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Supplemental Information

Intrahost Selection Pressures Drive Rapid Dengue Virus Microevolution in Acute Human Infections

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Title

Intrahost selection pressures drive rapid dengue virus microevolution in acute human infections

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Running Title

Intrahost virus microevolution in human dengue

Figure S1

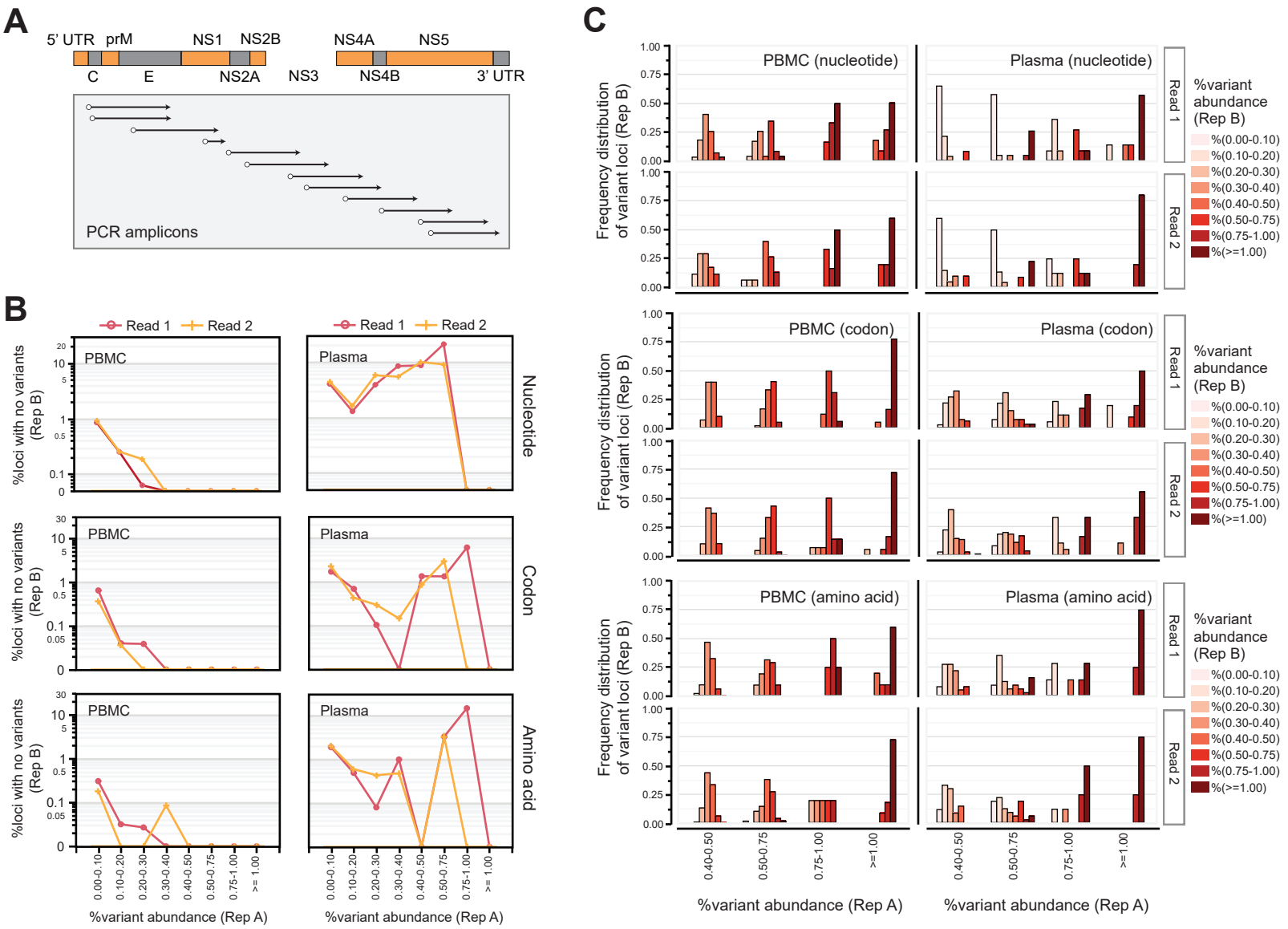

A
5' UTR
prM
NS1
NS2B
NS4A
NS5
C
E
NS2A
NS3
NS4B
3' UTR
PCR amplicons
B
Read 1
Read 2
PBMC
Plasma
Nucleotide
Codon
Amino acid
%loci with no variants (Rep B)
0.00-0.10
0.10-0.20
0.20-0.30
0.30-0.40
0.40-0.50
0.50-0.75
0.75-1.00
>= 1.00
%variant abundance (Rep A)
C
PBMC (nucleotide)
Plasma (nucleotide)
PBMC (codon)
Plasma (codon)
PBMC (amino acid)
Plasma (amino acid)
Frequency distribution of variant loci (Rep B)
Read 1
Read 2
%variant abundance (Rep B)
%(0.00-0.10)
%(0.10-0.20)
%(0.20-0.30)
%(0.30-0.40)
%(0.40-0.50)
%(0.50-0.75)
%(0.75-1.00)
%(>=1.00)
0.40-0.50
0.50-0.75
0.75-1.00
>=1.00
%variant abundance (Rep A)

Figure S2

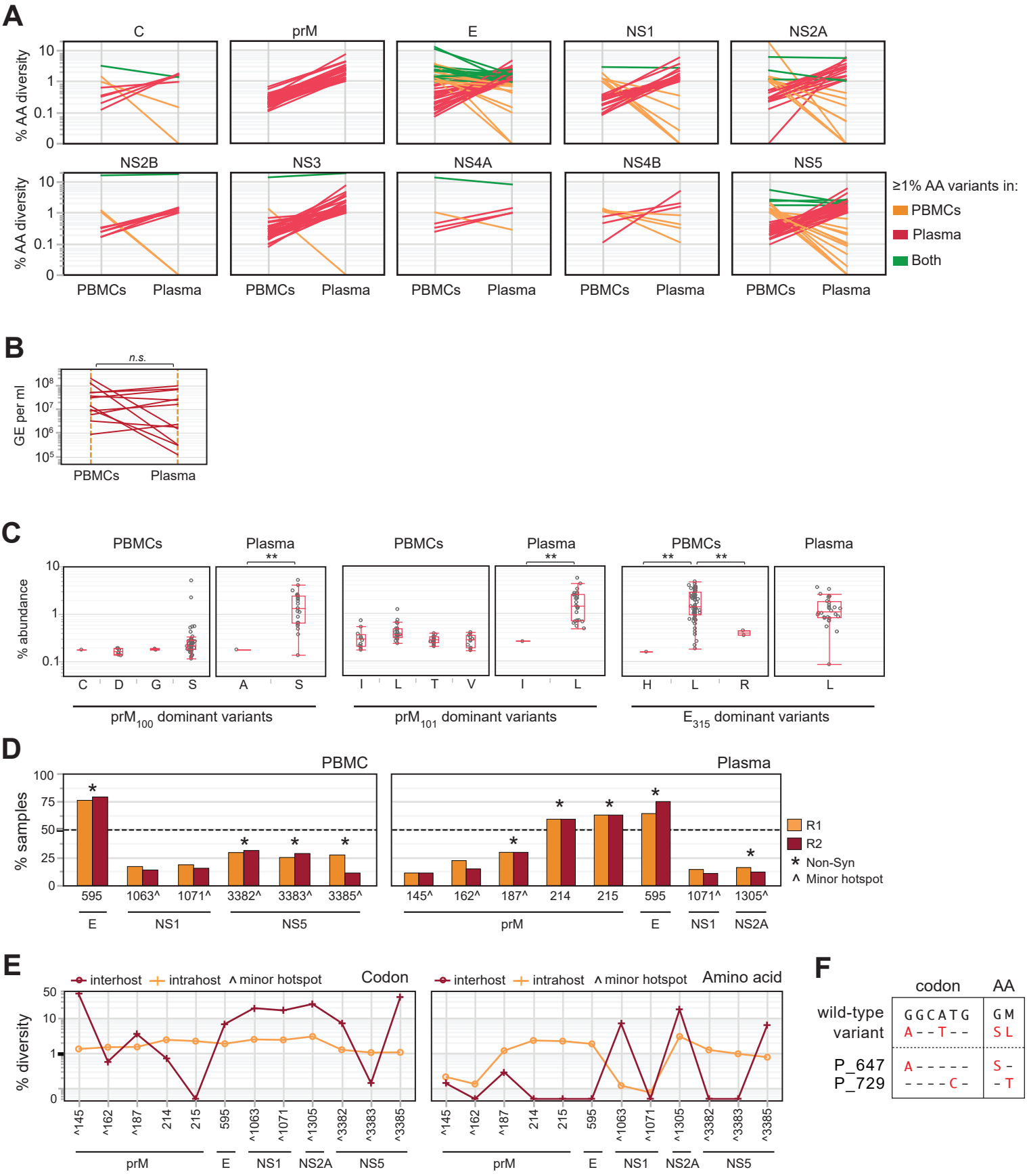

A
C
prM
E
NS1
NS2A
NS2B
NS3
NS4A
NS4B
NS5
% AA diversity
PBMCs
Plasma
≥1% AA variants in:
PBMCs
Plasma
Both
B
n.s.
GE per ml
C
% abundance
**
C D G S A S
I L T V I L
H L R L
prM100 dominant variants
prM101 dominant variants
E315 dominant variants
D
PBMC
Plasma
% samples
595 1063^ 1071^ 3382^ 3383^ 3385^
145^ 162^ 187^ 214 215 595 1071^ 1305^
E NS1 NS5
prM E NS1 NS2A
R1
R2
Non-Syn
Minor hotspot
E
interhost
intrahost
minor hotspot
Codon
Amino acid
% diversity
^145 ^162 ^187 214 215 595 ^1063 ^1071 ^1305 ^3382 ^3383 ^3385
prM E NS1 NS2A NS5
F
codon
AA
wild-type
variant
GGCATG
A--T--
GM
SL
P_647
P_729
A-----
----C-
S-
-T

Figure S3

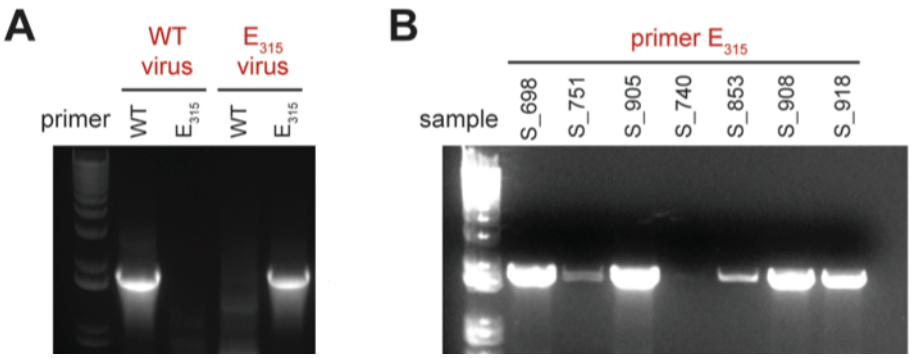

A
WT virus
E315 virus
primer
WT
E315
WT
E315
B
primer E315
sample
S_698
S_751
S_905
S_740
S_853
S_908
S_918

Figure S4

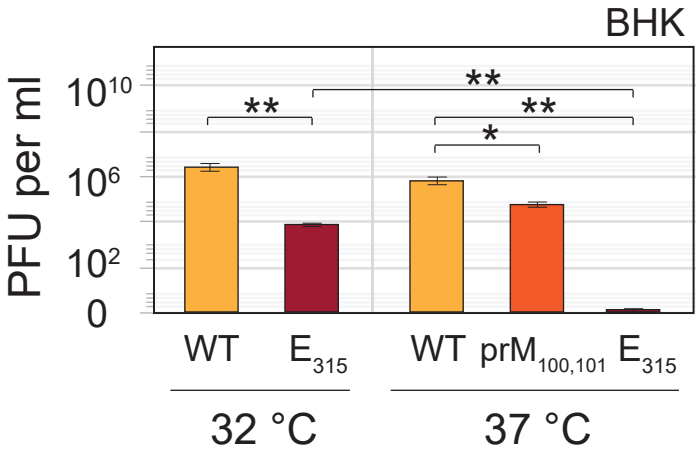
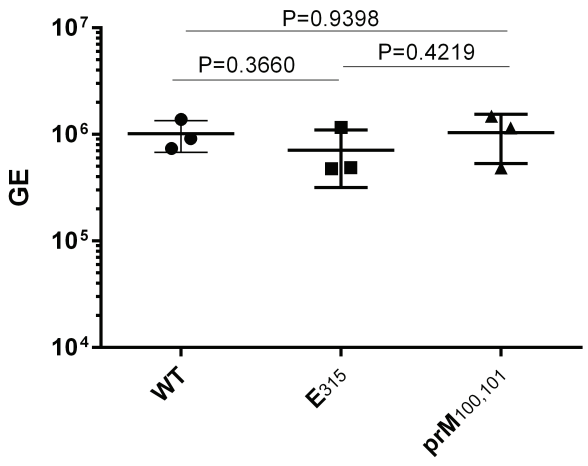


Figure S5



Supplemental Figure Legends

Figure S1. Defining thresholds for intrahost DENV-3 diversity, related to STAR Methods. (A) Sequence-directed capture of intrahost diversity in the DENV-3 coding region using twelve overlapping PCR amplicons. **(B)** For each subset of loci associated with discrete categories of percent variant diversity in replicate A (x-axis), the percent loci in replicate B exhibiting no variants is shown (y-axis). Data is pooled from replicate datasets for four PBMC (*left panels*) and three plasma (*right panels*) samples. Variant diversity is binned by read orientation (read 1 – red and read 2 - yellow), and by type of locus - nucleotide (*top panel*), codon (*middle panel*) or amino acid (*bottom panel*). The threshold for variant detection was chosen based on the minimum percent variant diversity in replicate A at which variants were also detected in replicate B (i.e., the % loci with no variants in replicate B equaled zero), for both reads 1 and 2. **(C)** Frequency distributions of loci in replicate B (y-axis) categorized by percent variant diversity (shades of red), for different cutoffs of percent variant diversity in replicate A (x-axis). Variant diversity is shown for replicate data from four PBMC (*left panels*) and three plasma (*right panels*) samples, and is binned by read orientation (read 1 or read 2) and type of locus - nucleotide (*top panel*), codon (*middle panel*) or amino acid (*bottom panel*).

Figure S2. Diversity profiles in PBMC and plasma samples at the gene and codon levels, related to Figure 1-3. (A) Gene-specific comparisons of percent amino acid variants in paired PBMC and plasma samples, colored by whether individual loci display ≥1% AA diversity in PBMC samples only (yellow), in plasma samples only (red), or in both PBMC and plasma samples (green). **(B)** Comparisons of genome equivalents per milliliter (GE per ml) of extracted RNA in paired PBMC and plasma samples. '*n.s.*' non-significant (Wilcoxon signed-rank test for paired samples). **(C)** Percent abundance of dominant non-synonymous variants observed in PBMC and plasma samples at the prM_{100}, prM_{101} and E_{315} hotspot loci, median ± SD, boxes represent 25^{th} and 75^{th} percentiles, and whiskers are 10^{th} and 90^{th} percentiles. ***P*<0.0001 (Wilcoxon test for unpaired samples). **(D)** All hotspot loci (i.e., loci with ≥1% variant abundance in ≥10% of samples) in PBMC (*left panel*) and plasma (*right panel*) samples in

both read orientations (read 1 in yellow, and read 2 in red). Locus numbering reflects codon position in the DENV-3 polyprotein such that the dominant hotspots prM_{100}, prM_{101} and E_{315} are assigned codon numbers 214, 215 and 595, respectively. Hotspots associated with non-synonymous variants (i.e., mutations that change amino acid sequence) are highlighted with asterisks (*). The dotted line indicates the threshold for identifying the prominent hotspots (i.e., presence of hotspot in ≥50% of samples) in prM and E. Minor hotspot variants are indicated with '^'. **(E)** Average percent intrahost (red) and percent interhost (yellow) diversity in codon (*left panel*) or amino acid (*right panel*) coordinates for each hotspot locus. Interhost diversity was calculated across all global DENV-3 isolates for which full-genome sequences were available (*n* = 690). Minor hotspot variants are indicated with '^'. **(F)** Intermediate and/or alternate nucleotide and amino acid variants for the $prM_{100,101}$ hotspot loci.

Figure S3. Detection of E_{315} variant virus using Reverse Transcription and PCR (RT-PCR), related to Figure 2. (A) RT-PCR using reverse primers specific for wild-type virus (WT) or E_{315} variant virus (E_{315}) yielded an amplified product that was 1865 nucleotide (nt) in length, from WT virus RNA template only, or E_{315} variant virus RNA template only, respectively. Both WT and E_{315} viruses were generated from infectious clones. **(B)** RT-PCR performed using the reverse primer specific for E_{315} variant virus (primer E_{315}) on RNA templates extracted from a randomly-chosen subset of 7 serum samples, yielded amplified product of 1865 nt from all samples.

Figure S4. Phenotype of wild-type, $prM_{100,101}$ and E_{315} variant viruses, related to Figure 4. Plaque forming units per milliliter (PFU/ml) of C6/36-passaged WT and E_{315} viruses at 32°C and 37°C, and $prM_{100,101}$ virus at 37°C, in BHK-21 cells. No plaques were detected for the E_{315} variant virus at 37°C. Data represent the mean ± SD. *P<0.01 and **P<0.0001 (t-test).

Figure S5. Entry capabilities of wild-type, $prM_{100,101}$ and E_{315} variant viruses, related to Figure 4. Entry assays were performed by incubating the WT, E_{315} variant, and $prM_{100,101}$ variant viruses with U937 cells at 37°C for two hours, followed by extensive washing. Viruses were quantified by qRT-PCR

and the Genome Equivalent (GE) values were plotted on the Y-axis, mean ± SD. *P*-values for the various comparisons were computed using a *t*-test.

Supplemental Tables

Table S1. Percent coverage across the DENV-3 genome for each patient sample, related to Table 1.

Sample type	Patient ID^#	Age in Years	Immune status	Disease severity	Sex	Sample collection date	% coverage (read 1)*	Average codon coverage (read 1)*	% coverage (read 2)*	Average codon coverage (read 2)*
PBMC	645	1	Primary	DF	M	09/06/2009	98.32	24357	98.10	20514
PBMC	698	7	Primary	DF	M	09/17/2009	99.71	23355	99.69	21379
PBMC	708	8	Primary	DF	F	09/25/2009	94.88	12353	94.30	10541
PBMC	716	3	Primary	DF	F	10/01/2009	98.73	7972.5	98.28	7655
PBMC	718	9	Primary	DF	F	10/04/2009	99.22	27086.5	99.22	24837.5
PBMC	729	14	Primary	DF	F	10/11/2009	99.10	19225	99.10	17433
PBMC	748	10	Primary	DF	F	09/25/2009	56.61	6716	55.50	6308
PBMC	751	10	Primary	DF	M	09/30/2009	98.33	26492	98.26	23950
PBMC	760	2	Primary	DF	M	10/05/2009	99.37	20094	99.36	18601.5
PBMC	765	5	Primary	DF	M	10/13/2009	99.72	29693.5	99.72	26486.5
PBMC	789	1	Primary	DF	M	10/13/2009	91.38	27174	90.88	25142.5
PBMC	828	5	Primary	DF	F	10/21/2009	90.18	8497	88.94	7891.5
PBMC	831	4	Primary	DF	F	10/20/2009	70.62	9696	66.54	7539
PBMC	832	3	Primary	DF	M	10/20/2009	98.13	29959	98.00	22980
PBMC	839	3	Primary	DF	F	10/23/2009	99.77	27094	99.66	22461.5
PBMC	844	2	Primary	DF	M	10/25/2009	99.49	23128	99.30	18748
PBMC	849	12	Primary	DF	M	10/26/2009	99.52	23127	99.47	19292
PBMC	905	6	Primary	DF	M	11/18/2009	99.70	20021	99.49	15207
PBMC	921	3	Primary	DF	F	11/28/2009	88.41	9714.5	87.74	8234
PBMC	922	7	Primary	DF	F	12/07/2009	88.73	8719	87.30	7408
PBMC	924	2	Primary	DF	M	12/07/2009	99.26	21616	98.99	17800.5
PBMC	927	6	Primary	DF	M	11/19/2009	99.71	18250	99.62	14752
PBMC	934	10	Primary	DF	M	11/28/2009	99.11	16879	98.93	14792.5
PBMC	938	9	Primary	DF	M	11/29/2009	99.72	32155.5	99.69	27281
PBMC	939	10	Primary	DF	F	12/07/2009	99.49	41328.5	99.43	35533.5
PBMC	944	8	Primary	DF	M	12/11/2009	99.88	22253	99.86	19311
PBMC	947	4	Primary	DF	F	12/12/2009	99.88	34318.5	99.88	30020
PBMC	950	4	Primary	DF	M	12/12/2009	98.68	32089	98.65	27836
PBMC	950R	4	Primary	DF	M	12/12/2009	98.57	28363	98.53	25061
PBMC	955	3	Primary	DF	M	12/19/2009	99.24	31680	99.24	27881.5
PBMC	955R	3	Primary	DF	M	12/19/2009	98.44	23452	98.40	21361
PBMC	956	1	Primary	DF	F	12/20/2009	99.89	34099	99.89	31041
PBMC	957	3	Primary	DF	F	12/18/2009	99.88	46762	99.88	42269.5
PBMC	957R	3	Primary	DF	F	12/18/2009	98.82	79693.5	98.81	69010.5
PBMC	1005	9	Primary	DF	M	01/17/2010	99.87	57352	99.86	50548
PBMC	1005R	9	Primary	DF	M	01/17/2010	98.67	30928	98.65	28224
PBMC	647	6	Primary	DHF	M	09/18/2009	77.77	5968	76.93	5009
PBMC	710	6	Primary	DHF	M	09/25/2009	99.52	20294	99.52	17935

PBMC	801	2	Primary	DHF	F	10/26/2009	98.58	32990.5	98.56	30312.5
PBMC	699	7	Primary	DSS	M	09/18/2009	97.54	15235	97.50	13659.5
PBMC	949	9	Primary	DSS	M	12/12/2009	99.64	22635	99.62	20165
PBMC	631	6	Secondary	DF	M	09/07/2009	66.10	6100	62.62	5751
PBMC	632	6	Secondary	DF	M	09/07/2009	98.05	18262	97.95	16261
PBMC	646	5	Secondary	DF	F	09/19/2009	95.93	5822	94.40	4982.5
PBMC	652	8	Secondary	DF	F	09/29/2009	96.80	8873	96.59	7706.5
PBMC	655	11	Secondary	DF	M	10/04/2009	65.73	6552	61.93	6103
PBMC	656	9	Secondary	DF	F	10/05/2009	99.03	18862	99.01	16160
PBMC	694	10	Secondary	DF	M	09/23/2009	98.53	23765	98.51	20980
PBMC	697	6	Secondary	DF	M	09/20/2009	99.69	15933	99.67	14163.5
PBMC	714	8	Secondary	DF	F	09/29/2009	98.24	26650	98.17	23905
PBMC	715	10	Secondary	DF	F	09/28/2009	96.94	20799	96.88	19146
PBMC	722	12	Secondary	DF	F	10/09/2009	75.16	3192	71.88	3037
PBMC	726	6	Secondary	DF	M	10/10/2009	98.94	18862	98.96	16855
PBMC	737	12	Secondary	DF	M	10/12/2009	98.98	25059	98.83	21842
PBMC	740	4	Secondary	DF	F	10/12/2009	99.44	14024	99.09	12111.5
PBMC	792	6	Secondary	DF	F	10/19/2009	57.97	6185	57.35	5689
PBMC	793	7	Secondary	DF	M	10/19/2009	99.22	20080	99.14	18858.5
PBMC	825	10	Secondary	DF	F	10/19/2009	99.63	41146	99.35	36957
PBMC	830	10	Secondary	DF	F	10/20/2009	99.24	16291.5	96.02	12751
PBMC	843	10	Secondary	DF	M	10/24/2009	95.98	44036	95.95	38892
PBMC	853	12	Secondary	DF	F	10/26/2009	99.72	22125	99.59	17492
PBMC	918	8	Secondary	DF	F	11/16/2009	99.12	14107	98.73	11335
PBMC	946	7	Secondary	DF	F	12/12/2009	99.71	22971.5	99.70	19424
PBMC	989	5	Secondary	DF	M	01/27/2010	99.59	11506.5	99.50	10864
PBMC	728	12	Secondary	DHF	M	10/09/2009	88.70	14226	88.67	12810
PBMC	731	5	Secondary	DHF	F	10/10/2009	86.95	36923	86.83	33841
PBMC	833	10	Secondary	DHF	M	10/22/2009	99.57	28447.5	99.43	23109.5
PBMC	848	8	Secondary	DHF	M	10/26/2009	99.58	22216.5	99.36	16733.5
PBMC	937	12	Secondary	DHF	M	11/29/2009	99.72	25700	99.71	21772
PBMC	959	4	Secondary	DHF	F	01/04/2010	98.59	26513.5	98.54	23619
PBMC	717	12	Secondary	DSS	F	10/04/2009	99.26	37144	99.26	30921
PBMC	847	10	Secondary	DSS	F	10/24/2009	97.57	28295	97.56	23012
Plasma	698	7	Primary	DF	M	09/17/2009	99.32	30860	99.04	24554
Plasma	718	9	Primary	DF	F	10/04/2009	99.65	22817.5	99.63	20161
Plasma	751	10	Primary	DF	M	09/30/2009	97.55	8484.5	97.45	7150
Plasma	763	7	Primary	DF	F	10/07/2009	88.76	15506	88.76	13640
Plasma	832	3	Primary	DF	M	10/20/2009	85.72	9447	77.49	19887
Plasma	844	2	Primary	DF	M	10/25/2009	99.34	28214	99.32	22459.5
Plasma	849	12	Primary	DF	M	10/26/2009	76.70	7388.5	76.56	5946.5
Plasma	863	6	Primary	DF	F	10/31/2009	50.75	41668	50.72	30857
Plasma	887	2	Primary	DF	M	11/09/2009	51.42	56349	51.35	48253
Plasma	897	4	Primary	DF	F	11/15/2009	97.34	11099	96.92	9293
Plasma	905	6	Primary	DF	M	11/18/2009	99.67	25622	99.67	23884
Plasma	909	5	Primary	DF	M	11/02/2009	97.46	40600.5	97.43	36203
Plasma	927	6	Primary	DF	M	11/19/2009	99.77	15265	99.71	13156

Plasma	938	9	Primary	DF	M	11/29/2009	99.92	26900.5	99.90	22439
Plasma	939	10	Primary	DF	F	12/07/2009	99.74	21931	99.72	20123
Plasma	944	8	Primary	DF	M	12/11/2009	99.82	36336	99.82	33207
Plasma	646	5	Secondary	DF	F	09/19/2009	99.80	9317	99.75	7654.5
Plasma	656	9	Secondary	DF	F	10/05/2009	99.79	23477	99.72	19973
Plasma	697	6	Secondary	DF	M	09/20/2009	89.64	29521	89.35	25767
Plasma	722	12	Secondary	DF	F	10/09/2009	99.47	21656	99.44	18554.5
Plasma	722R	12	Secondary	DF	F	10/09/2009	92.62	13972	91.91	12277.5
Plasma	737	12	Secondary	DF	M	10/12/2009	88.33	14093	88.27	11744
Plasma	740	4	Secondary	DF	F	10/12/2009	95.24	10473	92.92	9816
Plasma	742	9	Secondary	DF	F	10/13/2009	98.52	19365.5	98.43	16830
Plasma	830	10	Secondary	DF	F	10/20/2009	51.28	41408	51.33	36802
Plasma	853	12	Secondary	DF	F	10/26/2009	99.42	83233	99.35	73919
Plasma	868	13	Secondary	DF	M	11/02/2009	84.87	58334	83.43	51339
Plasma	908	9	Secondary	DF	F	11/04/2009	93.88	18502	91.27	16125
Plasma	918	8	Secondary	DF	F	11/16/2009	99.95	43136	99.96	40261
Plasma	936	14	Secondary	DF	M	11/30/2009	98.50	31324.5	98.50	27792
Plasma	936R	14	Secondary	DF	M	11/30/2009	99.71	28621.5	99.71	26402
Plasma	937	12	Secondary	DHF	M	11/29/2009	99.82	22978.5	99.81	20074
Plasma	847	10	Secondary	DSS	F	10/24/2009	99.74	27811	99.71	24865.5
Plasma	847R	10	Secondary	DSS	F	10/24/2009	99.90	57468	99.90	51485

^The suffix ‘R’ denotes replicate sample used in defining thresholds for percent intrahost diversity.

[#]All samples used for this study were from individuals who presented with DENV-3 infection during the 2009-2010 epidemic season in Nicaragua. Over 100 other clinical and laboratory parameters are available for these samples, but not shown due to space constraints.

*Only nucleotide loci with coverage of ≥1000 reads and locus-specific Sanger quality scores of ≥30 were considered for calculating genome coverage. Samples with ≥50% genome coverage are reported.

Table S2. Interhost diversity at hotspots for intrahost diversity, related to Figure 2.

Codon position	Primary[#] codons (intrahost)	Variant* codons (intrahost)	Primary[#] codons (interhost^)	Variant* codons (interhost^)	Primary[#] AA (intrahost)	Variant* AA (intrahost)	Primary[#] AA (interhost^)	Variant* AA (interhost^)
145	ATT, ATC	ATC, ATT	ATC	ATT, (ACT)	I	V, (T)	I	T
162	ACG, ACA	ACA, ACG	ACG	ACA	T	A	T	-
187	ACA	GCT, GCA, (ACT)	ACA	ACG, (GCA, GCG)	T	A	T	A
214	GGC	AGC	GGC	GGT	G	S	G	-
215	ATG	TTG	ATG	-	M	L	M	-
595	CAT	CTT	CAT	CAC	H	L	H	-
1063	AAT, AAC	AAC, AAT	AAC	AAT, AGC, (GAC, GAT)	N	S, (K, D, H, I, T)	N	S, D
1071	TTA, TTG	TTG, TTA	TTG	CTG, TTA	L	F, S, (*, V, I)	L	-
1305	GTG, ATG	GCG, GTG, ATG, GTA	GTG	CTG, ATG, (TTG, GTA)	V, M	A, M, V	V	L, M
3382	GAG	GGG	GAG	GAA	E	G	E	-
3383	GAG	GGG	GAG	GAA	E	G	E	-
3385	TCG	TTG, TCA	TCG	TCA, TTG, (ACA, TTA, CCA)	S	L	S	L, T, (P)

^For calculating interhost diversity, all available full-length DENV-3 genomes from global isolates (*n* = 690) were used

[#]Codons and amino acids with the highest prevalence are listed as ‘primary’

*Minor codons and amino acids are listed as ‘variant’ (in decreasing order of prevalence)

Table S3. Frequencies of various haplotypes linked to variants at the $prM_{100,101}$ and E_{315} hotspot loci, related to Figure 3.

Variant	Haplotypes linked to variant	Frequency (predicted)^	Frequency (observed)[#]	Number of samples with variant haplotype identical to wild-type consensus haplotype (percent of all samples with variant haplotype)
$prM_{100,101}$	C-G-C	0.702	0.688	3 (100%)
$prM_{100,101}$	T-G-C	0.170	0.195	2 (100%)
$prM_{100,101}$	C-A-C	0.093	0.104	2 (100%)
$prM_{100,101}$	C-A-T	0.001	0.013	1 (100%)
E_{315}	G-G-A	0.533	0.636	24 (100%)
E_{315}	A-G-A	0.304	0.195	7 (100%)
E_{315}	A-A-A	0.040	0.117	4 (100%)
E_{315}	A-G-G	0.017	0.052	4 (100%)

^Product of the frequencies of alleles at each of the three loci, calculated from Nicaraguan consensus DENV-3 genomes used in this study.

[#]Frequencies of haplotypes observed in Nicaraguan consensus DENV-3 genomes used in this study.

Table S4. Sequences of primers used for amplifying the DENV-3 genome, related to STAR Methods.

Sequence	Description	Tm	Amplicon#	Comments
GACTCGGAAGCTTGCTTAAC	DENV3_32_F	62.00	DENV3-Amplicon1	
TGATCCAAAGTCCCAAGCTG	DENV3_2196_R	61.18		
TCAATATGCTGAAACGCGTG	DENV3_128_F	60.81	DENV3-Amplicon2	
TGATCCAAAGTCCCAAGCTG	DENV3_2196_R	61.18		
AAGCTATGCATTGAGGGGAA	DENV3_1106_F	60.04	DENV3-Amplicon3	
GTATCGCAAGGGAGGRAGTG	DENV3_3378_R	59.69		
CATATGGCTGAAACTCCGAG	DENV3_2907_F	58.33	DENV3-Amplicon4	
CCAAGCACAAGACACCCATT	DENV3_3511_R	60.95		
GGARGTGGAAAGGTGGACAA	DENV3_3466_F	59.94	DENV3-Amplicon5	R=A or G
ATRCTGGCTGGGTCTGTGAA	DENV3_5393_R	58.16		
TTGACTGTTGCCTGGAGAAC	DENV3_3946_F	58.85	DENV3-Amplicon6	
CGTCTATGGCGGCYGACTT	DENV3_6073_R	59.46		
AAACGAACGCAGAACCAGAT	DENV3_5009_F	59.74	DENV3-Amplicon7	
GCGTACAATGTCCAGGCTGA	DENV3_6941_R	60.47		
CTCGTGTTGGAATGGGAGAG	DENV3_5417_F	60.17	DENV3-Amplicon8	
ACAYAAGGCCCATGATGTTC	DENV3_7389_R	60.79		
GTCAATCRCCCTTGATCTTG	DENV3_6366_F	60.60	DENV3-Amplicon9	R=G or A
ATGTTACCTGTRCCATTGGA	DENV3_8240_R	58.85		
CGGTRGATGGGATAATGACA	DENV3_7253_F	61.53	DENV3-Amplicon10	R=G or A
CTCCGGGTATCTTGGAAATG	DENV3_9139_R	59.38		
ATCCAATGGYACAGGTAACA	DENV3_8220_F	58.85	DENV3-Amplicon11	Y=T or C
GATTGTCCTCGATCCACACC	DENV3_10024_R	60.33		
ATGGATCTTATGAAGTCAAAGC	DENV3_8478_F	58.86	DENV3-Amplicon12	
ACTGTGGCTTAATRTGGCCT	DENV3_10302_R	59.62		