Re-introduction of dengue virus serotype 2 in the State of Rio de Janeiro after almost a decade of epidemiological silence

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Abstract

The Asian/American genotype of dengue virus serotype 2 (DENV-2) has been introduced in Brazil through the state of Rio de Janeiro around 1990, and since then it has been spreading and evolving, leading to several waves of dengue

epidemics that cause a major public health problem. Of particular interest has been the epidemic of 2008, whose highest impact was evidenced in the state of Rio de Janeiro, with a higher number of severe cases and mortality rate, compared to previous outbreaks. Interestingly, no circulation of DENV-2 was witnessed in this region during the preceding 9-year period. By that time, a new viral lineage of the Asian/American genotype was identified and pointed as responsible for the outbreak severity. The same scenario is repeating in 2019 in this state, however, only a few cases have been detected yet. With the aim to avoid an outbreak a great magnitude we employed phylogenetic studies combined with temporal and geographical features to determine the origin of the current viral strain, analyzing a region of 1626 nucleotides entailing the Envelope/NS1 viral genes. Our study reveals that the current strain belongs to the same lineage that caused the 2008 outbreak, however, it is phylogenetically distant from any Brazilian strain identified so far. Indeed, it seemed to be originated in Puerto Rico around 2002 and has been introduced into the state in late 2018. Taking into account that no DENV-2 case was reported over the last decade in the state, which represents a whole susceptible children generation, and the fact that a new viral strain may be causing current dengue infections, these results would be essential to strengthen dengue surveillance and disease control, avoiding the potential epidemiological consequences of virus spread.

Author Summary

By the time DENV2 was introduced into Brazil through the state of Rio de Janeiro in 1990, the first dengue haemorragic cases started to evidence as well. Years of seasonal outbreaks were followed by almost ten years oy epidemiological

48 silence in the state. However, in 2007 this serotype was re-introduced into the
49 state causing one of the worst dengue epidemics ever described in the country.
50 The same viral genotype was involved, however, a different viral lineage was
51 detected and pointed as responsible for the outbreak severity. This same
52 scenario could repeat nowadays in the state of Rio de Janeiro. Since new DENV-
53 2 cases are being detected in this region, we analyzed the identity and origin of
54 the viral strain obtained from two infected patients. Phylogeny combined with
55 temporal and geographical analyses of viral sequences demonstrated that the
56 strain causing 2019´s dengue cases belonged to the same lineage as the one
57 causing the outbreak in 2008, but to a different subgroup, and might have
58 originated in Puerto Rico and entered the state in recent times. This results may
59 help to prevent the recurrence of epidemics of great magnitude and may
60 represent as well a key starting point to strengthen Brazilian surveillance systems
61 and disease control.

62

63 **Introduction**

64 Arboviral infections have been re-emerging in Brazil over the last decades,
65 threatening the country and causing a constant burden for public health [1, 2].
66 Dengue virus (DENV) serotype 2 is one of the four different serotype of DENV,
67 an arbovirus that belongs to the *Flavivirus* family, and is transmitted to humans
68 by mosquitoes from genera *Aedes*. Besides Brazil, DENV is widely distributed
69 across tropical and subtropical areas of the world [3]. Human travelers and
70 extensive migrations have facilitated the spread of arbovirus potentially
71 threatening for human health through the globe [4, 5]. As a tourist magnet, the

72 state of Rio de Janeiro, in southeast Brazil, has been facing large arbovirus
73 epidemics year by year [6].

74 DENV-2 Asian/American genotype has been introduced into Brazil through the
75 state of Rio de Janeiro around 1990 [7], and brought along the onset of the first
76 cases of hemorrhagic fever and dengue shock syndrome. In 2008, in Rio de
77 Janeiro State alone, a DENV-2 outbreak caused more than 235.000 reported
78 cases, over 13000 hospital admissions and 263 deaths [8], which represented
79 increased disease severity and a higher rate of mortality, compared to previous
80 outbreaks [9]. This epidemic caused staggering human and economic costs, with
81 more than US$1 billion being spent at the national level on dengue prevention
82 and control [10]. Drumond et al demonstrated that the virus causing 2008
83 epidemic belonged indeed, to the same genotype of the virus that was first
84 introduced around 1990, but to a totally different lineage [11] with genetic
85 differences potentially associated with a more pathogenic clinical outcome of the
86 disease [12].

87 After that period, DENV-2 cases diminished gradually in the state, and different
88 DENV serotypes and arboviruses such as Zika, Chikungunya and Yellow fever
89 started to alternate its circulation. This serotype remained undetectable in the
90 state until 2018, when the Information System for Notifiable Diseases (SINAN)
91 and the Laboratory Sample Management System (GAL) registered 3 different
92 dengue cases caused by serotype 2 [13].

93 However, even though at a low rate, and below serotypes 1 and 4, DENV-2
94 maintained a basal and low circulation at national level, mainly in the north and
95 northeast regions of the country, where less than 2% of the notified dengue cases
96 belonged to serotype 2. This scenario abided between 2014 and 2016, but in

2017, even being the year with the lowest number of registered dengue cases nationally, the central-west region of the country started to show a growing predominance of DENV-2 circulation. During 2018, it kept on spreading at a low rate, obtaining on average, similar figures as the ones from 2017. However, throughout this current year, DENV-2 cases reached notification rates 282% superior as the preceding year, and its circulation has been already confirmed also in the southeast region of the country, including the state of Rio de Janeiro [14].

Considering that a whole new generation is naïve to this serotype infection and concerned about a potential new outbreak of great magnitude in the state, we sought to briefly analyze the virus phylogeny and its origin, to study the potential threats of this strain spread.

Materials and methods

Ethical statement

The strains analyzed in this study belong to the collection from the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, Brazil, and were obtained from human serum through the passive surveillance system from an ongoing Project approved by resolution number CAAE 90249218.6.1001.5248 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 2.998.362), Ministry of Health-Brazil. Samples were chosen anonymously, based on the laboratorial results.

Sera samples from infected patients from the municipalities of Vassouras and Volta Redonda were sent to the Central Laboratory of Rio de Janeiro LACEN/RJ

for diagnostic confirmation and DENV-2 serotype was detected by RT-PCR under Lanciotti´s protocol [15]. Genotyping was performed by the Regional Reference Flavivirus Laboratory at Instituto Oswaldo Cruz/FIOCRUZ in Rio de Janeiro.

RNA extraction, RT-PCR and sequencing

Briefly, viral RNA was extracted from 140 ul of serum samples using the QIAamp Viral RNA Mini Kit (Qiagen, CA-EUA), followed by a RT-PCR to amplify a small portion of 1639 nucleotides entailing the Envelope/NS1 region (1467-3106 according to AF489932 reference sequence) of the viral genome. For this purpose we used the QIAGEN OneStep RT-PCR Kit (Qiagen, CA-EUA) according to manufacturer's instructions, and primers pair 3A-4B, described elsewhere [16]. Thermocycling conditions consisted of a single step of 50°C for 30 minutes and 95°C for 15 minutes for reverse transcription, followed by 40 cycles of denaturation at 94°C (30 seconds), annealing at 63°C (60 seconds), extension at 72°C (2 minutes) and a final extension at 72°C (10 minutes). PCR products were confirmed by 1.5% agarose gel electrophoresis stained with SYBR Safe DNA gel stain (Invitrogen) and visualized under blue light. Subsequently, PCR specific bands were sliced from the gel and purified with the Qiagen Gel Extraction Kit (Qiagen, CA-EUA), following manufacturer's instructions. Quantification of cDNA was carried on with Qubit® fluorometer (Life Technologies) and finally sequenced by Sanger technique at the DNA sequencing Platform at Fiocruz Institute, Rio de Janeiro.

Phylogenetic analyses

The obtained sequences (Genbank accession numbers MK972823 and MK972824) were manually edited and aligned with BioEdit v7.2.5.0 software using

different DENV-2 reference sequences available at Genbank. Alignment of the final 378 sequences is available from the authors upon request. Evolutionary model was obtained with JModeltest v2.1.10 software [17] (GTR+I+G), chosen according the Akaike Information Criterion (AIC) and the Maximum Likelihood value (lnL), and phylogenetic trees were constructed using the Maximum likelihood method with RAxML v7.0 software. The robustness of the phylogenetic grouping was evaluated by bootstrap analysis with 1000 replicates. Phylogenetic tree was finally visualized in Figtree v1.4.3 software (available at http://tree.bio.ed.ac.uk/soft -ware/figtree/).

Spatiotemporal dispersion analyses

Based on phylogenetic results, sequences conforming the cluster of lineage IV of Asian/American genotype (n=137) with their respective epidemiological data were subset to carry on further temporal and geographical estimations of the evolutionary process. The time scale of the evolutionary process was assessed employing the sequences collection dates obtained from the Genbank annotations, and a GTR+I+G4 nucleotide substitution model, a relaxed uncorrelated lognormal molecular clock model, and a Bayesian Skyline coalescent tree prior. Migration events and their most reliable migration pathways were estimated using a non-reversible discrete phylogeography model with the Bayesian stochastic search variable selection approach, and a continuous-time Markov chain (CTMC) migration rate reference prior. The analysis was implemented in BEAST v1.8.1 software package [18]. The Markov Chain Monte Carlo analysis was run for 100 million generations and convergence was assessed in Tracer v1.7 (http://tree.bio.ed.ac.uk/software/tracer): 10% of the sampling trees were discarded as burn-in and accepted effective sample sizes

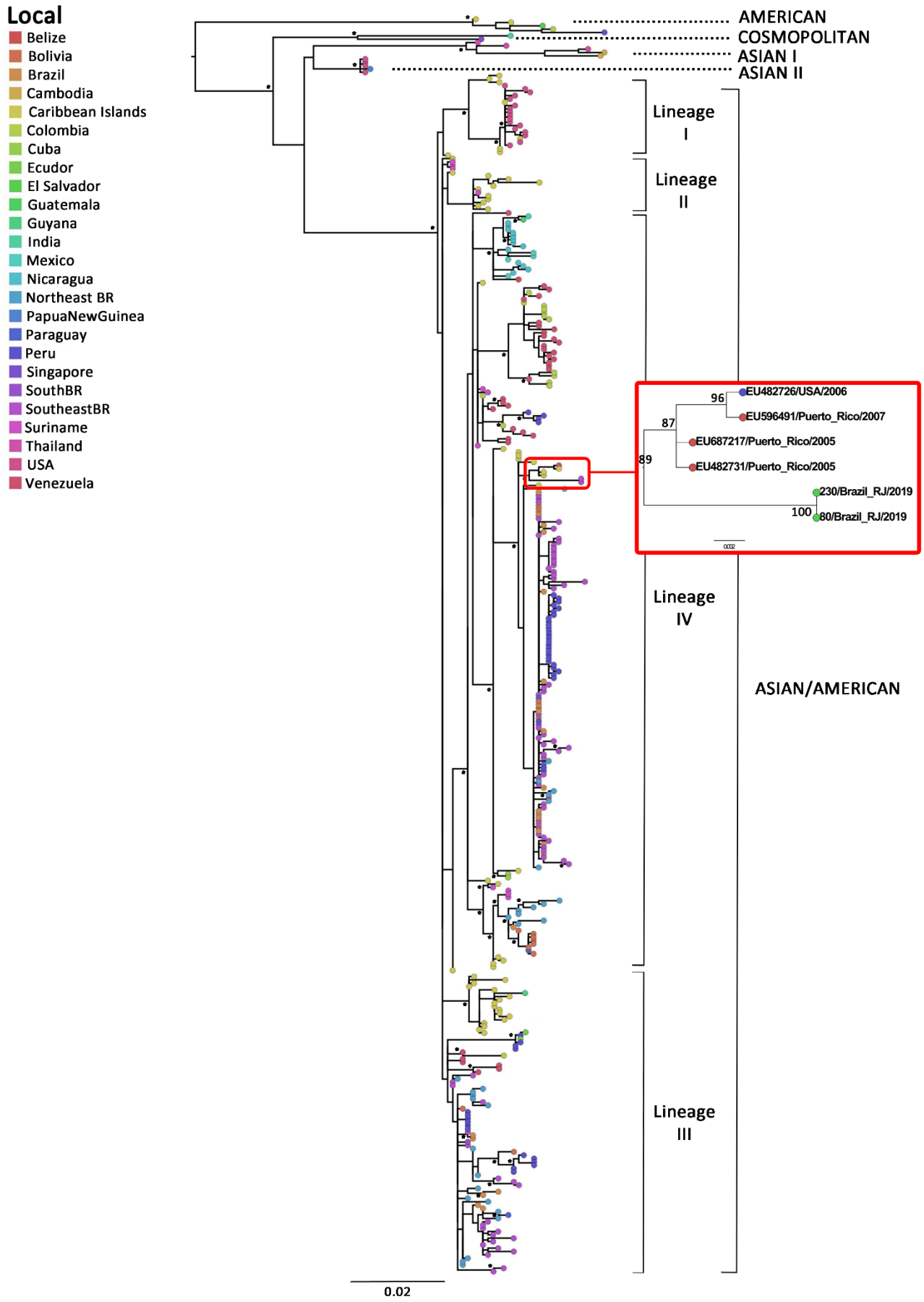
(ESS) values should be higher than 200. The 95% highest posterior density (HPD95%) interval was considered to estimate uncertainty for each estimated parameter. A maximum clade credibility tree (MCCT) was constructed with the TreeAnnotator v1.8.1 software (part of the BEAST package) after discarding 10% of the sampling, and visualized with the FigTree v1.4.3 program (available at http://tree.bio.ed.ac.uk/soft-ware/figtree/).

Results and discussion

Phylogenetic analysis showed that DENV-2 strains detected in patient sera from both municipalities belong to the same lineage of Asian/American genotype that caused the outbreak in 2008. However, it grouped in a totally separated cluster from sequences previously obtained from different locations of Brazil (Figure 1). The current lineage has been previously described by Mir et al. as lineage IV, which dominated the epidemics in South and Central America during the 2000s [19].

Figure 1 - Maximum likelihood phylogenetic tree of DENV-2 partial envelope and NS1 coding sequences. DENV-2 dataset includes sequences principally from the Asian/American (n=361), American (n=5), Asian I (n=5), Asian II (n=5) and Cosmopolitan (n=2) genotypes. Asian/American genotype includes sequences from the United States of America [USA] (n=19), Mexico (n=5), Nicaragua (n=13), Guatemala (n=1), Belize (n=1), Guyana (n=1), Suriname (n=11), Ecuador (n=2), Venezuela (n=35), Colombia (n=15), Peru (n=48), Bolivia (n=12), Paraguay

194 (n=5), Brazil [BR] (n=137, which includes 77 from the southeast region and 32
195 from the northeast) and from the Caribbean Islands which includes Jamaica
196 (n=1), Puerto Rico (n=13), Cuba (n=2), Dominican Republic (n=4) and the Lesser
197 Antilles islands (n=36) conformed by Trinidad and Tobago, the Virgin Islands,
198 Aruba, Dominica, Barbados, Grenada, Martinique, among others. Taxa are
199 represented with circles and colored according to the geographic origin of each
200 sequence as indicated at the legend (up left). The small cluster containing
201 isolates from the State of Rio de Janeiro in 2019 is zoomed-in. The asterisk in
202 the nodes corresponds to bootstrap values higher than 70, obtained with 1000
203 replicates. The scale bar indicates the genetic distances. The branch lengths are
204 drawn to scale with the bar at the bottom indicating nucleotide substitutions per
205 site.



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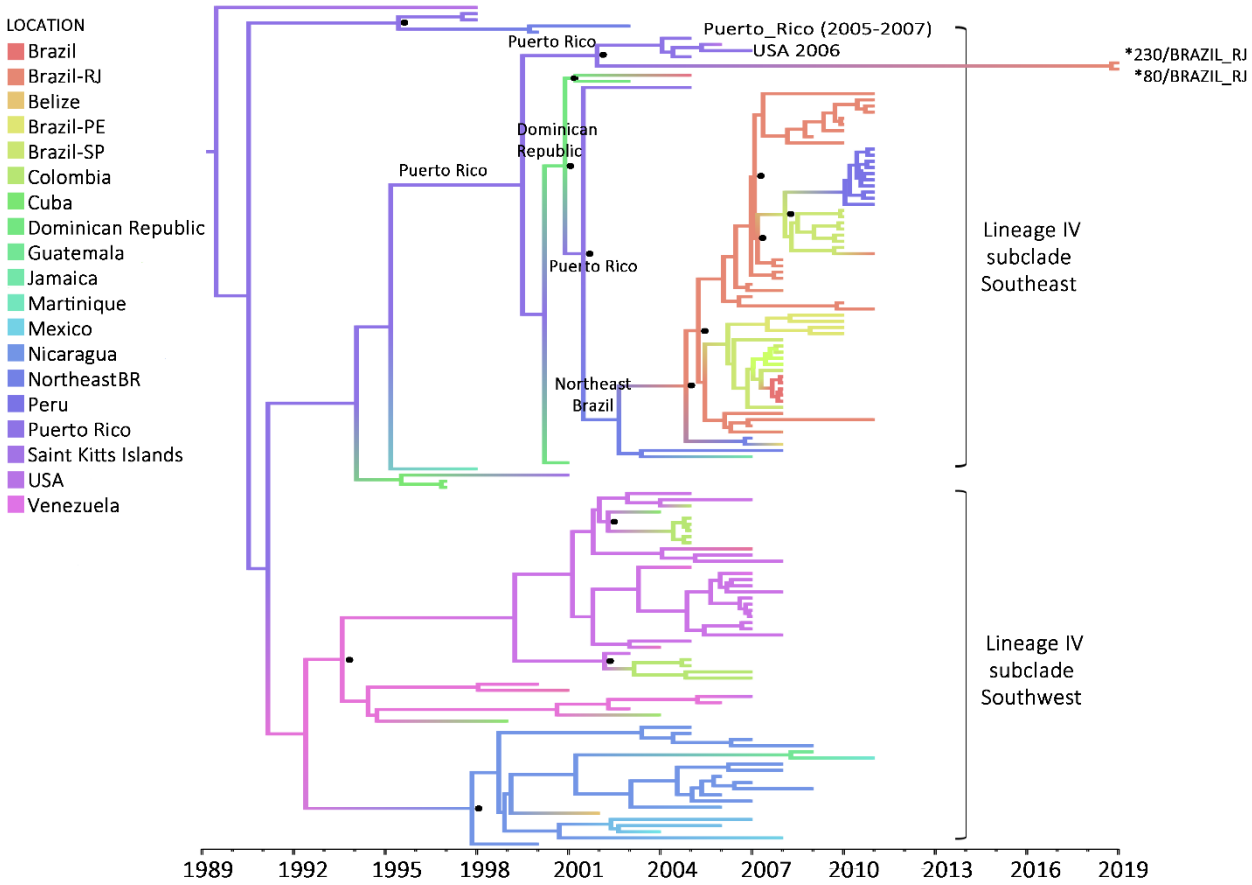
Surprisingly, this year´ sequences grouped together with sequences from Puerto Rico and the United States of 2005-2007, in a highly supported cluster (Figure 1). These findings suggest that a new viral strain may have been introduced into the state of Rio de Janeiro, which in effect, is genetically different from viral strains that kept on circulating basally in other regions of the country, at least in the viral covered region under study.

To have a more detailed picture of where this strain comes from, we conducted temporal and geographical analysis with a reduced dataset of 138 sequences conforming lineage IV group. These results suggest that the viral strain detected in our study originated in Puerto Rico (Posterior state probability [PSP]=0,81) in 2002 (95% HPD: 2000–2004) (Figure 2), and was then introduced in our state, where it started to spread in late 2018 (95% HPD: 2018–2019). To notice, no DENV-2 case has been diagnosed during the course of 2019 in the Caribbean countries [20], which clearly suggests that Rio de Janeiro´s 2019 strain could have been circulating elsewhere, up to its arrival into the state, or could have been replicating silently for some time up to rise to a detectable level. It would be likely that actually, the viral strain that entered into the state of Rio de Janeiro might well be introduced first into a different Brazilian state and spread then to Rio de Janeiro, based on the increasing DENV-2 reported cases of other states. As previously mentioned, since 2017, DENV-2 cases have been increasing and spreading through the central-west region, reaching the southeast region in 2018 and early 2019. The latter was responsible for 65.7% of the cases reported until March 2019 and is witnessing local epidemics in several municipalities of three of the four states that integrate it: Sao Paulo, Minas Gerais, and Espirito Santo; coincidentally, the three states that surround geographically the fourth integrant

of the group, the state of Rio de Janeiro [14]. Nevertheless, no molecular epidemiology analyses have been carried on to study genetic viral characteristics on those cases, and to confirm this statement we will need further sequencing yet unavailable. In fact, important caveats should be taken into account for the posed hypothesis: the limited number of available sequences of Latin America covering the region under study, and the lack of Brazilian sequences from this last 5 years period. These two aspects, if different, could be responsible for dissimilar estimations. The lack of DENV-2 Brazilian sequences over recent times goes hand-in-hand with the low circulation of this serotype across the country. Nevertheless, filling this information gap would be determinant to define when this new strain has actually entered into our territory.

Figure 2 - Time-scaled Bayesian Maximum Clade Credibility tree corresponding to the lineage IV of Asian/American genotype. Two major highly supported subclades are identified: southeast and southwest. Brazilian sequences are all included in the southeast subclade, and isolates under study are highlighted with an asterisk (*). Branches are colored according to the most probable location (legend shown on the left side) of their parental node inferred by discrete phylogeographical analysis. Location at key nodes involved in the introduction of subclade southeast into Brazil are highlighted. Posterior state probability values higher than 0,7 at key nodes are represented by a dot. All horizontal branch lengths are drawn to a scale of years. Time scale can be observed in the x-axis. The tree is automatically rooted under the assumption of a relaxed molecular clock.



Even though it is not the main focus of this note, the spatial and temporal origin of lineage IV estimated in our analysis is consistent with previous results [11, 19]. This lineage probably arose in Puerto Rico in middle 1989 (95% HPD=1985–1993) and became the dominant lineage in South and Central America from the early/middle 2000s onwards. However, little differences were detected for the subclade that spread through the southeast pathway. This subclade probably arose with the introduction of a virus from Puerto Rico (PSP=0,67) into the northeast region of Brazil around 2003 (95% HPD=2001-2004), moving then to the state of Rio de Janeiro (PSP=0,65) in 2005 (95% HPD=2004-2006), and spreading thereafter through the southeast region. This northeast passage however presented a low probability (PSP=0,3). Drumond and collaborators

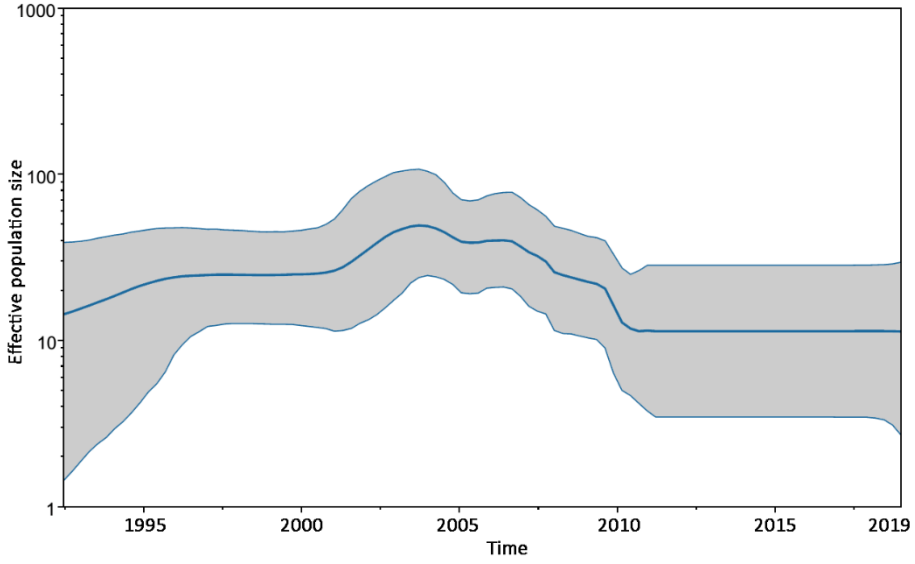
discussed in their work, a possible migration pathway through the north of the country before the arrival of this lineage into the southeast region. Thus, this observation was statistically unsupported [11]. For Mir and collaborators however, the circulation of this Southeast subclade, named in their work IV-SA4, started after the introduction of the virus from the Great Antilles directly into the southeast Brazilian region in 2004, moving then to the northeastern region, as well as to other countries in South America [19]. All the consistencies detected between our work and those already mentioned, represent little hallmarks that contribute credibility to the estimates made about this potential new viral strain detected in the State of Rio de Janeiro.

According to our estimations, the evolutionary rate of lineage IV was $9{,}5x10^{-4}$ substitutions/site/year (95% HPD=$7{,}7x10^{-4}$–$1{,}2x10^{-3}$ substitutions/site/year), exactly the same reported by Mir et al [19]. To notice, the strain giving rise to the virus isolates detected this year in the State of Rio de Janeiro, presented a nucleotide substitution rate at the lower limit of the 95%HPD interval, which would be consistent with slower evolution dynamics and a delayed detection.

On the other hand, our results regarding DENV-2 population dynamic of lineage IV showed a clear drop in population size between 2005 and 2010, and remained steady until this year (Figure 3). This observation could be consistent with the fact that other arboviruses different than DENV have been the responsible for the main epidemics of these last years in the American continent [21].

Figure 3 - Demographic reconstruction of lineage IV of Asian/American genotype. Changes in effective population size since the time of the most recent common

ancestor were estimated using the uncorrelated lognormal relaxed molecular clock and the Bayesian Skyline coalescent model. Middle blue line represented the mean value for the effective sample size, while grey areas the 95% confidence interval.



The phenomenon of DENV-2 lineage replacement across successive epidemic outbreaks has been well demonstrated my Mir and collaborators [19]. It should be considered that the circulation of lineage III (that dominated the Caribbean and South America in the 1990s) and IV in the State of Rio de Janeiro, was separated by an 8-year period. This might mean that, aside from the potential viral differences between both lineages itself [12], a generation of children born during this inter-lineage silent period had never be exposed to this serotype, by the time lineage IV entered the state. This fact could be somehow involved in the changes observed in 2007-2008 outbreak regarding the age-group affected. Thus, considering these evidences, strong surveillance and further analysis would be suggested as close future strategies for DENV-2 infection control and

316 prevention, to prevent a potential new epidemic that could be of great magnitude
317 like the one the state of Rio has already experienced a decade ago.

318

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323

324 **Author Contributions**

325 Conceived and designed the experiments: FdBN MCT AMBdF. Performed the
326 experiments: MCT. Analyzed the data: MCT FdBN. Contributed
327 reagents/materials/analysis tools: CAF SFdA AOC AMBdF. Wrote the paper:
328 MCT FdBN AMBdF.

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