is vital to have continuous epidemiological surveillance systems in place to detect the transmission of distinctive dengue virus genotypes of each serotype.

The objective of this study is to analyse dengue epidemic trends between 1990 and 2019, and to assess DENV serotypes and genotypes from the 2009 Mexican epidemic, specifically. Results will contribute to the development of a future surveillance program, integral to controlling the most virulent forms of this disease.

2. Material and Methods

2.1. Viruses

The serotypes of DENV used in this study were as follows: DENV-1 Hawaii, DENV-2 New Guinea C (NGC), DENV-3 H-87, and DENV-4 H-341. All control samples were supplied by the Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, USA. Additionally, sixty-five isolates of DENV virus were obtained from acute-phase plasma collected from patients with dengue fever or severe dengue through the Instituto Mexicano del Seguro Social (IMSS) from Veracruz State in 2009. Samples were anonymized, with only information regarding disease symptomology available. This study was approved by the Institutional Review Board of the IMSS (Commission of Scientific Research) and the Bioethical Commission for Research in Humans of the Center for Research and Advanced Studies of the National Polytechnic Institute (Comité de Bioética Para la Investigación en Seres Humanos, COBISH—CINVESTAV). All epidemiological data was obtained through the Mexican SINAVE (Dirección General de Epidemiología, 2020).

2.2. DENV infected cells and virus isolation

Aedes albopictus clone C6/36 cells were grown in minimal essential media (MEM), supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Cells were maintained at 28 °C without carbon dioxide (CO_2). After 18 hours of culture, cells (2×10^6/100 mm plate) were infected with 0.2 ml DENV-2 inoculum with an input MOI of 600 PFU/cell, and were incubated at 28 °C for 10 days.

Viruses were isolated as described by Cisneros et al. (2006), with a few modifications. After 18 hours of culture, C6/36 cells (2×10^6/15 ml tube) were infected with 0.01 to 0.1 ml of serum specimen per tube, diluted to 1.0 ml with medium, and incubated for 2 hours at 28 °C. After one wash, 3.0 ml of MEM was added, and cells were cultivated for approximately 15 days at 28 °C (passage number 1). Cells were observed daily and when a cytopathic effect was apparent from syncytium formation

and cellular lysis, cells were harvested and centrifuged at 3,000 rpm for 5 min. The pellet was then suspended in 0.6 ml of MEM and stored in aliquots of 0.15 ml at –70 °C. The supernatant (approximately 2.5 ml) was stored in 2 aliquots of 1.0 ml and one aliquot of 0.5 ml, at –70 °C. To obtain passage numbers two and three, C6/36 cells were incubated with 1.0 ml of the supernatant obtained from earlier passages, for 2 hours at 28 °C, following the same procedure as above. Serotypes among all samples were determined based on the isolates obtained from the first, second, or third culture passages.

2.3. RNA extraction procedure

RNA was extracted from the cell culture supernatant using TRIzol™ LS reagent (Gibco, Gaithersburg, MD) according to the manufacturer's recommendations. Isopropanol-precipitated RNA was recovered by centrifugation, and then air-dried. The resultant RNA pellet was suspended in 50 µl of diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO) treated water and used as a template for reverse transcription with polymerase chain reaction (RT-PCR).

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

The RT-PCR protocol described in Seah et al. (1995) was used to discern DENV serotypes. Synthetic oligonucleotide primer pairs were designed according to sequence data for the following DENV strains: 16681, New Guinea C, and Jamaica 1409 (Seah et al., 1995). The following genes were then amplified and sequenced: (1) protein C—nucleotide 139 (C-139) to prM-789 (prM-789) (Gardella-Garcia et al., 2008), and (2) NS3 from nucleotides 4,899 (DV1) to 5,067 (DSP1), 4,899 (DV1) to 5,279 (DSP2), 4,899 (DV1) to 5,174 (DSP3) or 4,899 (DV1) to 5,342 (DSP4), for serotypes DENV-1, DENV-2, DENV-3 and DENV-4, respectively (Seah et al., 1995). All assays were

performed using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts). A mixture of 5 µl of total RNA (0.1–0.5 µg), and 1 pmol/µl of each primer at nucleotide positions C-139 (forward, 5′-CAATATGCTGAAACGCGHG-3′) and prM-789 (reverse, 5′-CCTTCNGMNGACATCC-3′) was incubated at 65 °C for 5 min. After adding 25 µl of 2X Reaction Mix and 2 µl of SuperScript™ III RT/Platinum™ Taq with DEPC-treated water (total volume: 50 µl), RT was carried out at 50 °C for 60 min. This step was followed by incubation at 94 °C for 2 min to inactivate the reverse transcriptase. Afterwards, Platinum™ Taq was activated by incubation at 94 °C for 2 min to amplify the 629 bp fragment of C-pM. This was followed by 35 cycles with the following conditions: 94 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s, and a final extension of 72 °C for 10 min (storage at 4 °C). Strains of DENV -1, -2, -3 and -4 were used as positive controls for RT-PCR assays.

Serotypification was conducted according to the protocol of Seah et al. (1995): 10 cycles of 95 °C for 30 sec, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min and 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, with a final extension of 72 °C for 7 min (storage at 4 °C). In addition, the NS3 region—nucleotide positions 4,999 to 5,067 (NS3-169) for DENV-1, and 4,910 to 5,174 (NS3-265) for DENV-3, was obtained via an RT-PCR assay, as aforementioned, but with the following primers: (1) DV1 (forward, 5′-GGRACKTCAGGWTCTCC-3’) and DSP1 (reverse, 5’-AGTTTCTTTTCCTAAACACCTCG-3’) for DENV-1, (2) DV1 and DSP3 (reverse, 5’-TTAGAGTYCTTAAGCGTCTCTTG-3’) for DENV-3, and (3) DV1 and DSP4 (reverse, 5’ CCTGGTTGATGACAAAAGTGTTG 3’) for DENV-4.

2.5. Sequencing of PCR products

For automated sequencing, spin column-purified (Qiagen, Chatsworth, CA.) DNA fragments were sequenced using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems/ThermoFisher Scientifics, Waltham, MA). Sequencing was conducted using an Applied Biosystems Prism 3100, in a short capillary (47 cm × 50 µm inside diameter), and Performance Optimized Polymer 6 (Perkin-Elmer, Waltham, MA, and Applied Biosystems).

2.6. Phylogenetic analyses

The phylogenetic trees for DENV-1, DENV-2, DENV-3, and DENV-4 were inferred using the maximum likelihood method of Tamura and Nei (1993). For this method the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to each branch (Felsenstein, 1981). Initial trees for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using maximum composite likelihood (MCL) (Tamura et al., 2004). A discrete gamma distribution was used to model evolutionary rate differences among sites with 3 rate categories (+G, parameter = 0.2447). The final tree is drawn to scale, with branch lengths measured by number of substitutions per site. All above analyses were performed using MEGA7 (Molecular Evolutionary Genetics Analysis Version 7.0) software (Kumar et al., 2016).

Phylogenetic trees were then constructed using a Markov chain Monte Carlo integration (MCMC)-based Bayesian analysis with the minimum recommended parameters (uncorrelated log-normal relaxed clock, with a GTR substitution model with Gamma 4), implemented in BEAST v1.10.3 software (Suchard et al., 2018). MCL trees and Bayesian trees were compared to assess the extent of congruence between these methods.

2.7. Absolute percent difference between dengue fever and severe dengue cases in both Veracruz State and Mexico.

The percentage of variation between tandem epidemiologic years was calculated as follows:

$$\left[\left(\frac{final\ value}{initial\ value}\right) - 1\right] 100$$

Where *final value* is the number of cases (either dengue fever or severe dengue) in the year of interest, and *initial value* is the number of cases (either dengue fever or sever dengue) in the preceding year.

The absolute percent difference between dengue fever and severe dengue was calculated as follows:

$$\left(\frac{first\ value - second\ value}{(first\ value + second\ value)/2}\right) 100$$

Where *first value* is the number of cases of dengue fever and *second value* is the number of cases of severe dengue.

3. Results

3.1. Epidemiology of dengue in Mexico and serotyping of virus isolates

Cases of dengue are widely underreported and moreover, circulating serotype distribution is not well established, creating a need for a broader and more nuanced understanding (Gómez-Dantés and Willoquet, 2009). Figure 1 shows dengue and severe dengue incidence increase in Mexico between 1990 and 2019. The percentage of variation in number of dengue fever versus severe dengue cases in both Veracruz and

Mexico was calculated to assess spatiotemporal dengue patterns. Although percentage of variation generally fluctuates between each epidemiological year, according to Figure 2A, the rate of increase was greater from 2001 to 2002, 2003 to 2004 and 2011 to 2012 compared with other years. Figure 2B depicts the absolute percentage difference of dengue fever to severe dengue cases between 1990 and 2019 in both Veracruz State and Mexico. The relative percentage of severe dengue cases has increased compared to dengue fever cases in both Veracruz State and Mexico. The 2009 incidence of dengue fever was greatest in Veracruz compared to any other State in the country, evidence of the devastating effects of Hurricane Ida on mosquito-borne illness within a geographic area (DREF, 2009). If we compare the absolute percentage difference (proportion) in dengue fever versus severe dengue cases between Veracruz and Mexico (Figure 2B), the percentage difference remained similar between 1990 and 2000, followed by an increase in the percentage difference (greater sever dengue) beginning in 2000–2001 in Mexico, and 2003–2004 in Veracruz. Between 2007 and 2008 there is a sharp increase in the proportion of severe dengue cases in Veracruz that continues until 2009. In 2008 and 2014 there was a conspicuous dissonance in the proportion of severe dengue to dengue fever cases in Veracruz compared to Mexico. Both years exhibited a noticeable increase in severe dengue in Veracruz, separated by a sizeable recovery in 2010. The proportion of dengue fever to severe dengue cases in Veracruz compared to Mexico was very similar in the years 2004–2007, 2011–2012, and 2015–2017.

3.2. Isolation and serotyping of DENV

All samples were evaluated using the Mac-Elisa test, which is standard protocol of the Mexican Ministry of Health for diagnosing DENV infections (Cisneros-Solano et al., 2004). Patients whose clinical reports showed symptoms of severe dengue had laboratory results verified using an IgG-ELISA kit. Serotyping was performed via RT-

PCR using the RNA obtained from isolates in C6/36 cells and repeated at least twice. Out of 215 isolates from Oaxaca, 31 were diagnosed with severe dengue—74.5% were DENV-2, 6.4% were DENV-3, 6.4% were DENV-4, 4.2% were DENV-1, and 8.5% presented dual infections (DENV-2 with either DENV-1, -3, or -4).

3.3. Phylogenetic analysis of Veracruz isolates

Portions of the C139-prM789 or NS3-484 gene were amplified and sequenced as described in the Methods section. Fifteen sequences were obtained from the Veracruz 2009 isolates for the C-prM fragment (eight with dengue fever and eight with severe dengue) and seven sequences for the NS3 gene (all with dengue fever). Five sequences from the 2005 outbreak in Oaxaca were added, two of the DENV-2 Asian/American genotype and 3 of DENV-3, genotype III. Phylogenetic analyses were performed on the aforementioned sequences and furthermore prototype sequences of characterized isolates for DENV-1, -2, -3, and -4 (Domínguez-de-la-Cruz et al., 2020). Eleven sequences presented as the DENV-2, Asian II genotype (seven with dengue fever and seven with severe dengue), three presented as the DENV-2 Asian/American genotype (all with dengue fever), and two presented as DENV-4, genotype I (one with dengue fever and one coinfected with DENV-2 Asian II genotype with severe dengue). Genotypes I and II were assessed for DENV-1 and -3 using the NS3 gene (Domínguez-de-la-Cruz et al., 2020).

Phylogenetic analyses showed that C139-prM789 (Asian/American genotype) from Veracruz was most closely related to dengue strains from Oaxaca and Veracruz during the 2005 and 2006 outbreaks. Strains were closely related to those previously found in the Caribbean countries of Cuba, the Dominican Republic (DR), and Martinique (Mar) (France) (Domínguez-de-la-Cruz et al., 2020) (Figure 3). Strains from these regions were all grouped on the same branch of the constructed phylogenetic tree

(Figure 3). DENV-2 isolates from Veracruz (Asian II genotype) were most closely related to strains from Colombia and Thailand (Figure 3). DENV strains from Brazil, Venezuela and Jamaica formed an independent clade. Interestingly, analysis of C139-prM789 showed that isolates obtained in 2001 from Juchitán, Tonalá, Tuxtepec, Huatulco, and Salina Cruz, Oaxaca State shared the same clade as strains from Venezuela, Puerto Rico (USA), and Taiwan (Taiw) for the Asian/American genotype. A shorter fragment of the gene for the prM protein, including isolates from Guerrero State, Mexico (Asian II genotype) showed that isolates from Veracruz belonged to the same phylogenetic tree branch. This finding confirms the circulation of the DENV-2, Asian II genotype in Veracruz State (Domínguez-de-la-Cruz et al., 2020).

Phylogenetic analyses using C139-prM789 sequences revealed that both DENV-4 isolates from the 2009 dengue epidemic in Veracruz, Mexico present as genotype I, and are most closely related to strains from Philippines and China (Figure 4). Moreover, both isolates share a close evolutionary lineage with strains from the Amazonas State, Brazil (Figure 5). Notably, genotype I was not previously reported in Mexico. DENV-4 trees were constructed with sequences of 567 bp (Figure 4) and 363 bp (Figure 5) because Brazilian sequences were relatively shorter. DENV-4, genotype II, which has been previously reported in the Americas was not identified in this study (Bennett et al., 2003). Phylogenetic analyses using the NS3-484 gene showed that DENV-1 isolates presented as genotype V, and that they are most closely related to strains from Puerto Rico (USA) and Brazil. This particular genotype has been circulating in the Americas since approximately 2004 (Domínguez-de-la-Cruz et al., 2020). Phylogenetic analyses using the NS3-484 gene showed that DENV-3 isolates presented as genotype III, and that they share an affinity with strains from the 2005 dengue epidemic in Oaxaca. Such

genotypes have been circulating in the Americas since approximately 2001 (Domínguez-de-la-Cruz et al., 2020).

MCL trees were compared with Bayesian trees to assess the extent of congruence between these methods. Our results found that the topology of resultant trees was very similar. Bayesian analyses confirm the presentation of the Asian II genotype of DENV-2 (Domínguez-de-la-Cruz et al., 2020) and genotype I of DENV-4, in Veracruz (Figure 5).

4. Discussion

After broad campaigns between 1947 and 1970 by the Pan American Health Organization (PAHO) to eradicate *Ae. aegypti* in tropical and subtropical America, yellow fever and dengue were largely, though temporarily, eliminated (Brathwaite-Dick et al., 2012). Arbovirus surveillance eventually waned, leading to a rapid and widespread re-emergence of *Ae. aegypti* in environmentally suitable countries. An outbreak of DENV-1 infection occurred in Mexico in 1978. In 1984, eight DENV-4 severe dengue cases were reported, resulting in four deaths. By 1989, four more DENV-4 severe dengue cases were reported, resulting in 1 death, and in 1991, two more cases were reported. The emergence of DENV-2 in Mexico occurred in 1981 (30,000 cases) and was followed shortly thereafter by another outbreak in 1984 (23,000 cases). DENV-1, -2, and -4 were isolated by in 1994, and DENV-3 was first reported in Mexico in 1995 (Brathwaite-Dick et al., 2012). By 1997, all four serotypes of DENV were circulating in Mexico (Gardella-Garcia et al., 2008).

Phylogenetic analyses of DENV-1 and DENV-3 displayed the presentation of genotypes V and III, respectively, despite clustering with different clades. This is

suggestive of viral evolution. The DENV-2 Asian/American genotype has slowly replaced the American genotype. The geographic origin of the Asian/American genotype strain in Veracruz may have been introduced from nearby Caribbean islands. The DENV-2, Asian II genotype was identified in dengue fever (63.6%) and severe dengue (36.4%) cases. Strains isolated in the years 1983, 1984, 1992, 1994, and 1995 were identified as the American genotype. One 1996 isolate from Yucatán State was dubbed the ‘‘Cosmopolitan’’ genotype by Loroño-Pino et al. (2004). Mexican DENV-2 isolates from Yucatán and Oaxaca between 2000 and 2002, and Oaxaca and Veracruz between 2005 and 2006 have previously been reported to belong the Asian/American genotype (Díaz et al., 2006; Gardella-Garcia et al., 2009).

This study provides evidence for the introduction of the Asian II genotype of DENV-2, in Mexico during the 2009 Veracruz dengue outbreak. The Asian II genotype was first reported in Mexico in two DENV-2 isolates from 1997 in Guerrero, Mexico (GenBank accession number: AY449678, AY449679). According to phylogenetic analyses, this strain is closely related with DENV-2 strains collected from Cuba in 1981 (Rodriguez-Roche et al., 2015) (GenBank accession number: EU854293), and later Colombia (GenBank Accession Number: EU854293) and Asia (GenBank Accession Number: AF204178 and AF204177) (Domínguez-de-la-Cruz et al., 2020). The geographic origin of the Mexican DENV-2, Asian II genotype may be Guerrero State and/or the Caribbean islands. This study has also described the introduction of DENV-4 genotype II, previously reported in Manaus, Brazil in 2008 (de Melo et al., 2009; Figueiredo et al., 2008;). Although DENV-4 is less prevalent than other dengue serotypes in Mexico, it has been shown to be involved in the development of severe dengue following secondary infections, and hence of significant epidemiological importance (Ahamed et al., 2019; Guo et al., 2017; Soo et al., 2016; Suppiah et al.,

2018). This study identified two DENV-4, genotype II isolates, one from a patient coinfected with DENV-3, genotype III, and diagnosed with severe dengue.

The increased frequency of severe dengue in Veracruz coincides with the permanence of the DENV-2 Asian/American genotype and the emergence of both the DENV-2, Asian II genotype, and DENV-4, genotype I. The DENV-2, Asian II genotype, and DENV-4, genotype I have been reported in Acapulco and Chilpancingo, Mexico, as well as Brazil (Kubiszeski et al., 2020; de Melo et al., 2009; Figueiredo et al., 2008). The novel genotypic/lineage variations in DENV-2 and DENV-4 may have influenced the magnitude and severity of Veracruz dengue epidemics in 2009, since the severe dengue patients were infected by the novel DENV-2 Asian II genotype. This suggests that these particular genotypes cause more severe disease directly (Figure 2B). Prior studies have shown the potential for novel genotypic/lineage variations to have epidemiological affects (Ahamed et al., 2019; De La Cruz-Hernández et al., 2013; Gardella-Garcia et al., 2008; Zhang et al., 2005). Relative increases in severe dengue cases in particular may be due to the circulation of specific genotypes during certain years that cause an increase in dengue infection severity and incidence. Once the infection caused by that specific genotype passes through the population, herd immunity is achieved, reducing the incidence of infection. If a different, novel genotype begins to circulate, however, it will re-infect the population, generating another spike in either incidence, disease severity, or both (OhAinle et al., 2011; Cologna et al., 2005; Hang et al., 2010).

Apart from direct host-, vector-, and virus-related determinants, ecological factors such as climatic variation (i.e. seasonality), and weather events (i.e. flooding, hurricanes, etc.) are integral to the fitness and ultimate distribution of both dengue vectors and novel DENV strains. Our results show that increases in dengue fever and

severe dengue cases overlapped with the climatological and infrastructural fallout of Hurricane Ida in 2009. Hurricanes in Veracruz in 2007 (Hurricane Dean) and 2012 (Hurricane Ernesto) (CONAGUA, 2019), were also found to coincide with an uptick in severe dengue cases (Figure 2). Although such observations are indeed in agreement with previous reports showing a causative association between severe hurricane years and arbovirus infection rates, further statistical analyses will have to be conducted before drawing any conclusions regarding the data from this study in particular (Messina et al., 2015; Morin et al., 2013). A future synthesis of demographic, sociocultural, ecological and infrastructural elements with evolutionary virology is paramount to further elucidating dengue epidemiology in the Americas.

5. Conclusions

In 2009, Veracruz State was affected by intense rains due to Hurricane Ida, causing flooding and infrastructural damage, leaving thousands without homes, and creating an ideal mosquito breeding (DREF, 2009). Approximately 42,000 cases of dengue infection were confirmed that year in Veracruz, representing a 30% increase from the previous year (2008). The emergence of novel genotypes is due to an overall increase in dengue cases, leading to a higher probability of genetic divergence. Findings show that the predominant DENV serotypes circulating during this outbreak were: (1) DENV-2, followed by (2) DENV-3, (3) DENV-1, and (4) DENV-4. Five cases of DENV-2, genotype I were coinfected with serotypes -1, -3, or -4. The primary DENV genotypes found in Veracruz during the 2009 epidemic were:

(A) The Asian II and Asian/American genotypes (DENV-2)

(B) Genotype III (DENV-3)

(C) Genotype V (DENV-1)

(D) Genotype I (DENV-4).

Severe dengue cases were identified mainly in subjects infected with the DENV-2, Asian II genotype, and DENV-4, genotype I. Results suggest a possible association between the emergence of novel DENV genotypes with both an increase in DENV incidence and frequency of severe dengue. To corroborate this potential relationship, annual testing should be conducted in a large sample size every year following the introduction of novel genotypes to a particular geographic region. Routine serological and nucleic acid detection methodologies should be employed to this end.

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Author Disclosure Statement:

The authors have no potential conflicts of interest.

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Figure Legends

Figure 1. Dengue virus incidence in Veracruz State and Mexico. Reported incidence (Annual new cases per 100,000 individuals) of dengue fever and the proportion of severe dengue cases across Veracruz State and Mexico between 1990 and 2019

Figure 2. (A) Number of cases and percentage of variation of dengue fever and severe dengue between 1990 and 2019, and (B) Absolute percentage differences between dengue fever and severe dengue cases between 1990 and 2019 in both Veracruz State and Mexico. (A) The total number of cases of dengue fever and severe dengue in Veracruz State and Mexico is displayed in bars and the percentage of variation in lines. D-MEX and D-VER represent dengue cases from Mexico and Veracruz, respectively; SD-MEX and SD-VER represent severe dengue cases from Mexico and Veracruz, respectively. (B) Displays the absolute percentage difference between the number of cases of dengue fever and severe dengue in both Veracruz State

and Mexico between 1990 and 2019, according to the formula described in the Material and Methods section. Lower values represent a higher proportion of severe dengue. Table 1 displays all corresponding, graphed values.

Figure 3. Maximum-likelihood phylogenetic tree of 150 DENV-2 CpM gene sequences (380 nucleotides). The midpoint rooted phylogenetic tree includes 13 new sequences in Asian/American and Asian II genotypes, labelled in bold. All samples are from the State of Veracruz, Mexico. The phylogenetic tree was obtained using MEGA 7 as described in the section of Methods. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to branches. Positions of sequences from Mexico are indicated using a dot, from the Americas using a triangle, and novel sequences from Veracruz State in bold. Horizontal branch lengths are proportional to the bar representing number of nucleotide substitutions/sites. MX: Mexico; MZ/OAX: Mexico, Oaxaca; MX/VER: Mexico, Veracruz; US/PR: United States, Puerto Rico; VE: Venezuela; NI: Nicaragua; COL: Colombia; EC: Ecuador; BR: Brazil; Ph, Philippines; China, Chin. DENV strains are named as follows: GenBank accession number/strain/country/year/serotype/genotype (Domínguez-de-la-Cruz et al., 2020).

Figure 4. Maximum-likelihood phylogenetic tree of 150 DENV-4 CprM gene sequences (567 nucleotides). Details of phylogenetic tree analysis and graphical features are the same as those provided in the Figure 3 legend. This phylogenetic tree includes two novel sequences of genotype I, labelled in bold. Positions of sequences from Mexico are indicated using a dot, from the Americas using a triangle, and novel sequences from Veracruz State in bold. MX: Mexico; MZ/OAX: Mexico, Oaxaca; MX/VER: Mexico, Veracruz; US/PR: United States, Puerto Rico; VE: Venezuela; NI: Nicaragua; COL: Colombia; EC: Ecuador; BR: Brazil.

Figure 5. Maximum clade credibility (MCC) tree of 132 DENV-4 CprM gene sequences (363 nucleotides). This phylogenetic tree includes two new sequences of genotype I, labeled in bold. Positions of sequences from Mexico are indicated using a dot, and from the Americas using a triangle. Horizontal branch lengths are proportional to the bar representing the probability of coalescence. DENV strains are named as follows (GenBank accession number/serotype/country/year/genotype). MX: Mexico; MZ/OAX: Mexico, Oaxaca; MX/VER: Mexico, Veracruz; US/PR: United States, Puerto Rico; VE: Venezuela; NI: Nicaragua; COL: Colombia; EC: Ecuador; BR: Brazil; Ph, Philippines; China, Chin.

Tables

Table 1. Number of cases and incidence of dengue fever/severe dengue in Veracruz State and Mexico between 1990 and 2019.

Credit author statement:

M-d-LM conceived and designed research; EHG participated in the elaboration of the project and obtained the isolates; EHG, MM-G, CAB-C, ED-de-la-C performed research; GPR performed the phylogenetic analysis; ED-de-la-Cruz participated in the data analysis and submission of sequences to the GenBank repository; RED, ED-de-la-C, and ADB participated in all data analysis; and M-d-LM wrote the paper; RED participated in the manuscript edition; all authors contributed reviewing and approved the manuscript.

Table 1. Number of cases and incidence of dengue fever/severe dengue in Veracruz State and Mexico between 1990 and 2019.

Year	Number of Cases						Incidence (per 100,000)					
	Veracruz State			Mexico			Veracruz State			Mexico		
	Dengue Fever	Severe Dengue	*Total*	Dengue Fever	Severe Dengue	*Total*	Dengue Fever	Severe Dengue	*Total*	Dengue Fever	Severe Dengue	*Total*
1990	160	0	160	1663	6	1669	10.71	0.00	10.71	11.09	0.00	11.09
1991	143	0	143	1931	1	1932	4.43	0.00	4.43	6.72	0.00	6.72
1992	32	0	32	1102	0	1102	4.86	0.00	4.86	13.74	0.00	13.74
1993	166	0	166	791	0	791	6.87	0.00	6.87	3.28	0.00	3.28
1994	2462	0	2462	7868	0	7868	37.44	0.07	37.51	8.95	0.03	8.98
1995	5503	79	5582	14396	355	14751	99.68	1.14	100.82	39.33	0.59	39.92
1996	5297	358	5655	19835	884	20719	105.22	7.27	112.49	37.62	1.56	39.18
1997	10563	155	10718	51021	954	51975	152.2	2.2	152.4	55.48	1.03	56.51
1998	2147	28	2175	15181	225	15406	33.66	0.6	34.26	24.17	0.39	24.56
1999	2331	10	2341	23725	220	23945	32.7	0.2	32.9	23.4	0.2	23.6
2000	568	7	575	1706	50	1756	8.02	0.11	8.13	1.72	0.07	1.79
2001	2344	14	2358	4643	312	4955	31.35	0.2	31.55	4.6	0.31	4.91
2002	2357	98	2455	13131	2159	15290	32.71	1.36	34.07	12.95	2.11	15.06
2003	988	95	1083	5018	1419	6437	14.95	3.05	18	5.01	1.7	6.71
2004	4250	1570	5820	6243	1959	8202	58.42	21.58	80	5.93	1.86	7.79
2005	3901	636	4537	17487	4418	21905	53.47	8.72	62.19	16.43	4.15	20.58
2006	7265	1066	8331	22566	4426	26992	95.57	21.13	116.7	22.94	4.81	27.75
2007	12608	2645	15253	42936	9433	52369	174.09	36.52	210.61	40.59	8.92	49.51
2008	2066	2051	4117	27964	7560	35524	28.49	28.37	56.86	26.26	7.11	33.37
2009	3412	2978	6390	44565	11396	55961	136.08	40.91	176.99	112.18	10.6	122.78
2010	867	302	1169	22352	9336	31688	13.76	4.4	18.16	33.89	6.18	40.07
2011	996	651	1647	10970	4608	15578	17.2	11.04	28.24	14.12	5.88	20
2012	7531	5041	12572	32662	17706	50368	193.15	69.82	262.97	56.37	16.01	72.38
2013	4941	3858	8799	43663	18667	62330	62.47	48.78	111.25	89.51	16.74	106.25
2014	2060	1866	3926	23374	8647	32021	25.8	23.37	49.17	19.51	7.22	26.73
2015	2884	876	3760	21201	5464	26665	35.84	10.89	46.73	17.52	4.52	22.04
2016	1833	391	2224	14112	3683	17795	22.61	8.64	31.25	11.54	3.1	14.64
2017	1031	324	1355	11334	2794	14128	12.63	3.96	16.59	9.18	2.26	11.44

2018	2239	467	2706	8229	4477	12706	27.24	5.69	32.93	6.6	3.59	10.19
2019	9195	1707	10902	27884	13621	41505	111.12	20.63	131.75	22.14	10.82	32.96

The number of cases and incidence of dengue fever and severe dengue in Mexico and Veracruz State were collected from the official website of the Mexican SINAVE (Dirección General de Epidemiología) [2] during the period from 1990 to 2019. The incidence was calculated as the number of annual new cases per 100,000 individuals.

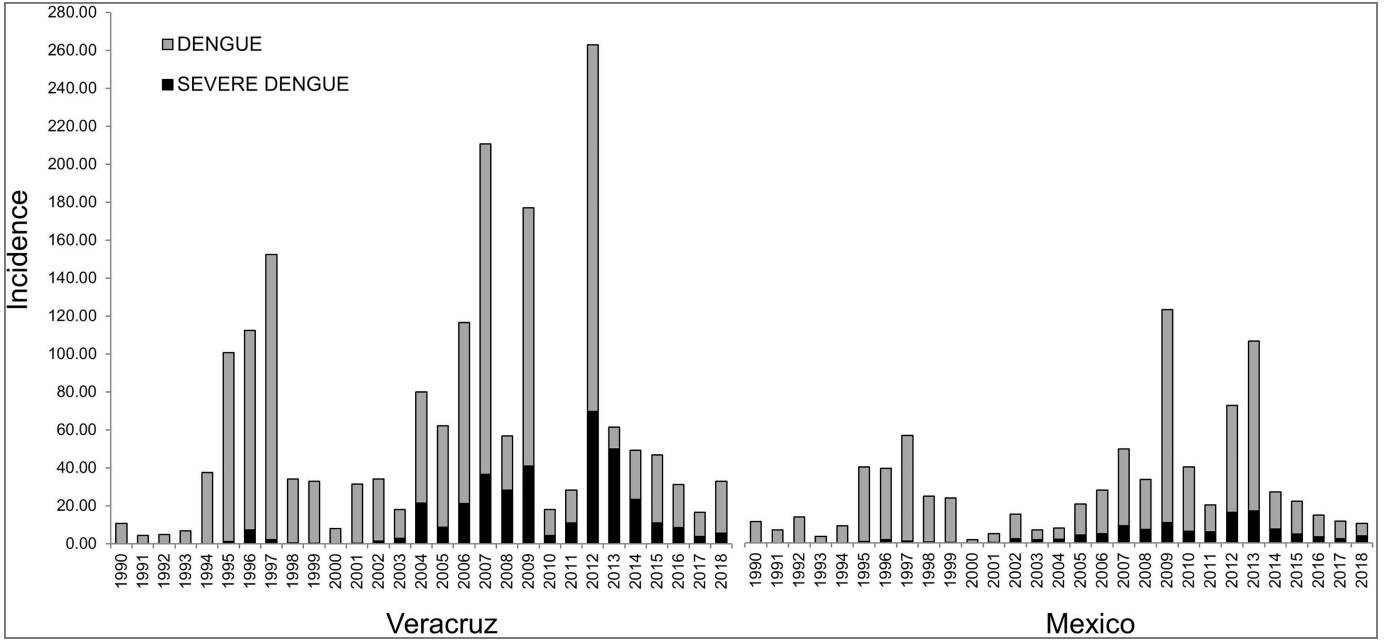
Highlights (Maximum 85 characters, including spaces, per bullet point).

-Novel DENV-2 and DENV- 4 genotypes have emerged in Veracruz State, Mexico.

-DENV-2 genotype Asian II and DENV-4 genotype I were both identified.

-A genotype replacement event was observed with DENV-2 and DENV-4 in Mexico.

-The concurrent introduction of new genotypes may increase the overall incidence of severe dengue.


DENGUE
SEVERE DENGUE
Incidence
280.00
260.00
240.00
220.00
200.00
180.00
160.00
140.00
120.00
100.00
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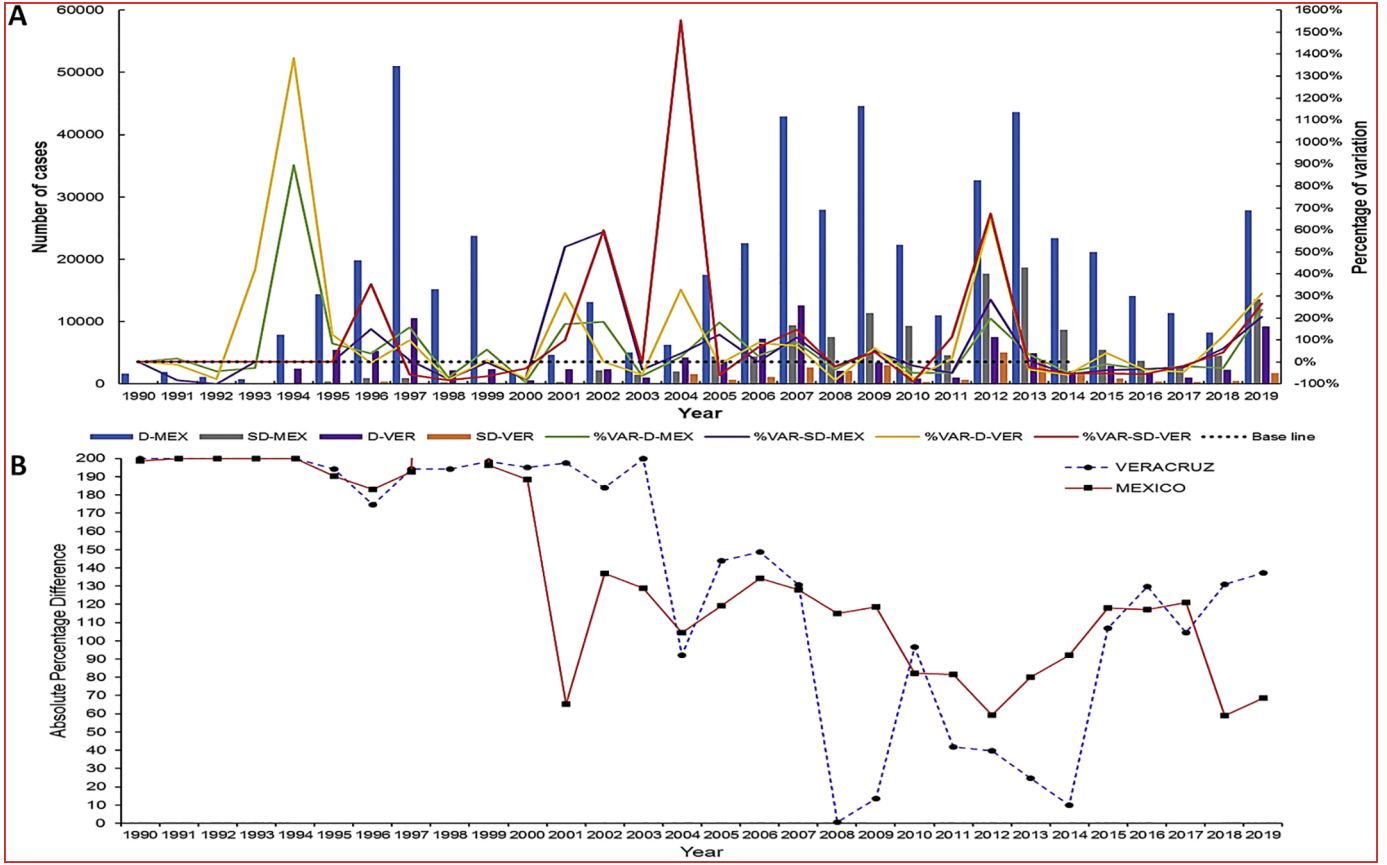
1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018
Mexico

Figure 1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Number of cases
Percentage of variation
Year
D-MEX
SD-MEX
D-VER
SD-VER
%VAR-D-MEX
%VAR-SD-MEX
%VAR-D-VER
%VAR-SD-VER
Base line
B
Absolute Percentage Difference
VERACRUZ
MEXICO
Year

Figure 2

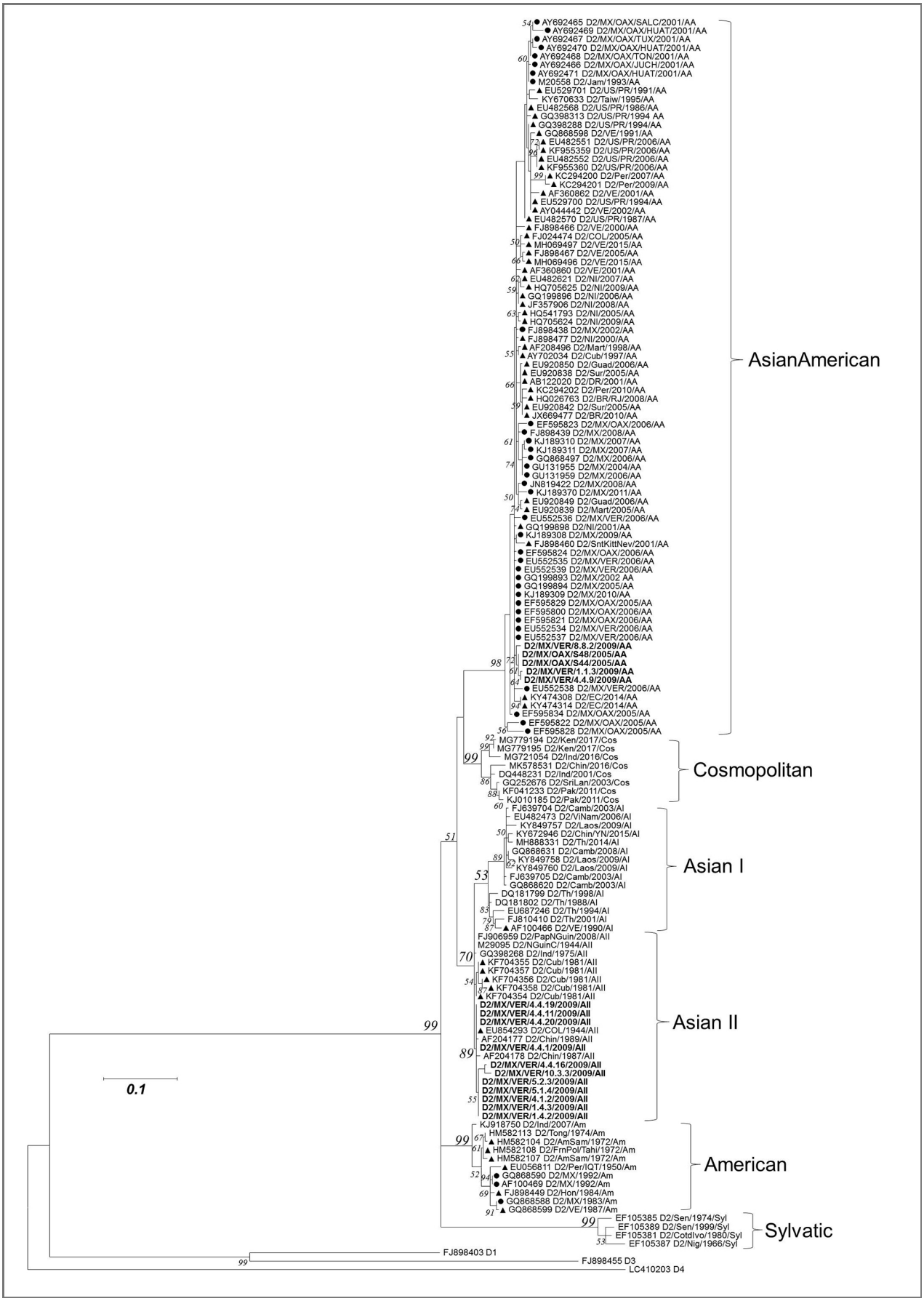
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Figure 3

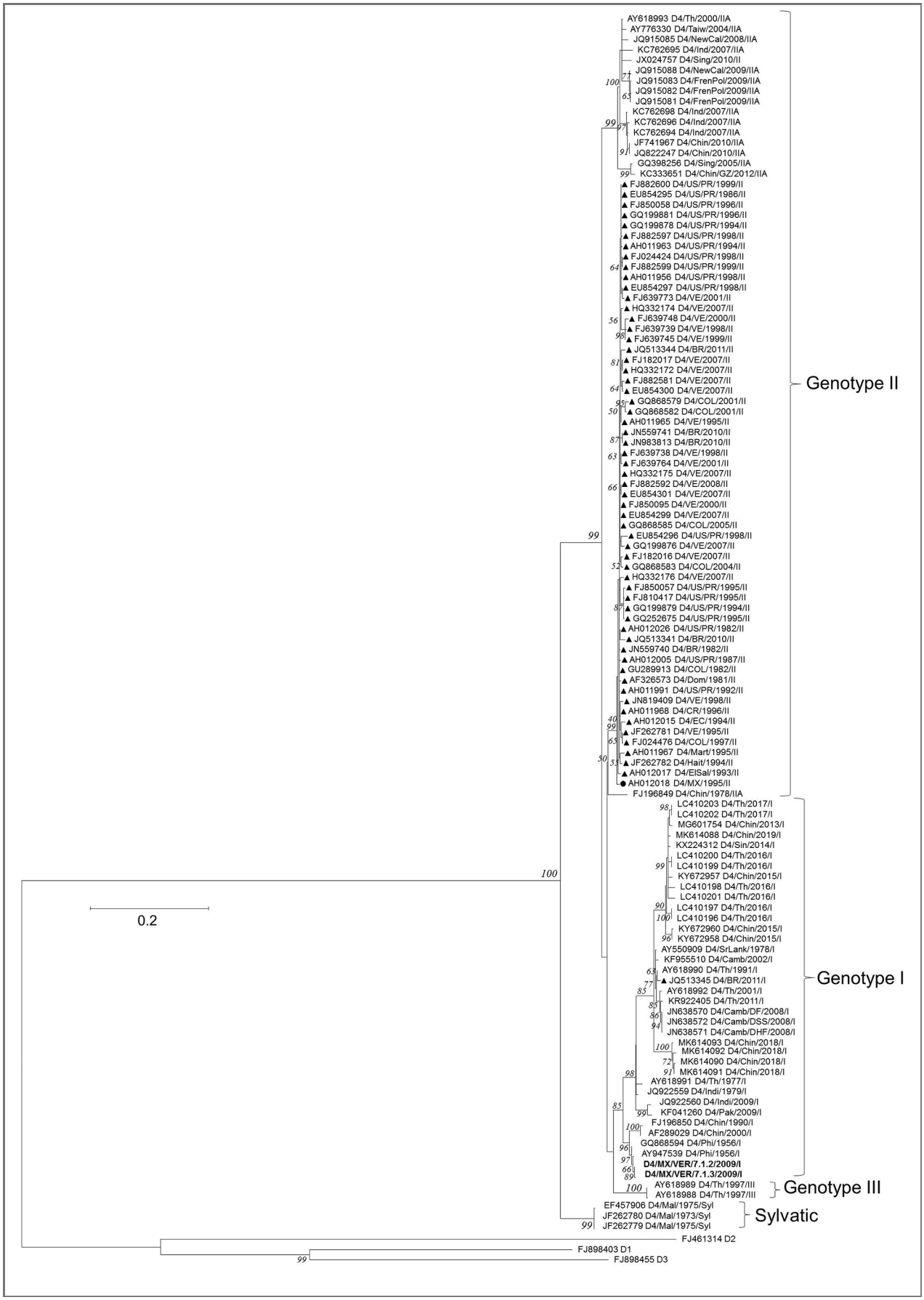
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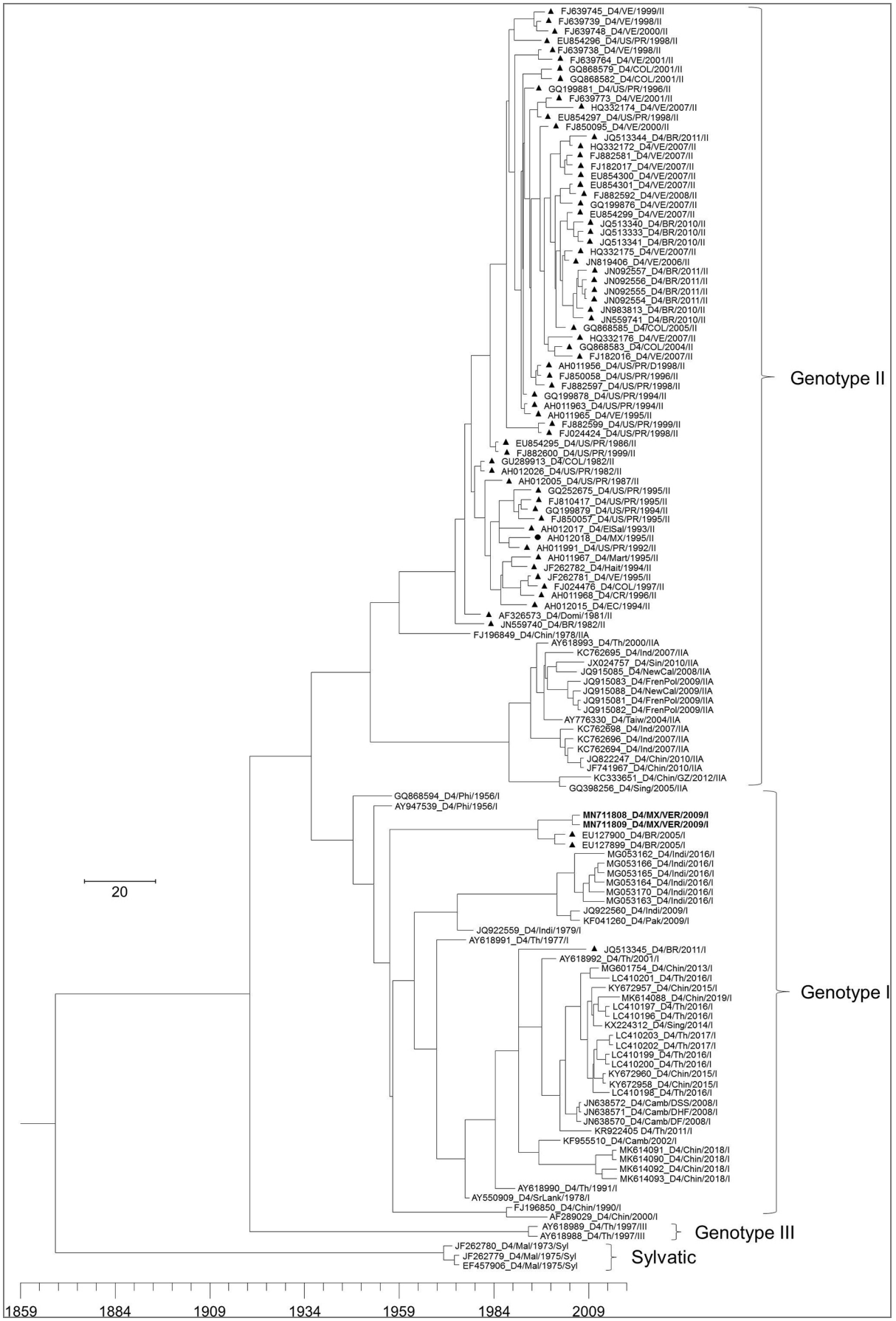
Figure 4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2009

Figure 5