SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Bayesian Analyses

To determine the time of the most recent ancestor (TMRCA) of the Nicaraguan dengue epidemics, Maximum Clade Credibility (MCC) trees were generated using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST 1.6.1 (*1*). For all analyses, the exact date of sampling was utilized, and the model was parameterized using the GTR+Γ_4 model of nucleotide substitution, a different substitution rate for each codon, a strict molecular clock, and a Bayesian Skyline tree prior with five coalescent-interval groups. All MCMC chains were run for sufficient length (~150M generations sampled every 10,000) to ensure stationary parameters, with statistical error reflected in values of the 95% highest probability density with 10% removed as burn-in. The comparison of results across three replicate computes and between strict versus relaxed molecular clock models showed similar results for TMRCA estimates and tree topology. ML bootstrap-supported clades were consistent with well-supported MCC clades.

Selection Analyses

We implemented several different tests in PAML (*2*) to test for positive selection. The yn00 program was implemented for pairwise tests of both individual sequences as well as the ancestral nodes as per Yang and Nielsen (*3*), Nei and Gojobori (*4*), Li, Wu and Luo (*5, 6*), and Pamilo and Bianchi (*7*). The codeml program was implemented to test for selection by allowing for dN/dS ratios to vary both by site across the genome and by site and branch, specifying for the latter set of models each of the branches leading to (1) the Nicaraguan ancestor, (2) the NI-1 clade ancestor, and (3) the NI-2B clade ancestor. Because of the computational cost of the site and branch-site models, we replaced certain clades of sequence with their inferred ancestors. In all analyses, the outgroup American (4 sequences) and Asian-1 (9 sequences) clades were collapsed to their ancestors. For branch-site models differentiating the branch leading to the Nicaraguan clade, we also collapsed the Nicaraguan clade to its ancestor. For all site models and for branch-site models differentiating the NI-1 and NI-2B clades, we collapsed the NI-1, NI-2A, and NI-2B clades to their ancestral sequences. Asian-American sequences outside of the Nicaraguan clade were always retained as original sequences. For site-specific models, we used codeml with

model = 0 and NSsites = 0 1 2 7 8, which implements tests of Nielsen and Yang (*8*). For branch-site-specific models, we used model = 2 and NSsites = 2 (with variable omega or fixed omega = 1) which implements the test of Yang and Nielsen (*9*) with modifications by Zhang et al. (*10*).

Viral Replication Assays

U937/DC-SIGN cells were obtained from A. de Silva (University of North Carolina, Chapel Hill). For viral replication assays, U937/DC-SIGN cells were cultured in RPMI/10% FBS plus P/S and plated at 2.5 x 10^4 cells/well in 20µL in a 96-well round-bottom plate. 3.5 x 10^7 GE/well of Nicaraguan DENV-2 viral isolates (NI-1 or NI-2B) in 125µL were added to each well. Following a 1-h incubation, 100µL media (RPMI/2%FBS plus P/S) was added to each well, plates were incubated for 48 h and supernatants were pooled from triplication infections for quantitation of viral replication by plaque assay. K562 cells were plated at 2.5 x 10^4 cells/well in 20µL in a 96-well round-bottom plate. 1.5 x 10^7 GE/well of Nicaraguan DENV-2 viral isolates (NI-1 or NI-2B) in 50µL were added to WHO tetravalent serum diluted 1:15 in RPMI/10% FBS plus P/S. Following a 1-h incubation, virus/serum mixtures were added to cells, incubated for 2 h, washed once in medium and plated in 200µL RPMI/2% FBS plus P/S for 48 h, at which time supernatants were harvested for plaque assay. For viral replication assays, viral output in supernatants was assayed by plaque assay on BHK cells as previously described (*11*). Competition assays in U937/DC-SIGN cells were performed as described in Materials and Methods for C6/36 cells and iDCs.

Patient Viremia

Patient viremia was quantitated by extracting viral RNA from 140µL of acute patient serum (QIAamp Viral RNA Kit) and eluting in 60µL Elution Buffer. Two µL of viral RNA was used for Taqman qRT (Verso One-Step qRT; Thermo Scientific) by adapting an NS5 qRT-PCR protocol (*12*) to the Nicaraguan DENV-2 sequence. Primers and probe used for detection of Nicaraguan DENV-2 viral RNA were as follows: Forward 5'-ACAAGTCGAACAACCTGGTCCAT-3', Reverse 5'- GCCGCACCATTGGTCTTCTC-3', and Probe 5' FAM-TGGGATTTCCTCCCATGATTCCACTGG-TAMRA 3'. Cycling conditions were 50°C for 30 min and 95°C for 15 min, followed by 40 2-step cycles of 15 sec at 95°C and 1 min at 60°C. An RNA standard was constructed by reverse-transcribing the amplicon generated

from a Nicaraguan DENV-2 isolate and cloning into the TOPO pCR2.1 vector (Invitrogen). Plasmid containing the amplicon in the correct orientation was digested downstream of the amplicon, transcribed using T7 RNA polymerase (Promega), digested with DNase, and quantitated at A260nm. GE per ng RNA was calculated and used to establish the standard curve. RNA levels as low as 123 GE per well could be reliably detected; 123 GE (equivalent to $2.6x10^4$ GE/mL sera) was used as the limit of detection of the assay. All undetectable values or values lower than the limit of detection in the assay were assigned a value of 2.6×10^4 GE/mL serum. RNA extracted from a Nicaraguan DENV-2 virus isolate passaged in C6/36 cells was aliquoted and used as a positive control on each plate to monitor assay precision. Values for samples processed in multiple valid assays were averaged.

Focus Reduction Neutralization Test (FRNT)

FRNT (Focus Reduction Neutralization Test) neutralization assays were performed using Vero-76 cells in 24-well plates, with 5×10^4 cells/mL plated in 500µL one day prior to the assay. Serial 4-fold dilutions of patient sera, starting at 1:10, were incubated with 30µL of Nicaraguan DENV-1 (N1265) or DENV-3 (N7236) isolates for 1 h at 37°C. Two hundred and fifty µL of medium was aspirated from the wells of the 24-well plates, and 25µL of virus/serum mixture was added to duplicate wells. Following 1-1.5 h of incubation at 37°C, 0.5 mL overlay (2% carboxymethylcellulose, 1X α-MEM, 2% FBS) was added per well. Foci were developed 6 days post-infection by fixing in ice-cold 10% methanol for 10 min, blocking in 5% non-fat dry milk (NFDM) in PBS for 10 min, incubating with monoclonal antibody 4G2 (1µg/mL) directed against DENV E protein for 45 min, washing 2X with PBS, incubating with secondary antibody (goat anti-mouse antibody conjugated to HRP; Jackson Immunoresearch) at a dilution of 1:1000 in 5% NFDM/PBS for 40 min, washing 2X in PBS, and developing foci with ~300µL/well colorimetric substrate (TrueBlue Peroxidase Substrate; KPL) for ~10 min. Foci from duplicate wells were counted and averaged, and relative infection was calculated as compared to average duplicate 'virus only' positive control wells processed on each plate.

SUPPLEMENTAL FIGURES

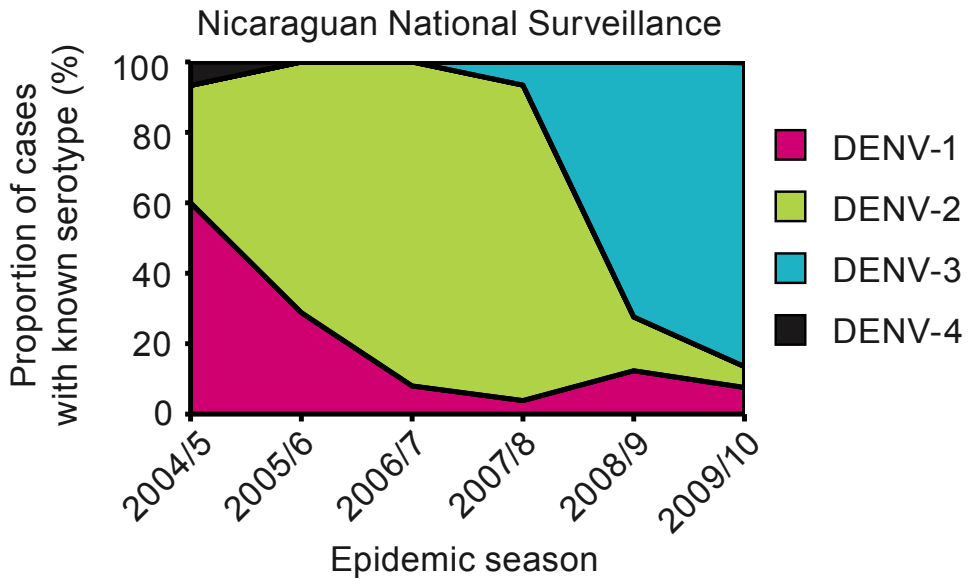
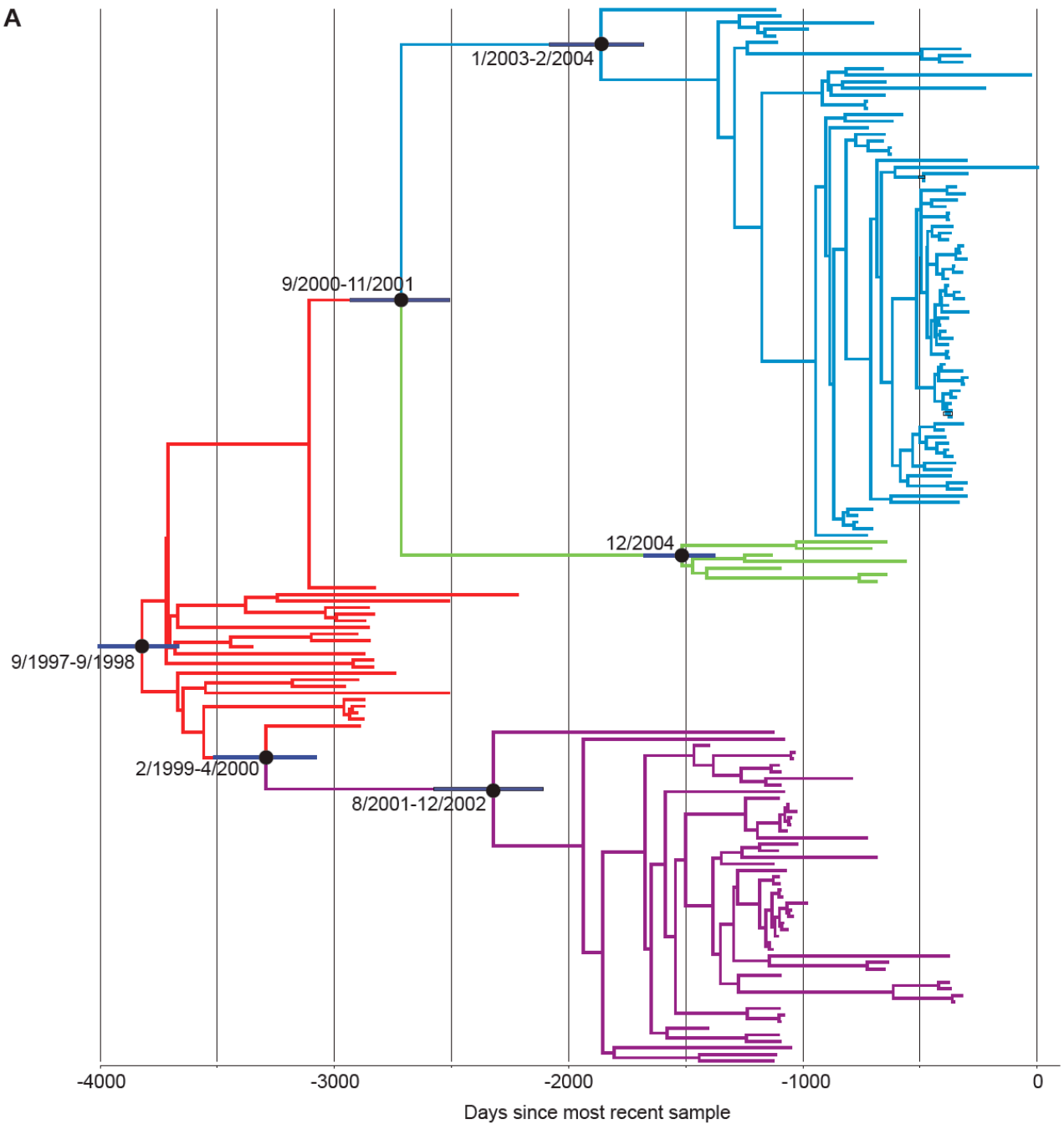
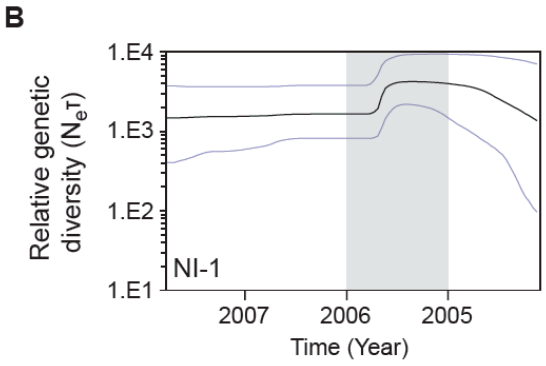


Fig. S1. National Dengue Surveillance data in Managua, Nicaragua. Data available from the Ministry of Health in Nicaragua on the serotype of dengue-positive cases in Managua, Nicaragua, during the same years in which the data from our studies was derived. Serotypes are colored as in Figure 1.


A
1/2003-2/2004
9/2000-11/2001
12/2004
9/1997-9/1998
2/1999-4/2000
8/2001-12/2002
-4000
-3000
-2000
-1000
0
Days since most recent sample


B
Relative genetic diversity (N_eT)
1.E4
1.E3
1.E2
1.E1
NI-1
2007
2006
2005
Time (Year)

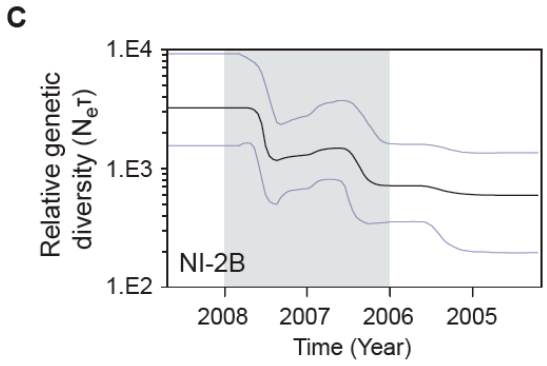

C
Relative genetic diversity (N_eT)
1.E4
1.E3
1.E2
NI-2B
2008
2007
2006
2005
Time (Year)

Fig. S2. Nicaraguan DENV-2 evolution. **(A)** Maximum clade credibility (MCC) tree of 159 full-length DENV-2 genomes sampled between 1999 and 2008 in Nicaragua. Only nodes with a posterior probability of ≥0.60 are shown. Major clades are colored as in Figure 2. Time (x-axis) is measured in days from the most recent sample (day 0; sampled on 2008.09.24). **(B and C)** Bayesian skyline plots, showing changes in relative genetic diversity through time ($N_e\tau$) for clade NI-1 (*N*=50) (**B**) and NI-2B (*N*=80) (**C**). Gray shading indicates seasons where each clade was dominant. A decline in clade NI-1 population diversity is observed concurrently with an increase in clade NI-2B population diversity.

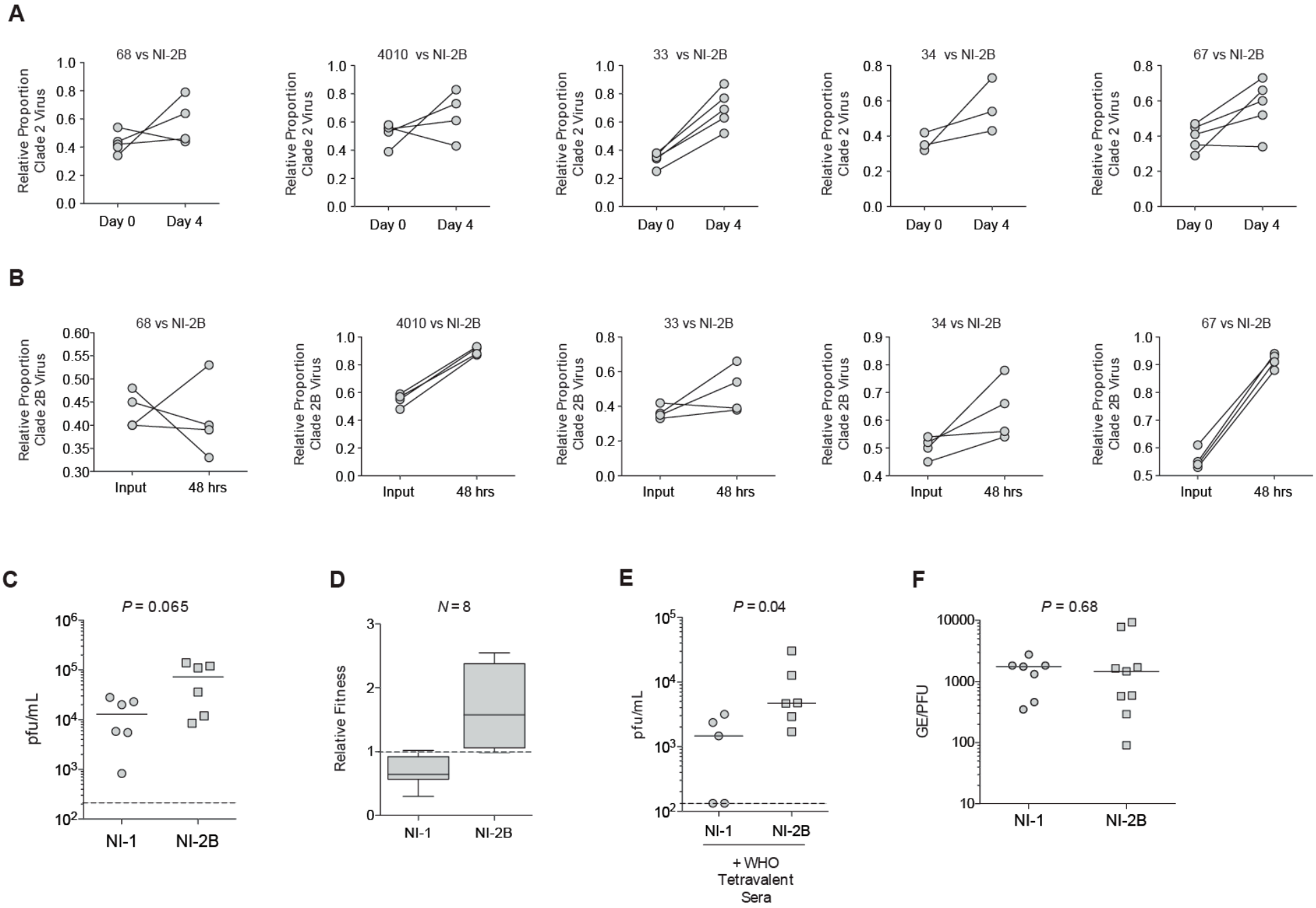

A
68 vs NI-2B
4010 vs NI-2B
33 vs NI-2B
34 vs NI-2B
67 vs NI-2B
Relative Proportion Clade 2 Virus
Day 0
Day 4
B
68 vs NI-2B
4010 vs NI-2B
33 vs NI-2B
34 vs NI-2B
67 vs NI-2B
Relative Proportion Clade 2B Virus
Input
48 hrs
C
P = 0.065
pfu/mL
NI-1
NI-2B
D
N = 8
Relative Fitness
NI-1
NI-2B
E
P = 0.04
pfu/mL
NI-1
NI-2B
+ WHO Tetravalent Sera
F
P = 0.68
GE/PFU
NI-1
NI-2B

Fig. S3. Fitness of NI-1 and NI-2B isolates in vitro. (A, B) Competition of NI-1 viral isolates ("68", "4010", "33", "34" and "67") with NI-2B viruses in C6/36 cells (**A**) and iDCs (**B**). The relative proportion of the NI-2B viruses as compared to the NI-1 virus is plotted at the time of infection (Day 0) and the time of collection of collection of viral supernatants (**A**: Day 4 for C6/36 cells; **B**: 48 h for iDCs). **(C and D**) In vitro replication of NI-1 and NI-2B isolates in a human monocytic cell line stably-expressing DC-SIGN, U937/DC-SIGN. *P*-values were calculated using the Mann Whitney test. (**C**) Viral output (pfu/mL) 48 h after infection with six NI-1 (circles) and six NI-2B viruses (squares). (**D**) Pairwise competition of two NI-2B viruses with four NI-1 viruses, with relative fitness calculated from relative proportions of each virus after 48 h in culture. (**E**) Antibody-mediated infection of human K562 cells, a cell line only infectable in the presence of enhancing antibody. A 1:30 dilution of WHO tetravalent serum was incubated with five NI-1 viruses (circles) and six NI-2B viruses (squares) for 1 h prior to infection of K562 cells. **(F)** The Genome Equivalent (GE) to Plaque-Forming Unit (PFU) ratio was compared for seven NI-1 and nine NI-2B viral isolates ($P = 0.68$; Mann-Whitney).

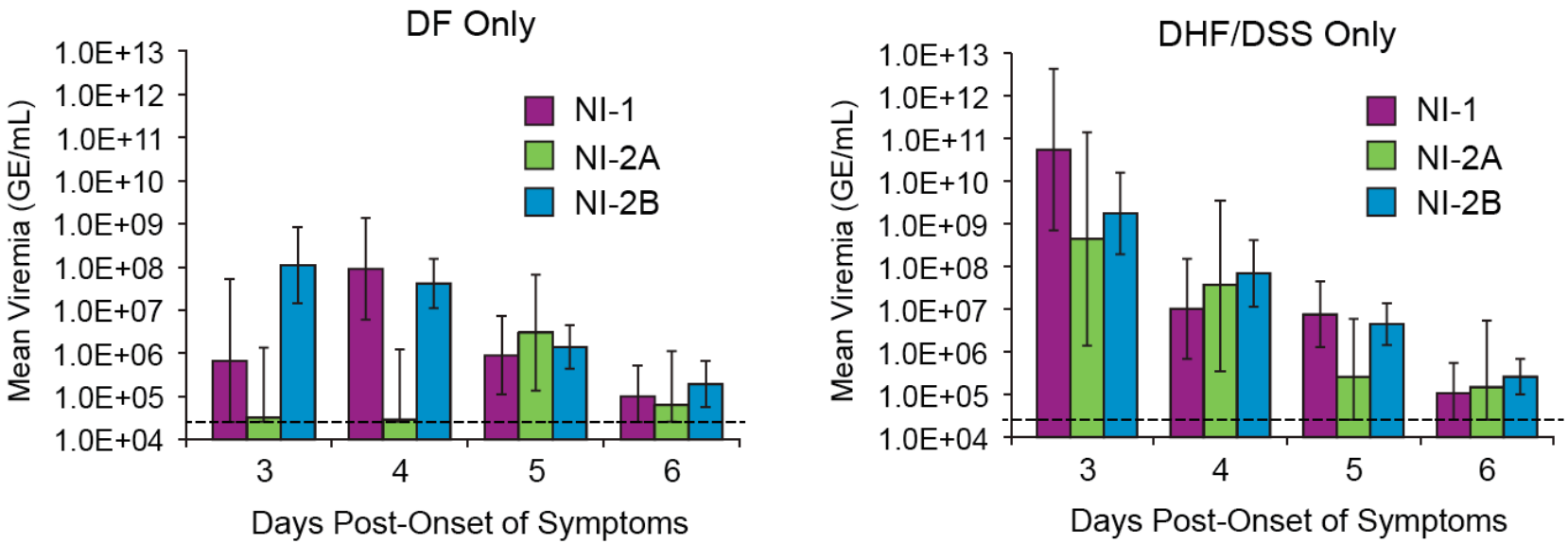


Fig. S4. Patient viremia by clade and day post-onset of symptoms in DF and DHF/DSS cases. Viremia levels in children with NI-1, NI-2A or NI-2B infections in sera collected on days 3 to 6 post-onset of symptoms. *N* =202 samples from 116 patients. GE were determined using qRT-PCR directed to NS5. Shown are estimated mean values with 95% confidence intervals. The limit of detection of the assay (2.6 x 10^4 GE/mL sera) is shown with a dashed line. Error bars were adjusted to the limit of detection. Mean viremia (GE/mL) is shown for DF cases only (left panel) and for DHF/DSS cases only (right panel) by clade (NI-1, white bars; NI-2A, light gray bars; NI-2B, dark gray bars) (P = 0.0003, mixed effects repeated measures model, comparing the effect of the interaction of day of sample, disease severity, and clade with "no effect"). Clades are colored as in Figure 4.

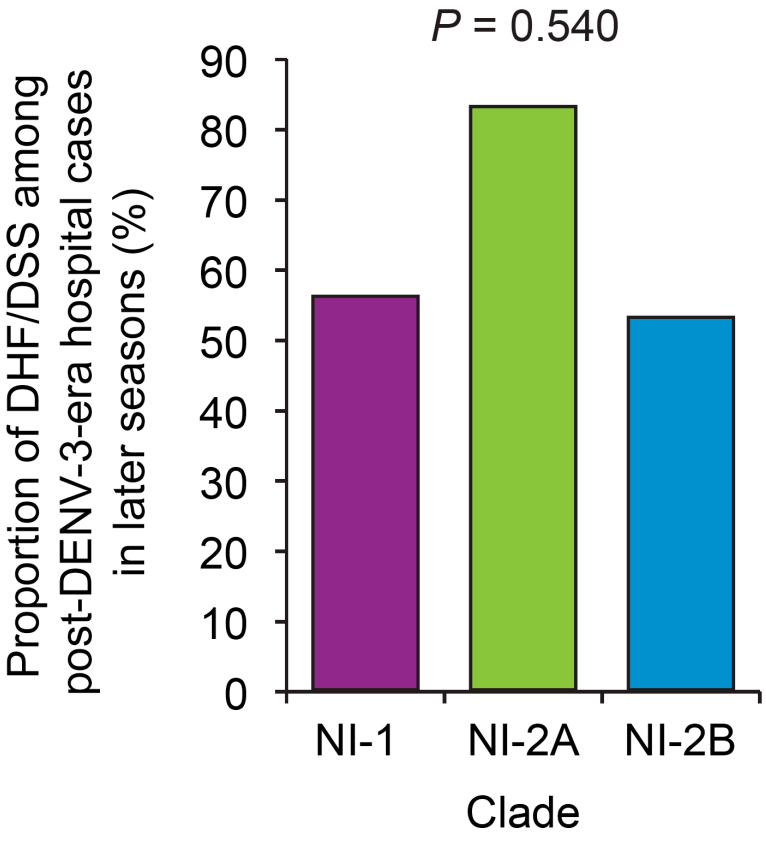


Fig. S5. Cases of severe dengue (DHF/DSS) by clade in "post-DENV-3-era" Hospital cases in later seasons. P = 0.54; Fisher's exact test, comparing the proportion of DENV-2 Hospital cases in the later seasons (2006/7–2008/9) that are DHF/DSS, NI-1 versus NI-2A versus NI-2B, among children from the "post-DENV-3-era" (1999+) birth group.

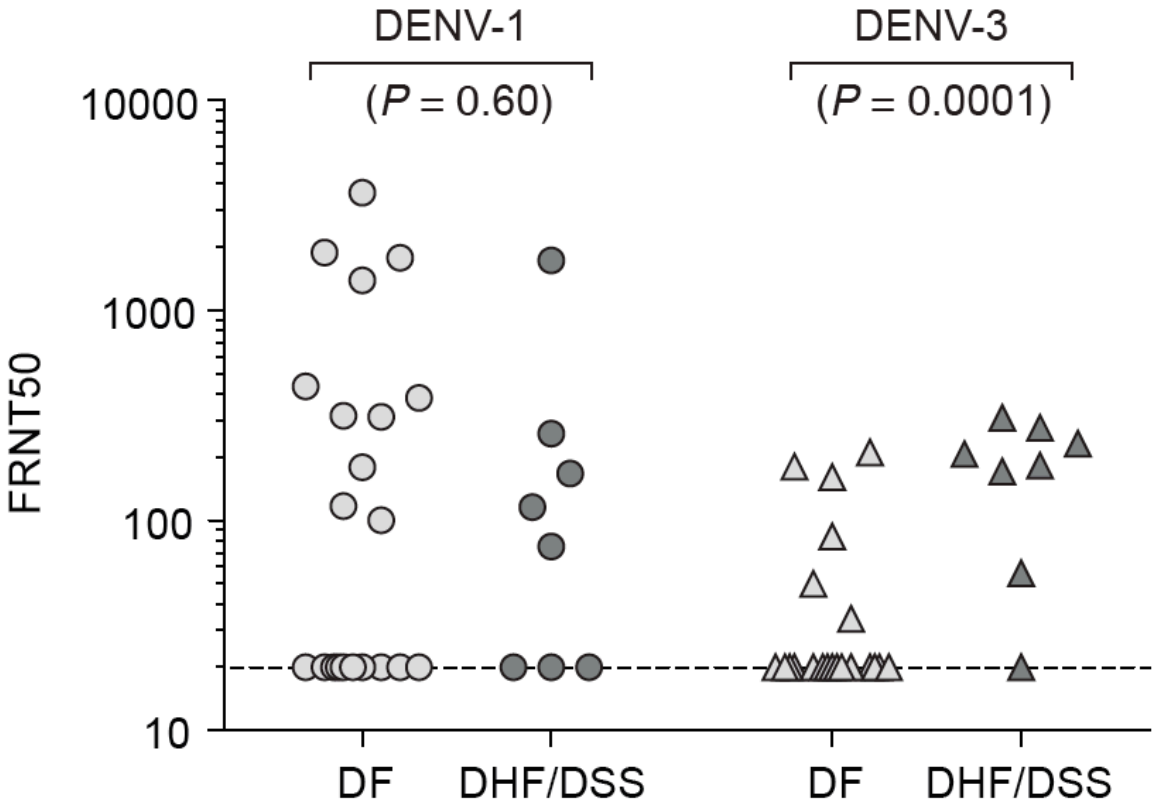


Fig. S6. Focus Reduction Neutralization Test (FRNT) analysis of Cohort sera using Nicaraguan DENV isolates. DENV-1, circles, $P = 0.60$; DENV-3, triangles, $P = 0.0005$; Wilcoxon Rank-Sum test, comparing FRNT titers in DF versus DHF/DSS cases in a nested case control study within the Hospital study.