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ORIGINAL ARTICLE

Population dynamics of DENV-1 genotype V in Brazil is characterized by co-circulation and strain/lineage replacement

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Abstract Following successive outbreaks of dengue fever caused predominantly by dengue virus (DENV) 2 and 3, DENV-1 is now the primary serotype circulating in Brazil. We sequenced and analyzed Brazilian DENV-1 genomes and found that all isolates belong to genotype V and are subdivided into three lineages, which were introduced during four different events. The first introduction occurred in 1984-85, the second in 1997-99, and the third and fourth occurred from 2004 to 2007. These events were associated with an increase in genetic diversity but not with positive selection. Moreover, a potential new recombinant strain derived from two distinct lineages was detected. We demonstrate that the dynamics of DENV-1 in Brazil is characterized by introduction, movement, local evolution, and lineage replacement. This study strengthens the relevance of genotype surveillance in order to identify, trace, and control virus populations circulating in Brazil and Latin America.

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Introduction

The species *Dengue virus* (DENV) includes four genetically and antigenically distinct serotypes, termed DENV-1 through DENV-4, that are members of the family *Flaviridae*, genus *Flavivirus*. As with other flaviviruses, DENV has a single-stranded positive-sense RNA genome of 10,700 nucleotides that is surrounded by a nucleocapsid and covered by a lipid envelope containing viral glycoproteins. The RNA genome contains a single open reading frame that encodes a precursor polyprotein, which is co- and post-translationally cleaved into three structural (C, prM and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins [1]. Infection by any DENV serotype can cause a wide range of clinical symptoms that vary from an acute self-limited febrile illness, the classic dengue or dengue fever (DF), to life-threatening syndromes characterized by hemorrhage and capillary leakage, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [1, 2]. DENV causes 25 to 100 million cases of DF and 250,000 cases of DHF/DSS per year worldwide. Moreover, it is estimated that 2.5 to 3 billion people annually are at risk of infection in more than 100 countries [3].

Brazil has the highest incidence of dengue cases in the Americas. In 2010, more than 1,000,000 cases of dengue were reported, with the highest number of DFH/DSS cases

(16,540 cases) and deaths (673 cases) [4, 5]. DENV-1 was initially introduced into Brazil in 1981, during an outbreak in the Northern Region. A second major outbreak in Rio de Janeiro (Southeast region) was reported in 1986, and from there, DENV-1 spread to the Northeast and Midwest regions of the country. Subsequently, DENV-2, DENV-3 and DENV-4 were introduced into Brazil [6–9].

DENV-1 was the most predominant serotype in Brazil in the 80's but was replaced by DENV-2 in the 90′s, which was subsequently replaced by DENV-3 in 2000 [4, 10]. The replacement or introduction of a new DENV serotype/genotype/lineage is usually succeeded by an increase in the number of incidences and often by substantial outbreaks [11–13]. The replacement of the predominant DENV serotypes circulating in the country results in transmission cycles during alternating years with higher and lower incidences, but always with an overall increase in the number of cases per year [10, 14]. Additionally, there has been an expansion in the range of the epidemics in each successive year, with an increase in the number of severe dengue cases, mainly in children and teenagers [15]. The largest DENV epidemics to date occurred in 2010 and were predominantly caused by DENV-1 [5].

Within the DENV-1 serotype, five genotypes have previously been reported, based on analysis of the envelope (E) gene [16, 17]. Earlier phylogenetic studies of Brazilian and American DENV-1 isolates demonstrated that Brazilian DENV-1 isolates belong to genotype V, lineage I, suggesting that only one significant event of DENV-1 introduction has occurred in the country [18, 19]. However, the appearance of more than one lineage of DENV-1 circulating in Brazil was reported recently [13]. Moreover, the detection of three recombinant isolates, including two Brazilian isolates, suggests that recombination among South American DENV-1 sequences is not a rare event in the evolution of this serotype [19].

In this study, we sequenced a DENV-1 viral genome obtained from serum of a patient diagnosed with DF from São José do Rio Preto, São Paulo, Brazil, in 2008. Phylogenetic and molecular evolutionary analyses demonstrate that different lineages of DENV-1 have been introduced and have circulated in different regions of Brazil at different times. The introduction of new strains resulted in lineage replacement and an increase in genetic diversity of DENV-1, and it also favored the occurrence of recombination events among these Brazilian isolates.

Materials and methods

Strain and sample preparation

The strain BR/BID-V3490/2008 was obtained from a serum sample in 2008 from a patient with DF from São José do Rio Preto, São Paulo, Brazil. One hundred forty µl of serum was used for RNA extraction using a QiAmp Viral RNA kit (QIAGEN). The cDNA was produced in a 20-µl reverse transcription reaction containing 1 µl Superscript III Reverse Transcriptase (Invitrogen), random hexamers (1 µl of 50 ng/µl stock); specific 3′ reverse primer (1 µl of 10 µM stock) and 5 µl of template RNA. The 5′ primer was used for specific priming of the RT reaction of samples of dengue 1. Twenty microliters of viral cDNA was diluted in 800 µl of water as template for 96 specific PCR reactions [20]. The 10-µl PCR reaction contained 3 µl of template, 0.03 µl of pfuUltra II polymerase 1 (5 U/µl) (Stratagene), 100 mM dNTPs (Applied Biosystems), and 4 µl of a mixture of forward and reverse primers (0.5 µM stock). The primers were synthesized with M13 sequence tags so that PCR amplicons could be sequenced using universal M13 forward and reverse primers. The PCR reactions produced 96 overlapping amplicons, each 500-900 nucleotides (nt) in length, which were subsequently sequenced bidirectionally using the Big Dye chemistry on ABI3730xl DNA sequencers according to Applied Biosystems protocols.

Sequence analysis

All DENV-1 complete genome sequences available in GenBank were retrieved (April, 2011) and aligned using MAFFT [21]. A maximum-likelihood tree was constructed by RAxML using the GTR (General Time Reversible) model of nt substitution with the gamma model of rate heterogeneity [22]. Also, 350 non-parametric bootstrap replicates were performed. After this initial analysis, 59 sequences were selected for posterior analyses of amino acid (aa) content of the viral protein and for phylogenetic and molecular evolutionary analysis. All complete genome sequences from Brazilian DENV-1 were analyzed here, including our new sequence and 10 other sequences available from GenBank that had not been analyzed previously (Online Resource 1). Nucleotide sequences comprising the whole coding region of DENV-1 genome (10,176 nt, from the first nt of the anchored capsid protein gene to the last nt of the NS5 gene) were used for further phylogenetic analysis. maximum-likelihood trees were constructed using DENV-1 genome sequences, using General Time Reversible and Gamma distribution (GTR+*G*) implemented in MEGA 5 [23]. Rates of variation among sites were estimated for each dataset, and six discrete gamma categories were used to model evolutionary rate differences among sites. The reliability of branching patterns was tested using 1000 bootstrap replicates. Phylogenetic analysis was also performed using 235 sequences containing 239 nt from the E and non-structural NS1 junction from the five different genotypes of DENV-1.

Analysis to detect possible recombination events among DENV-1 isolates was performed using the methods implemented in the Recombination Detection Program 3 package [24]. Initially, default parameters were used, and possible recombination events detected by at least five methods and supported by phylogenetic analysis (maximum-likelihood trees using GTR+G, with six gamma categories, 500 bootstrap replicates) were further analyzed to corroborate the results.

Estimates of the evolutionary divergence within and among different lineages were calculated based on the whole coding region of the DENV-1 genome (10,176 nt). For that, the mean distance, given by the number of base substitutions per site from all sequence pairs within or among different lineages, was computed using the Tamura-Nei model implemented in Mega 5 [23], and values were further converted into percentages. Using CODEML, implemented in PAML, different codon models were used to check if the non-synonymous (dN)/ synonymous (dS) ratio was variable among the lineages containing Brazilian strains [25].

Time of the most recent common ancestor (MRCA) for some lineages was calculated using BEAST package v.1.6.1 with Markov chain Monte Carlo algorithms (MCMC) [26]. Input files for BEAST were created with the BEAUTi graphical interface [27] based on 90 genome sequences or 99 partial sequences (2325 nt, from capsid to E gene), excluding the recombinant sequences (Online Resource 1). The calibration point was the year each strain was isolated/obtained. Different runs were performed using the nucleotide substitution model GTR+G (four gamma categories), the Bayesian skyline plot (BSL), under strict or relaxed (uncorrelated lognormal) molecular clock, estimating the nucleotide evolutionary rate or using the previous estimated rate for DENV1 of 7.50×10^{-4} substitutions per site per year, as described previously [28]. The molecular clock and rate that best fit the data was chosen by Bayes factor (BF) comparison, performed with Tracer v.1.5.0 [29]. Fifty million chains were run, and the first five million steps were discarded. The trees were sampled at each 1000 steps, resulting in 45,000 trees, which were summarized in a maximum clade credibility (MCC) tree using TreeAnotator v.1.6.1 [30], which was then visualized in FigTree v.1.3.1 [31]. Convergence of parameters was verified with Tracer, and uncertainties were addressed as the 95 % highest probability density (HPD) intervals.

Results

Sequence analysis

Analysis was first carried out using 59 complete genome sequences, and different lineages were defined based on the branching patterns of the phylogenetic tree (supported by bootstrap values ≥98 %). The phylogenetic tree revealed the presence of one main cluster that gathers most of the Latin America DENV-1 isolates (lineages 1 to 5) and another smaller cluster that contains Brazilian and Virgin Island isolates (lineage 6). The Brazilian isolates were placed within lineages L1, L3 and L6 (Fig. 1). Within the Latin America cluster, lineage L1 grouped the Brazilian isolate BR/BID-V2401/2008 and other isolates from Venezuela and Colombia. Lineage L2 grouped isolates from Nicaragua, Mexico and Venezuela. Lineage L3 grouped Brazilian DENV-1 isolates from different regions of the country, isolated from 1990 to 2001: Den1BR/90, DF01-HUB01021093, BR/97-111, BR/BID-V2378/2001, BR/01-MR, BR/97-409, BR/97-233, SB 01057805(DF02), and the Argentinian isolate ARG0028. Lineage L4 grouped isolates from Argentina and Puerto Rico, while lineage L5 grouped isolates from Puerto Rico and French Guiana (Fig. 1).

Outside the Latin America cluster, lineage L6 grouped Brazilian DENV-1 isolated from 2000 to 2008, from the Northern and Southeast regions of the country. These isolates were more closely related to an isolate from the Virgin Islands (VG/BID-V2937/1985). Moreover, BR/BID-V3490/2008 and other Brazilian isolates clustered within L6 were more closely related to isolates from Reunion Island (Reunion 191/04) and Singapore (SG/05K4147DK1/2005) than to other isolates from Latin America or Brazil (Fig. 1). The mean similarity values among Brazilian DENV-1 isolates in lineage L6 compared to Virgin Islands, Reunion and Singapore isolates were 99.26 % (±0.057), 97.16 % (±0.15) and 97.36 % (±0.12), respectively.

The estimates of evolutionary divergence within each lineage containing Brazilian and Latin American isolates ranged from 0.70 (±0.04) to 1.17 % (±0.08) (Fig. 1). The estimates of evolutionary divergence among lineages L1 to L6 ranged from 2.30 % (±0.12) to 4.50 % (±0.22) (Table 1). A large number of transitions over transversions was observed (kappa value = 11.037). The dN/dS ratio diverged among different lineages containing Brazilian and Latin America isolates, and they were all evolving under purifying selection, as observed by the omega values (dN/dS), ranged from 0.053 to 0.151 (Fig. 1). Phylogenetic analysis of partial sequences of the E and non-structural NS1 junction (239 nt) demonstrated that all Brazilian isolates clustered within genotype V (data not shown).

Thirty-one conservative and/or non-conservative amino acids (aa) substitutions were observed in the analysis of the deduced polyprotein sequences, characterizing and distinguishing the lineages containing the Brazilian and Latin American strains (Table 2). Twelve aa substitutions were unique to all isolates belonging to lineage L6 and were not observed in any other lineage from Latin America

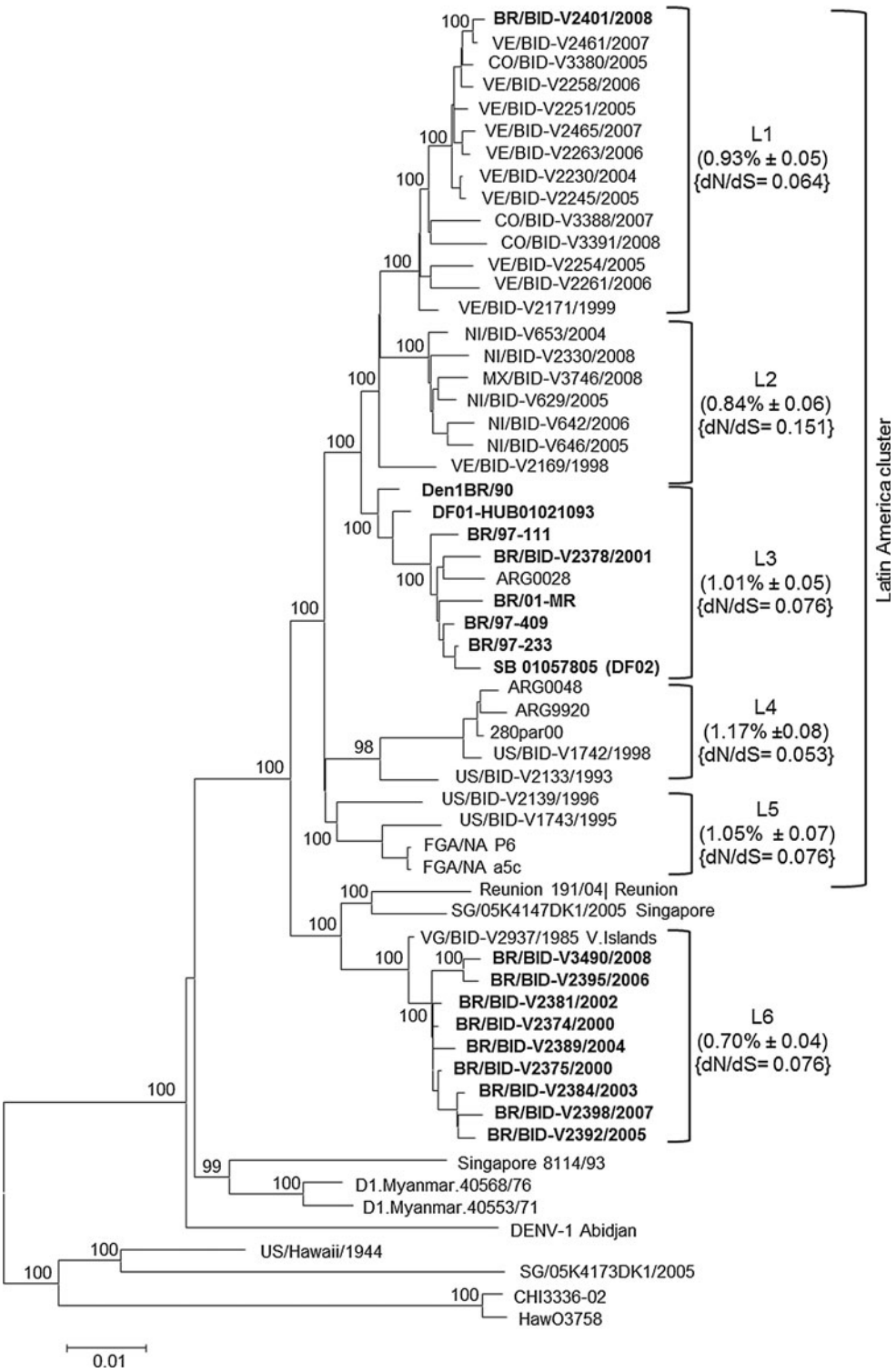

Fig. 1 Phylogenetic analysis of DENV-1 based on the complete genome sequence. The evolutionary history was inferred using the maximum-likelihood method, using the general time-reversible model for nucleotide substitution with discrete gamma distribution to model evolutionary rate differences among sites (6 categories [+ G, parameter = 0.2785]). The tree with the highest log likelihood (-42583.6960) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 59 nucleotide sequences with a total of 10,176 positions in the final dataset. A total of 1000 bootstrap replicates were run, and some values are represented as percentage in respective nodes. The evolutionary divergence (%) within each lineage is shown in parentheses, and the dN/dS ratio for each lineage is presented in brackets. The lineages and Latin America cluster are indicated, and the Brazilian DENV-1 sequences are shown in bold. Evolutionary analysis was conducted in MEGA5 [23] and PAML [25]

(Table 2). Among the Brazilian isolates, the E protein contained several aa substitutions including conservative and non-conservative substitutions at positions 33, 82, 299 (located in domain I of the E protein), 340, 348, 396 (located in domain III of the E protein), 430, 438 and 444 (located in region EH2 from the stem region of the E protein) (Table 3). All 13 cysteine residues of the E protein were conserved in all sequences, as were the asparagine residues in positions 67 and 153 (data not shown).

Recombination analysis using different methods in the RDP3 package did not detect any evidence of recombination in our isolate BR/BID-V3490/2008, but a possible new

Table 1 Estimates of evolutionary divergence among dengue virus 1 lineages from Brazil and Latin America, based on complete genomes

Lineage	**Evolutionary Divergence** (%)				
	L1	L2	L3	L4	L5
L2	2.43 (±0.13)				
L3	**4.50 (±0.22)**	3.98 (±0.21)			
L4	2.30 (±0.12)	2.31 (±0.14)	4.41 (±0.24)		
L5	3.84 (±0.18)	3.35 (±0.18)	4.45 (±0.21)	3.70 (±0.19)	
L6	**3.09 (±0.15)**	2.51 (±0.14)	**3.84 (±0.22)**	3.02 (±0.18)	3.18 (±0.15)

The mean distance is given by the number of base substitutions per site, which was determined by averaging over all genome sequence pairs within and between groups and then used to estimate evolutionary divergence expressed as percentage. Standard error estimates are shown in parentheses, and these were obtained by a bootstrap procedure (1000 replicates). The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.38). The estimates of evolutionary divergence between lineages containing Brazilian isolates are shown in bold. Analysis was conducted using the Tamura-Nei model implemented in Mega 5 [23]

recombination event was detected in the sequence of Brazilian isolate SB 01057805(DF02). Moreover, evidence of three other recombination events was identified in Brazilian isolates DF01-HUB01021093 and BR/01-MR and the Argentinian isolate ARG 9920 (Fig. 2a). It seems that SB 01057805(DF02) originated from viruses similar to BR/97-409 (assigned as the major parental strain) and to BR/BID-V2395/2006 (designated as the minor parental strain). The recombination breakpoints were located at positions 6,108 and 6,780 (considering the whole genome sequence), comprising the region containing the NS4 gene (Fig. 2b). This event was supported by different methods with the following *P*-values (using Bonferroni settings for multiple comparison correction): 4.43×10^{-12} (RDP), 5.36×10^{-11} (GENECONV), 4.26×10^{-12} (BootScan) 2.68×10^{-3} (SiScan) 3.93×10^{-2} (Chimaera) and 4.21×10^{-2}, (MaxChi). Moreover, phylogenetic trees reconstructed by the maximum-likelihood method (GTR + Gamma six categories, 500 bootstrap replicates) (data not shown) also supported the recombination event in the SB 01057805(DF02) isolate.

Coalescent analysis was performed with different datasets (90 complete genomes and 99 partial sequences), excluding the recombinant sequences. The nucleotide substitution rate of 7.5×10^{-4} and strict molecular clock resulted in better values for the Bayes factor, and these results (from three independent runs) are presented here. Moreover, the effective sample sizes for all model parameters reached values >200. A similar topology was observed in the reconstructed maximum clade credibility tree based on the complete genome, and a posterior probability value of 1.0 was observed for different nodes, including those defining lineages 1 through 6 (Fig. 3). Among the lineages from Latin America and/or Brazil, the oldest was L5, in which the MRCA dates at 1978 (30.51 years before 2009, 95 % HPD = 28.69 - 32.40). L2 grouped strains from Nicaragua, Mexico and Venezuela, which the MRCA dates at 1990 (18.98 years before 2009, 95 % HPD = 17.83 - 20.19) and the MRCA of L4 dates at 1983 (26.21 years before 2009, 95 % HPD = 24.74 - 27.65) (Fig. 3).

Among the lineages containing Brazilian isolates, L3 was the oldest, which the MRCA dates to 1984 (24.61 years before 2009, 95 % HPD = 23.46 - 25.81), followed by L6, with the MRCA dating to 1984 (24.56 years before 2009, 95 % HPD = 24.18 -24.98). When we considered only the Brazilian isolates belonging to L6 (denoted L6-BR), their MRCA dated to 1998 (11.01 years before 2009, 95 % HPD = 10.29 - 11.78). L1 is the most recent lineage containing Brazilian isolates, with the MRCA dating to 1993 (15.93 years before 2009, 95 % HPD = 15.15 - 16.72). L1 can be subdivided into two populations (denoted L1a and L1b), with the Brazilian isolate BR/BID-V2401/2008 placed within L1a. In addition, isolates within L1b have an arginine residue at position 396 of the deduced envelope protein, distinguishing them from L1b and all other isolates. The MRCA of this latter population dates to 1995/96 (13.64 years before 2009, 95 % HPD = 12.95 -14.46), while the MRCA of L1b dates to 1994 (15.09 years before 2009, 95 % HPD = 14.41 - 15.82) (Fig. 3).

When phylogenetic trees were constructed and coalescent analysis was performed using 99 partial sequences (2325 nt from the capsid to the E gene), similar clustering patterns were observed, with minor differences (Fig. 4). Two Brazilian isolates, 31807/BR/RJ/1986, and 31768/BR/RJ/1986 cluster within L3, but the Brazilian prototype Den1BR/90 was excluded from this lineage, although the latter was still placed inside the Latin America cluster. The MRCA of L3 (excluding Den1BR/90) dates to 1985 (1984 - 86, 26.17 years before 2011, 95 % HPD = 25.45 - 26.99). Within lineage L1, two populations are observed, and the Brazilian isolates 0122_2011/BR/RJ/2011 and 15_2010/BR/RJ/2010 group within L1b, while isolate BR/BID-V2401/2008 clusters within L1a. The

Table 2 Amino acids substitutions characterizing different lineages of dengue virus 1 from Brazil, Latin America, Singapore (strain SG/05K4147DK1/2005), and Reunion Island (strain Reunion 191/04)

Protein	AA position	Lineage						Strain	
		L1	L2	L3	L4	L5	L6	Singapore	Reunion
prM/M	143	**A**	A	**A (G)**[1]	A	A	***V****	V	V
	145	V	V	V	I	V	V	V	V
	169	R	Q	R	R	R	R	R	R
	236	**R**	R	**K**	K	K	**K**	R	K
E	39	N	N	N	D	N	N	N	N
	57	V	I	V	V	V	V	V	V
	299	**M**	T	**T**	M	M	**M**	M	M
	340	**S**	S	**S (L)**[2]	S	S	**L**	S	S
	341	T	T	T	A	T	T	I	T
	430	**V**	V	**V**	V	V (M)[3]	***L****	V	V
	438	**V**	V	**V**	V	V	***I****	I	T
NS1	77	**H**	H	**H**	H	H	***Y****	Y	Y
	112	K	K	K	R	K	K	K	K
	146	**G**	N	**E**	D	D	**D**	D	D
	224	**T (I)**[4]	T	**I**	I	I	**I**	I	I
	293	**N**	N	**S (N)**[5]	N	N	***Y****	N	N
NS2A	18	**M**	M	**M**	M	M	***I****	L	L
	139	**E**	E	**E**	E	E	***D****	D	D
	155	**V**	I	**I**	I	I	**I**	I	I
	156	**K**	K	**K**	K	K	***R****	K	K
	168	**T**	T (A)[6]	**T**	M	M	**M**	M	M
	171	**V (I)**[7]	A	**V**	V	V (A)[8]	**A**	A	T
NS2B	4	**L**	L	**L**	L	L	***I****	I	I
	11	**I**	I	**I (V)**[9]	I	I	**V**	V	V
	63	**Q**	Q	**E**	E	E	**E**	E	E
	70	**A**	T	**T**	T	T	**T**	T	T
NS3	170	A	A	A	T	A	A	A	A
	474	**V**	V	**V**	V	V	***I****	V	V
NS4A	89	**T**	T	**T (M)**[10]	M	M	**M**	M	M
NS4B	17	**Y**	H	**H**	H	H	**H**	H	H
	24	**Q**	Q	**Q**	H	H	**H**	H	H
	153	**A**	A	**A (T)**[11]	A	A	**T**	A	A
NS5	135	**M**	M (I)[12]	**M (I)**[13]	T	T (I)[14]	**T**	I	I
	370	**T**	T	**A**	A	A	**A**	A	A
	503	L	L	L	F	L	L	L	L
	585	**N**	N	**N**	N	N	***S****	S	S
	629	**L (S)**[15]	L	**F (L)**[16]	L	L	**S**	L	L
	640	**E**	E (G)[17]	**E**	E	E	***K****	K	K
	669	I	I	I	V	I	I	I	I
	678	**T**	**T**	I	I	I	**T**	T	T

The table shows amino acid substitutions observed in all isolates belonging to different lineages from Latin America (established using the predicted polyprotein sequence of 59 isolates used for phylogenetic analysis and displayed in Fig. 1). Columns referring to lineages that contain Brazilian isolates are shown in bold. The amino acids that differed among the lineages containing Brazilian isolates are in bold. Letters in italics and marked with a (*) are the aa substitutions observed in the Brazilian lineage L6, but not in other lineages. Amino acids unique to L6 and/or strains from Singapore (SG/05K4147DK1/2005) and Reunion Island (Reunion 191/04) are underlined. The following exceptions were observed:[1]BR/01-MR presented a G, [2]ARG0028 and BR/01-MR presented a L, [3]US/BID-V2139/1996 presented a M, [4]VE/BID-V2230/2004 and VE/BID-V2245/2005 presented an I, [5]Den1BR/90 and DF01-HUB01021093 presented a N, [6]NI/BID-V642/2006 presented an A, [7]BR/BID-V2401/2008 presented an I, [8]US/BID-V2139/1996 presented an A, [9]ARG0028 presented a V, [10]BR/BID-V2378/2001and SB 01057805 (DF02) presented a M, [11]ARG0028 presented a T, [12]NI/BID-V642/2006, [13]BR97_409 presented an I, [14]US/BID-V2139/1996 and US/BID-V1743/1995 presented a T, [15]VE/BID-V2251/2005 presented a S, [16]Den1BR/90, DF01-HUB01021093 and SB 01057805 (DF02) presented a L, and [17]NI/BID-V2330/2008 presented a G

Table 3 Summary of amino acid differences in the predicted E protein amongst Brazilian dengue virus 1 isolates

Lineage	Isolate	Amino acid position and localization in protein E								
		33 DI	182 DI	299 DI	340 DIII	348 DIII	396 DIII	430 EH2	438 EH2	444 EH2
	Prototype Den1BR90	V	A	T	S	T	K	V	V	T
L1	BR/BID-V2401/2008	.	.	M	.	.	.	.	.	.
	15/BR/RJ/2010	.	.	M	.	.	R	.	.	.
	0122/BR/RJ/2011	.	.	M	.	A	R	.	.	.
L3	31768/BR/RJ/1986	.	.	.	.	.	.	.	.	.
	31807/BR/RJ/1986	F	.	.	.	.	.	.	.	.
	BR/97-233	.	T	.	.	.	.	.	.	.
	BR/97-409	.	.	.	.	.	.	.	.	.
	BR/97-111	.	.	.	.	.	.	.	.	.
	Den1BR/90	.	.	.	.	.	.	.	.	.
	BR/01-MR	.	.	.	L	.	.	.	.	.
	DF01-HUB01021093	.	.	.	.	.	.	.	.	.
	SB01057805(DF02)	.	T		.	.	.	.	.	.
	BR/BID-V2378/2001	.	.	.	.	.	.	.	.	.
L6	BR/BID-V2375/2000	.	.	M	L	.	.	L	I	.
	BR/BID-V2374/2000	.	.	M	L	.	.	L	I	.
	BR/BID-V2381/2002	.	.	M	L	.	.	L	I	.
	BR/BID-V2384/2003	.	.	M	L	.	.	L	I	.
	BR/BID-V2389/2004	.	.	M	L	.	.	L	I	.
	BR/BID-V2392/2005	.	.	M	L	.	.	L	I	A
	BR/BID-V2395/2006	.	.	M	L	.	.	L	I	.
	BR/BID-V2398/2007	.	.	M	L	I	.	L	I	.
	BR/BID-V3490/2008	.	.	M	L	.	.	L	I	.
	55-2009ES	.	.	M	L	.	.	L	I	.
	1435RJ/2009	.	.	M	L	N	.	L	I	.
	1433RJ/2009	.	.	M	L	N	.	L	I	.
	188/BR/RJ/2010	.	.	M	L	.	.	L	I	.
	19/BR/RJ/2010	.	.	M	L	.	.	L	I	.
	20/BR/RJ/2010	.	.	M	L	.	.	L	I	.

D I, domain I; D III, domain III; EH-2, α-helical domain 2 from the stem region of the envelope protein. Dots indicate identical amino acids in Brazilian DENV-1 prototype strain (Den1BR/90)

MRCA of L1a and L2b date to 1996 (14.93 years before 2011, 95 % HPD = 13.86 -16.32) and 1995 (15.67 years before 2011, 95 % HPD = 14.87 - 16.54), respectively. L1a and L1b share a common ancestor that dates to 1994 (16.62 years before 2011, 95 % HPD = 15.54 - 17.73). Outside the Latin America cluster, six new Brazilian isolates group within L6 with the MRCA dating to 1984 (26.77 years before 2011, 95 % HPD = 26.13 - 27.58)]. Considering only the Brazilian isolates (L6-BR) within L6, their MRCA dates to 1998/99 (12.41 years before 2011, 95 % HPD = 11.52 - 13.47). Within L6-BR, isolates from the Southeast Region, obtained from 2008 to 2010, share a common ancestor from 2006/07 (Fig. 4).

Discussion

According to Gonçalvez et al. [16], the phylogenetic trees reconstructed using full and partial genome sequences indicate that all Brazilian DENV-1 isolates belong to genotype V; however, they do not form a monophyletic group. In our phylogenetic trees, one main cluster containing the majority of the isolates from Latin America was observed, as has been demonstrated previously [13, 19, 32], although some of the Brazilian isolates were located outside this cluster. The presence of different lineages of DENV-1 circulating in Latin America has been demonstrated [32], but until recently [13], all of the Brazilian

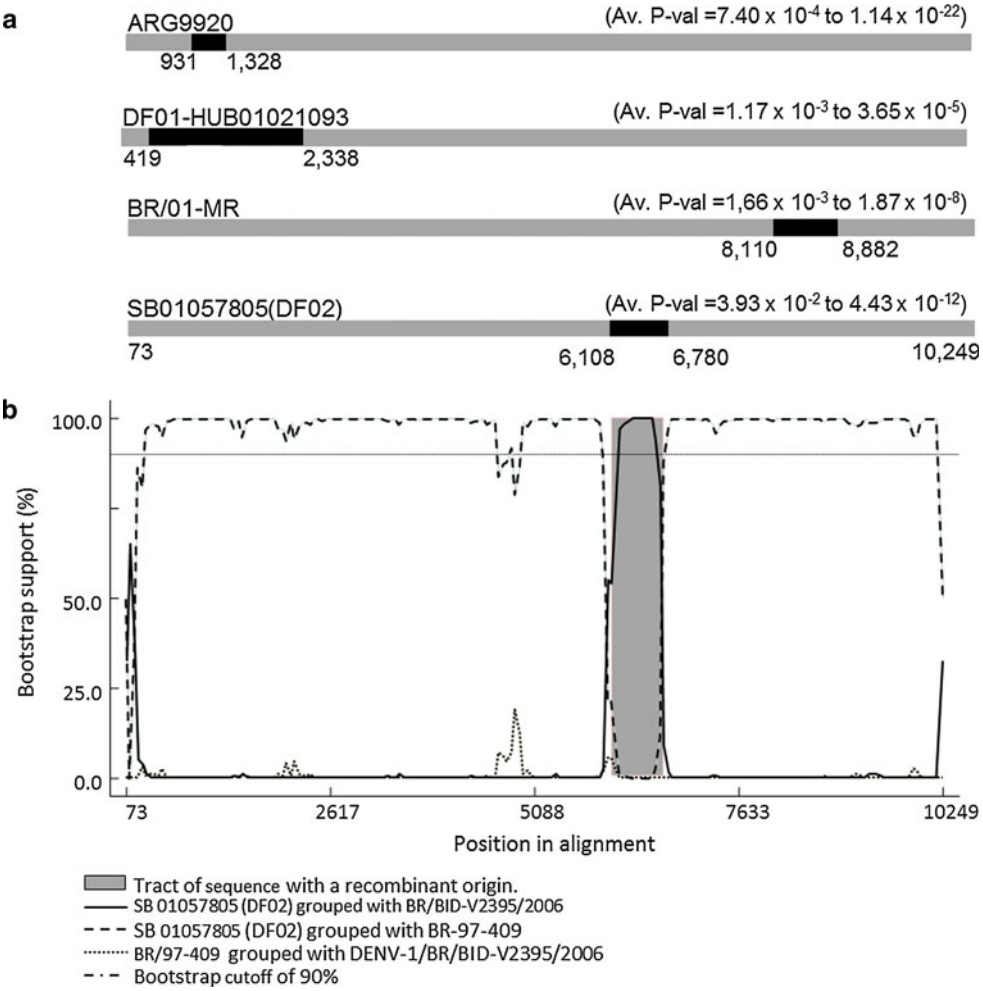

Fig. 2 Evidence of recombination in dengue virus 1 (DENV-1) isolates from Brazil and Argentina. (**a**) Schematic diagram of the dengue virus 1 genome (grey bar) showing the potential recombinant regions (black bar) detected in four DENV-1 isolates. The most probable recombination breakpoints (specified by Bootscan analysis) are shown beside the recombination regions. The name of the recombinant isolate is shown above the grey bar (left side). The range of average P-values (Av P-val) for recombination signals detected by at least five methods is shown above the grey bar (right side). (**b**) Bootscan plot showing the recombination event signal in the SB01057805(DF02) sequence. The analysis involved 90 nucleotide sequences with a total of 10,176 positions in the final dataset (positions 73 to 10,249 of DENV-1 genome). The analysis was conducted using Recombination Detection Program 3 [24]

isolates have grouped inside a single lineage with low genetic diversity, which was thought to be the result of a single introduction event of DENV-1 in the 80's, at the time of the first reported DENV-1 cases in Brazil [18, 19, 33, 34]. Our analysis, based on complete genome sequences, reinforces the evidence that different lineages of DENV-1 genotype V have been introduced and have established circulation in Brazil.

The introduction of different DENV-1 lineages into Brazil was not associated with neutral or positive selection. Although the dN/dS ratio varied among lineages, they were all evolving under strong purifying selection. Similar values of dN/dS ratios were observed previously when different genes/genomes of DENV were analyzed [35, 36]. A study carried out with Cambodian samples revealed several potentially important amino acid substitutions and high purifying selective pressure. According to the authors, some non-synonymous mutations may be involved in important roles in protein structure and function [37]. The majority of nucleotide sites in the genomes of arboviruses, including DENV, are evolving under purifying selection due to pressures associated with virus replication in human and mosquito cells [38]. Selective pressure within the vectors could also lead to genetic diversity and changes in the epidemiological panorama, as is the case in Thailand, where the alternation of DENV-1 clades has enhanced dengue transmission by the vectors [39].

Analysis of deduced amino acid sequences revealed conservative and non-conservative substitutions that characterized lineages of DENV-1 circulating in Latin America. Moreover, most of the amino acid substitutions were conservative and unique to lineage L6 and to isolates from Singapore (SG/05K4147DK1/2005) and Reunion Island (Reunion 191/04), supporting a closer relationship between these isolates. The deduced E sequences of the Brazilian isolates were quite conserved. It is interesting to note that non-conservative amino acid substitutions were observed in domain III of the E protein, distinguishing the Brazilian isolates belonging to different lineages. This domain is an immunoglobulin-like domain that plays important roles in flavivirus attachment and antibody neutralization [40, 41]. The 12 cysteine residues involved in formation of disulfide bridges as well as two asparagine residues located at positions 67 and 153 of the envelope protein of all isolates

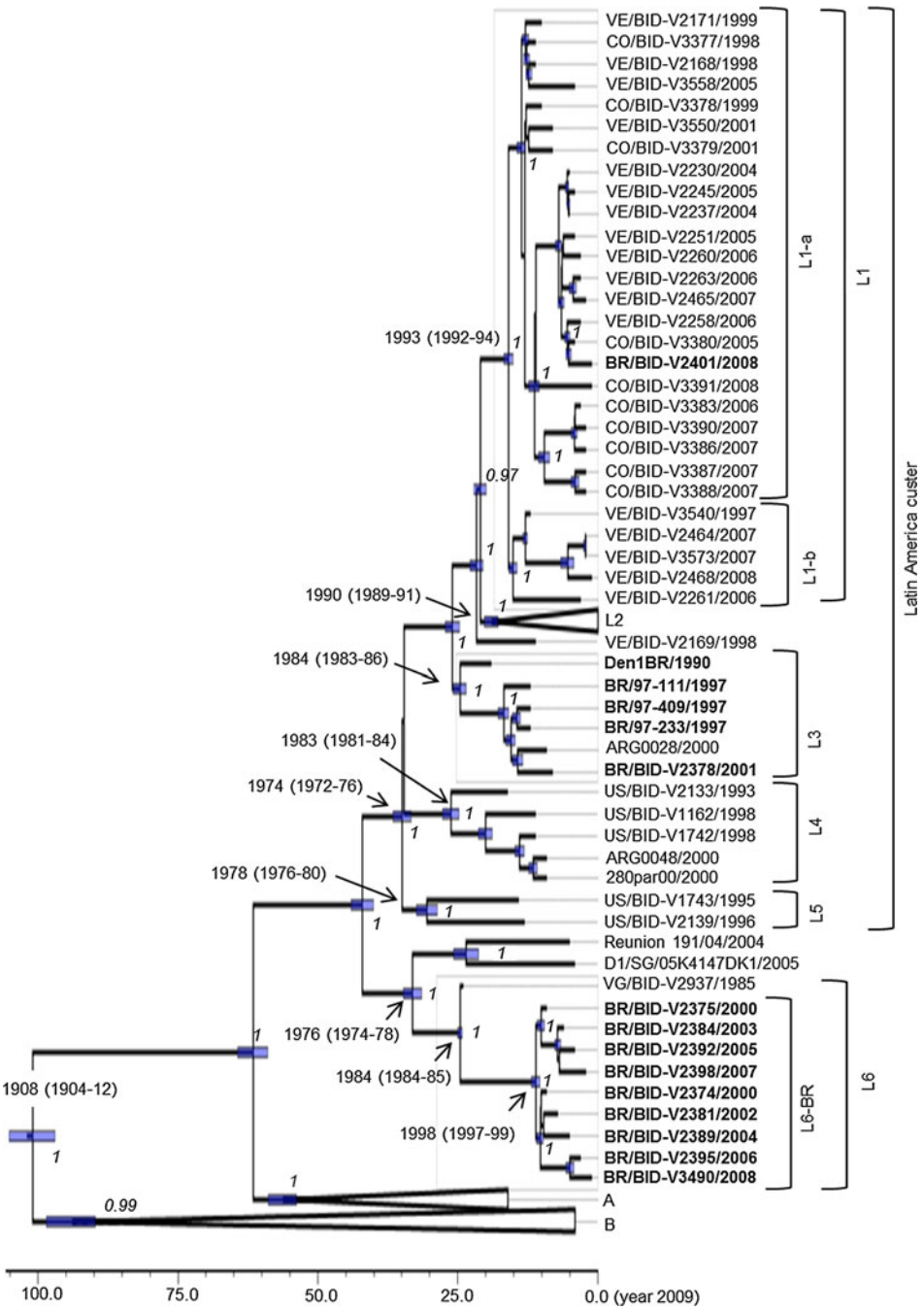

Fig. 3 Bayesian coalescent analysis of Brazilian DENV-1 based on the complete genome sequence. The maximum clade credibility tree was inferred using 90 DENV-1 genome sequences. The time of the most recent common ancestor (MRCA) was estimated using the year of isolation as the calibration point, under the strict molecular clock, with the general time-reversible model, with discrete gamma distribution and an estimated nucleotide substitution rate of 7.5×10^{-4}. The posterior probabilities are shown near some nodes. The extent of the 95 % highest posterior density (95 % HPD) intervals for each divergence time is represented by grey bars in the nodes. The estimated years that the MCRA was estimated to exist are shown for some nodes with 95 % HPD displayed in parentheses. The origin value of the reverse-scale axis corresponds to the year 2009. Lineages are delimitated by square brackets, and Brazilian DENV-1 isolates are shown in bold. For clarity purposes, L2, A and B were collapsed. L2 contained isolates from Mexico: MX/BID-V3658/2006, -V3657/2006, -V3664/2006, -V3665/2007, -V3732/2007, -V3743/2007, -V3746/2008, -V3757/2008, -V3761/2008, -V3746/2008; from Nicaragua: NI/BID-V1223/2007, -V2341/2006, -V2342/2006, -V2652/2008, -V5067/2009, -V5071/2009, -V540/2005, -V5504/2009, -V602/2005, -V630/2005, -V669/2004, -V2330/2008, -V629/2005, -V642/2006, -V646/2005, -V653/2004 and isolates from Venezuela: VE/BID-V2162/1997, -V3544/1998, -V3549/2000. A contained isolates Singapore 8114/93, D1.Myanmar.40568/76, D1.Myanmar.40553 and DENV-1 Abidjan. B grouped isolates US/Hawaii/1944, SG/05K4173DK1/2005, CHI3336-02 and HawO3758. Three independent runs were performed using programs from BEAST package v.1.6.1, BEAUTi, Tracer v.1.5.0, TreeAnotator v.1.6.1 and FigTree v.1.3.1 [26, 27, 29–31]

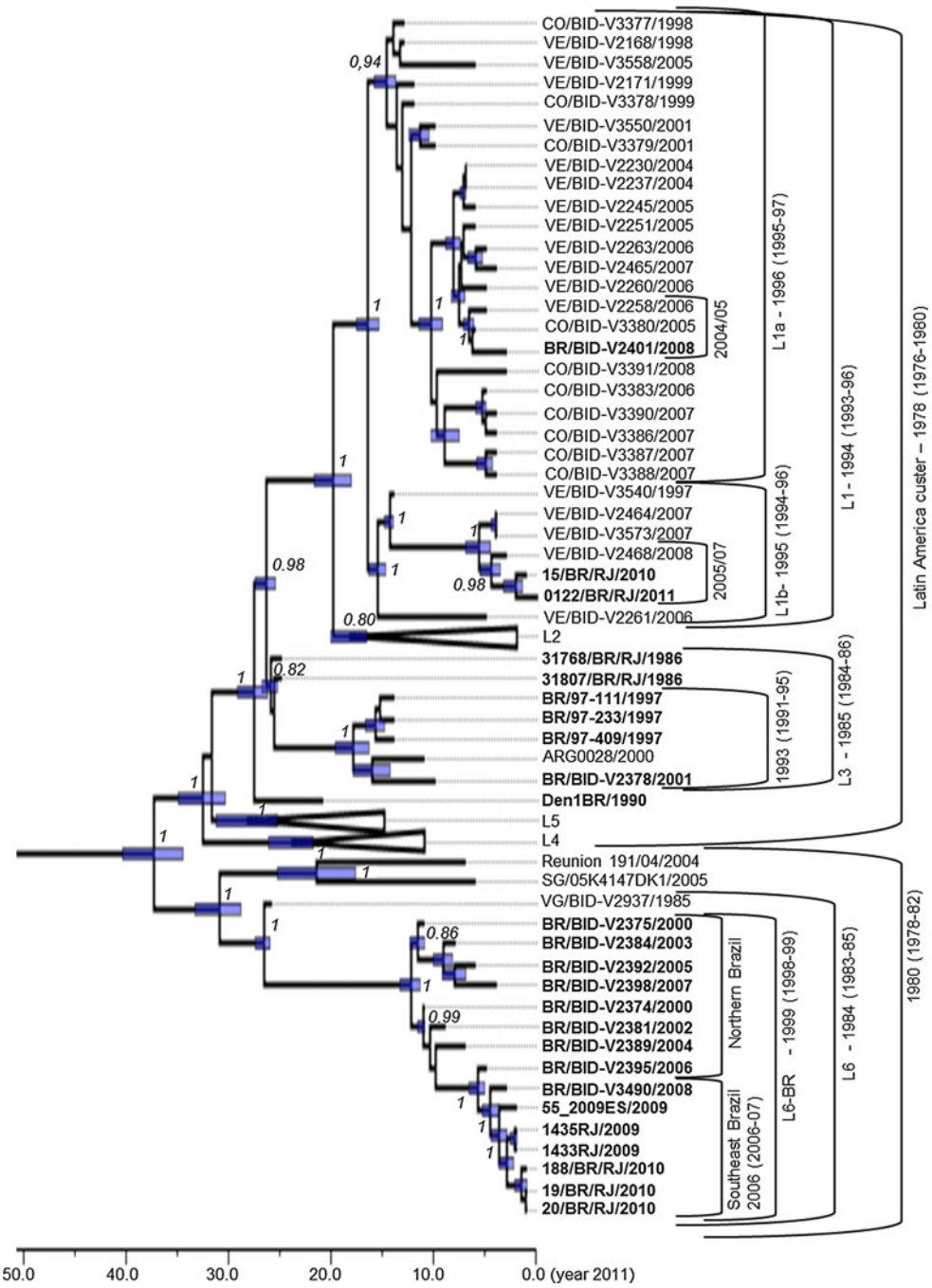

Fig. 4 Bayesian coalescent analysis of Brazilian DENV-1 based on partial genome sequence. The maximum clade credibility tree was inferred using 99 DENV-1 partial sequences (2,325), and a subtree is displayed here. The time of the most recent common ancestor (MRCA) was estimated, using the year of isolation as the calibration point, under the strict molecular clock, with the general time-reversible model, with discrete gamma distribution and an estimated nucleotide substitution rate of 7.5×10^{-4}. The posterior probabilities are shown near some nodes. The extent of the 95 % highest posterior density intervals for each divergence time is represented by grey bars in the nodes. The estimated years that the MCRA was estimated to exist are shown for some nodes with upper and lower intervals in parentheses. The origin value of the reverse-scale axis corresponds to year 2011. Lineages are delimitated by square brackets, and Brazilian DENV-1 isolates are shown in bold. For clarity purposes, L2, L4 and L5 were collapsed, and each grouped the same isolates as shown in Figure 3. Analysis was performed using programs from BEAST package v.1.6.1, BEAUTi, Tracer v.1.5.0, TreeAnotator v.1.6.1 and FigTree v.1.3.1 [26, 27, 29–31]

were conserved, reinforcing the importance of these amino acids in the envelope protein structure. Asparagine residues at positions 67 and 153 are two potential N-linked glycosylation sites within neutralizing epitopes of the envelope protein. Mutations leading to amino acids substitutions in these sites could have an effect on virus-mediated membrane fusion and virulence [42, 43]. While the genetic divergence within lineages containing Brazilian strains can be considered low, it was higher between lineages, demonstrating that the introduction of different lineages into Brazil contributed to raising the genetic diversity among DENV-1 strains circulating in the country. A recent study in Singapore revealed a high viral diversity that was likely due to multiple introductions as well as local evolution and diversity of circulating DENV in the country, which offers ample opportunity for selection of strains of higher fitness, thereby increasing the risk of outbreaks despite a low *Aedes* population [12].

Our analyses, which included 11 new DENV-1 complete genome sequences, enabled us to detect four incidences of recombination in three Brazilian isolates and one Argentinian isolate, confirming previously described results [19].

The detection of one new potential inter-genotypic recombinant strain was possible due to the inclusion of a greater number of genome sequences of Brazilian isolates in our analysis. This recombinant Brazilian isolate was SB 01057805(DF02), whose parental strains resemble isolates from the Northern Region of the country, belonging to lineages L3 and L6. Both strains assigned as the major and minor parental strains (BR/97-409 and BR/BID-V2395/2006, respectively) were obtained from human hosts and amplified in C6/36 cells [44]. However, there is no available information regarding the original host of SB01057805(DF02). This strain was amplified *in vitro* in an *Aedes albopictus* C6/36 cell line (three passages) to achieve sufficient viral titers for RNA isolation [19]. It is important to note that no information is available with regard to the cloning and sequencing of the recombinant viruses or whether they could represent mixed populations. However, the co-circulation of different DENV populations, including different genotypes, is a factor that increases the chances of the occurrence of mixed infections both in the mosquito vector and in the human host [45, 46], which in turn could favor the occurrence of recombination, as observed in Brazil, where different DENV-1 lineages from genotype V have been co-circulating. Although it is not possible to determine whether recombination has occurred in the mosquito vector, the human host or *in vitro* (during virus replication in C6/36 cells), or when these events might have occurred among the Brazilian DENV-1 isolates, recombination events have already been described in natural populations, including intra and inter-genotypic recombinants, as described for DENV-1 and DENV-2 [19, 45, 47–49]. Although it is likely that most recombinant events are deleterious and thus eliminated by purifying selection, some of them could result in an increase in the fitness of the virus. These events could have important implications for virus evolution, virulence, and diagnosis and for the development of vaccines and therapeutic drugs [45, 47, 49].

The coalescent analysis demonstrated that the MRCA of all isolates is placed at 1908 (1904-12) (Fig. 3), which is in agreement with data that suggests the end of the 19th and the beginning of the 20th century as the probable beginning of the DENV-1 epidemic transmission in humans [33]. The MRCA of the Latin America cluster dates to 1974 (1972-76), prior to the first report of DENV-1, which occurred in the Americas, in Jamaica, in 1977 [32, 50]. Our results are in agreement with a previous study that placed the MRCA of DENV-1 in the Americas at 1973 (1970-75) [50].

L3 is the oldest Brazilian lineage and clusters isolates from different periods and regions of the country, including the prototype strain Den1BR/90 in addition to one strain from Argentina, in agreement with previous results [13, 19]. When we performed the analysis with shorter sequences (2325 nt), Den1BR/90 was placed outside of lineage L3, possibly due to the use of a greater number of sequences; similar results have been observed in another study [13]. Based on median values, the MRCA of L3 dates to 1984/85,which suggests that L3 does not share a common origin with isolates introduced into the country in the early 80′s, at the time of an outbreak of DENV-1 reported in the Northern Region of Brazil [6]. On the other hand, they may have a common origin with isolates that caused an outbreak in 1986 in Rio de Janeiro. Indeed, isolates in L3 share a common ancestor with 31807/BR/RJ/1986 and 31768/BR/RJ/1986 (isolated in 1986 in Rio de Janeiro) that date to 1985 (1984-86).

Interestingly, L6, the lineage containing the majority of Brazilian isolates, was placed outside the Latin America cluster and also grouped with isolate VG/BID-V2937/1985, from the Virgin Islands. The most common recent ancestor of the Brazilian isolates from L6 (L6-BR) was probably introduced into Brazil's Northern Region in 1998/99, establishing its circulation in that area and subsequently diversifying into two populations. It is likely that in 2006/07, a DENV virus isolate from the Northern Region was transported to the Southeast Region and gave rise to a separate population in this area of the country through local evolution over time.

In contrast, a different relationship was observed in lineage L1 where three Brazilian isolates separated into two populations (supported by phylogenetic and coalescent analysis and by the amino acid difference detected in codon 396 of the envelope protein sequence) with two isolates (0122_2011/BR/RJ/2011 and 15_2010/BR/RJ/2010) from Rio de Janeiro/Southeast Region being closely related to each other but not related to isolate BR/BID-V2401/2008 from the Northern Region. BR/BID-V2401/2008 shares a common ancestor with two strains, one from Colombia and one from Venezuela, that date to 2004/05. The two Rio de Janeiro isolates share a common ancestor with a Venezuelan strain (VE/BID-V2254/2005) that dates to 2006 (2005/07). The tree topology and coalescent analysis suggest that those strains were introduced into Brazil by two parallel events in different regions of the country and are now evolving independently.

Regardless of which dataset (partial or complete genome) is used, it is evident that L3 does not contain viruses isolated after 2001. This may be a result of surveillance sampling methods or represent a lineage replacement event by the newer lineages of L6 or L1. With the introduction of more recent isolates from L1 (2004-2007) into Brazil, it appears that the L6-BR lineage has replaced the Brazilian L3 lineage, since the L6-BR lineage was introduce in 1998/99 and was first detected in 2000 and 2001, while the L3 lineage was introduced in the 1980 s and has not been detected since 2001.

We observed a gap of 1 to 2 years in the MRCAs of most lineages and populations between the estimated year of MRCA and the year the virus was isolated. This was particularly evident for L3, L6, L1a, L1b, L6-BR and isolates within L6-BR obtained in the Southeast Region of Brazil. This pattern is similar to the pattern observed by Allicock et al. [51], who demonstrated that the MRCAs of all DENV serotypes were estimated to exist for several years (between 2 and 4 years, or 2 years in the case of DENV-1) prior to the first reported outbreaks for each serotype. These findings can be explained by the possibility of viruses remaining undetected until a threshold number of infections/disease incidences is reached that can be detected by the limited surveillance systems of most countries in the Americas [51].

This study reinforces the evidence that different lineages of DENV-1 have been introduced into Brazil, generating an increase in the genetic diversity of these viruses. In addition to lineage replacement, as observed for L2, DENV-1 dynamics in Brazil is characterized by co-circulation and generation of genetically distinct viruses as a result of local evolution or due to exogenous virus introduction at different times or during the same period. Presently, there are at least two different lineages circulating in different regions of Brazil. Co-circulation of multiple lineages and the new evidence of recombination events increase the possibility of generating even more diverse isolates with distinct biological properties, which may lead to incidences of more severe disease. This possibility is especially important in a situation where DENV-1 is recirculating among other DENV serotypes/genotypes. Our results also demonstrate that the main sources of exogenous viruses are other Latin America countries, reinforcing the need for genotype surveillance in order to detect and trace virus populations that are circulating in Brazil and Latin America.

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