

1 **Early identification of dengue virus lineage replacement in Brazil using**
2 **portable genomic surveillance**

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35 **Abstract**

36 Over 400 million people are estimated to be at risk of acquiring dengue virus (DENV).
37 Despite efforts to mitigate the impact of DENV epidemics, the virus remains a public health
38 problem in the Americas: more than one million DENV cases were reported in the continent
39 between January and July 2019 DENV was first detected in Brazil in 1982, and Brazil has
40 reported 88% (1,127,244 cases) of all DENV cases in the Americas during 2019 to date. São
41 Paulo state in the southeast of Brazil has reported nearly half of all DENV infections in the
42 country. Here we characterised the genetic diversity of DENV strains circulating in São
43 Paulo state in 2019, at the epicentre of the ongoing DENV epidemic. Using portable
44 nanopore sequencing we generated 20 new DENV genome sequences from viremic patients
45 with suspected dengue infection residing in two of the most-affected municipalities,
46 Araraquara and São José do Rio Preto. We conducted a comprehensive phylogenetic analysis
47 with 1,630 global DENV strains to better understand the evolutionary history of the DENV
48 lineages that currently circulate in the region. The new outbreak strains were classified as
49 DENV2 genotype III (American/Asian genotype). Notably, phylogenetic analysis indicated
50 that the 2019 outbreak is the result of a novel DENV lineage that was recently introduced to
51 Brazil from the Caribbean region. Our genetic analysis further indicates that the introduction
52 and onwards spread of the outbreak lineage (named here DENV2-III BR-4) indicates a new
53 DENV2 lineage replacement in Brazil. Dating phylogeographic analysis suggests that
54 DENV2-III BR-4 was introduced to Brazil in or around early 2014, possibly from the
55 Caribbean region. Our study describes the early detection of a newly introduced and rapidly-
56 expanding DENV2 virus lineage in Brazil.

57

58 **Key words:** dengue 2, outbreak, genomic surveillance, lineage replacement

60 **Author Summary**

61 Dengue is the most important mosquito-borne viral disease of humans. The disease is caused
62 by the dengue virus (DENV) that is classified within genus *Flavivirus*. DENV infections are
63 caused by 4 serotypes (DENV 1-4) that are genetically related but antigenically distinct.
64 Dengue infection results in a variety of symptoms that range from mild fever to dengue
65 hemorrhagic fever and/or dengue shock syndrome (DHF/DSS). Clinical outcomes are
66 associated with different types of infection, viral serotypes, genotypes, lineages, and host
67 genetic factors. As a re-emerging infectious disease, DENV has become a serious threat to
68 public health in the Americas, and particularly in Brazil, where it was introduced in the 1980s
69 and became well established due to the country-wide re-infestation of the *Aedes aegypti*
70 mosquito vector species. During the first six months of 2019, 1,282,183 DENV cases were
71 reported in the Americas, with Brazil reporting a staggering 1,127,244 (88%) of all dengue
72 cases in the continent. To date, no information exists on the genetic composition of the
73 DENV lineage or lineages causing the current epidemic. Here we use portable sequencing to
74 rapidly generate virus genome data from cases occurring in two different are severely-
75 affected municipalities in São Paulo state, Brazil. We find that the 2019 dengue outbreak in
76 Brazil is caused by a newly introduced DENV serotype 2 genotype III (Asian/American) that
77 seems to be replacing previously-circulating DENV2 lineages. We discuss the potential
78 implications of our results regarding the current outbreak in the context of previous outbreaks
79 in the same region.

81 **Introduction**

82 Over 400 million people are estimated to be at risk of acquiring dengue virus (DENV, genus
83 *Flavivirus*, family *Flaviviridae*) [1], a mosquito-borne virus transmitted in tropical and
84 subtropical areas by competent urban vectors such as the mosquitoes *Aedes aegypti* and
85 *Aedes albopictus* [2]. DENV is classified into four distinct virus lineages named serotypes 1
86 to 4 (DENV1-4). Within each DENV serotype there is some degree of genetic variation, and
87 at least 19 DENV genotypes have now been described [3]. Increasing human mobility has
88 facilitated the co-circulation of multiple dengue serotypes in the same region [4], a pattern
89 known as hyperendemicity. In such regions, DENV epidemiological dynamics are complex
90 and typically characterized by virus genotype replacement every 7-10 years [5-9]. Clade
91 replacement is typically associated with an increased number of cases and cases with severe
92 disease. Certain genotypes and lineages seem to be more frequently associated with severe
93 disease outcomes [8, 10, 11].

94

95 DENV was first detected in Brazil in 1982 [12]. Since then, it has become a serious public
96 health concern due to its high incidence in the country and association with severe dengue
97 illnesses [13]. Co-circulation of dengue serotypes has been observed throughout Brazil [14,
98 15], particularly in highly populated areas of the southeastern region that includes the federal
99 states of São Paulo, Rio de Janeiro, Minas Gerais and Espírito Santo. Between 1995 and
100 2015, Brazil reported nearly 8 million DENV cases, which comprises 55% of all cases
101 reported in the Americas during this period [12]. Over the last thirty years, the southeast
102 region of Brazil has reported 2225 dengue-related fatal cases, representing 43% of all
103 dengue-related deaths in the country [13].

104 In the first half of 2019 (1st Jan to 30th Jun) Brazil has already reported 1,127,244 dengue
105 cases [12]. Importantly, this number is nearly 8-fold higher than in the previous year and

106 corresponds to 89% of all the dengue cases reported in the Americas over the same period.
107 The number of severe cases (n=710) and dengue-related deaths (n=366) also increased by at
108 least 2.3-fold in comparison with 2018.

109

110 The Southeast region of Brazil has reported 65.7% of all dengue cases identified in the
111 country [16]. São Paulo state is the most highly densely populated state and the main socio-
112 economic hub in Brazil; previous studies suggest the state was an important source location
113 for the spread of DENV4 in the country [17]. Here, we characterise the genetic diversity of
114 circulating DENV in two municipalities of São Paulo state. We generated virus genome
115 sequences from the ongoing outbreak using a well-established portable genomic approach
116 [18, 19]. In an attempt to better understand the origin and dynamics of the 2019 outbreak, we
117 conducted comprehensive genetic analyses to understand the relationship between the current
118 epidemic strains and those that circulated in previous outbreaks in the Americas.

120 **Methods**

121 Brazil is organized into 26 federal states and 1 federal district. São Paulo state is the most
122 populous Brazilian state and comprises 615 municipalities. São José do Rio Preto is the 11th
123 most populated municipality (450,657 inhabitants), and Araraquara the 32nd most populated
124 municipality (230,770 inhabitants) in the state (www.ibge.gov.br). In each municipality, the
125 number of dengue suspected cases is notified by local public health secretaries to the Centro
126 de Vigilância Epidemiológica "Prof. Alexandre Vranjac" (CVE), part of São Paulo's State
127 Health Secretary. As part of dengue surveillance efforts in São Paulo state, samples are
128 collected from patients suspected of acute DENV infection and tested for DENV by real-time
129 quantitative reverse transcription PCR (qRT-PCR) by several research centers and public
130 health institutions, including Adolfo Lutz Institute. Monthly numbers of dengue cases per
131 serotype are then aggregated by CVE.

132

133 To assess the genetic diversity of dengue cases circulating in São Paulo state, we selected 20
134 qRT-PCR positive samples DENV serotype 2 from patients in two municipalities, Araraquara
135 and São José do Rio Preto. The majority of the samples (19 out of 20) was collected between
136 January and the end of April 2019; however, to investigate whether the same lineage was
137 circulating before 2019, we also included one sample from São José do Rio Preto collected in
138 early June 2017. Samples had mean RT-qPCR cycle-threshold values of 19.8 (range: 16.4 -
139 25). Diagnostic details and symptoms are shown in **Table S1**.

140

141 Residual anonymized clinical diagnostic samples from Araraquara were obtained following
142 ethical approval by Hospital das Clínicas - University of São Paulo's Institutional Review
143 Board (CAPPesq) (number 3.156.894). São José do Rio Preto samples were obtained from
144 virological surveillance routine, within the study approved by University of São José do Rio

145 Preto Institutional Review Board approval #48982/2012. We used residual anonymized
146 clinical diagnostic samples, with no risk to patients, which were provided for research and
147 surveillance purposes within the terms of Resolution 510/2016 of CONEP (Comissão
148 Nacional de Ética em Pesquisa, Ministério da Saúde; National Ethical Committee for
149 Research, Ministry of Health).

150

151 The 20 qRT-PCR–positive DENV2 samples were subjected to viral genomic amplification at
152 the Institute of Tropical Medicine, University of São Paulo, Brazil. Genome sequencing was
153 conducted using the portable nanopore MinION sequencing platform, which has been used
154 previously in Brazil during outbreaks of Zika virus and yellow fever virus [9-11]. Sequencing
155 was performed using a multiplex PCR primer scheme designed to amplify the entire coding
156 region of DENV2 as previously described [12].

157

158 RNA was extracted and reverse-transcribed to cDNA using Superscript IV First-Strand
159 Synthesis System (Thermo Fisher Scientific, MA, US) and random hexamer priming. Then,
160 multiplex PCR was performed to generate overlapping amplicons of the whole genome of the
161 targeted DENV2 strain. DENV2 genome amplification consisted of 35 cycles of PCR
162 according to the reaction mix and thermocycling described by Quick et al [19]. AmpureXP
163 purification beads (Beckman Coulter, High Wycombe, UK) were used to clean up PCR
164 products, which were then quantified by Qubit dsDNA High Sensitivity assay on a Qubit 3.0
165 instrument (Life Technologies). Sequencing libraries were generated using the Genomic
166 DNA Sequencing Kit SQK-LSK108 (Oxford Nanopore Technologies), by pooling, in
167 equimolar proportions, a total of 250 ng of PCR products previously barcoded using the
168 Native Barcoding Kit (NBD103, Oxford Nanopore Technologies, Oxford, UK). The libraries
169 were loaded onto an Oxford Nanopore flow cell R9.4 (FLO-MIN106) and sequencing data

170 were collected for 30 hours. The median number of mapped reads was 45,013 reads per
171 sample, and the generated consensus genomes had a mean coverage of 81% of the genome at
172 20x minimum sequencing depth. Sequencing statistics for each sample are shown in **Table**
173 **S1**. Raw and processed data are available on GitHub (<https://github.com/arbospread>).

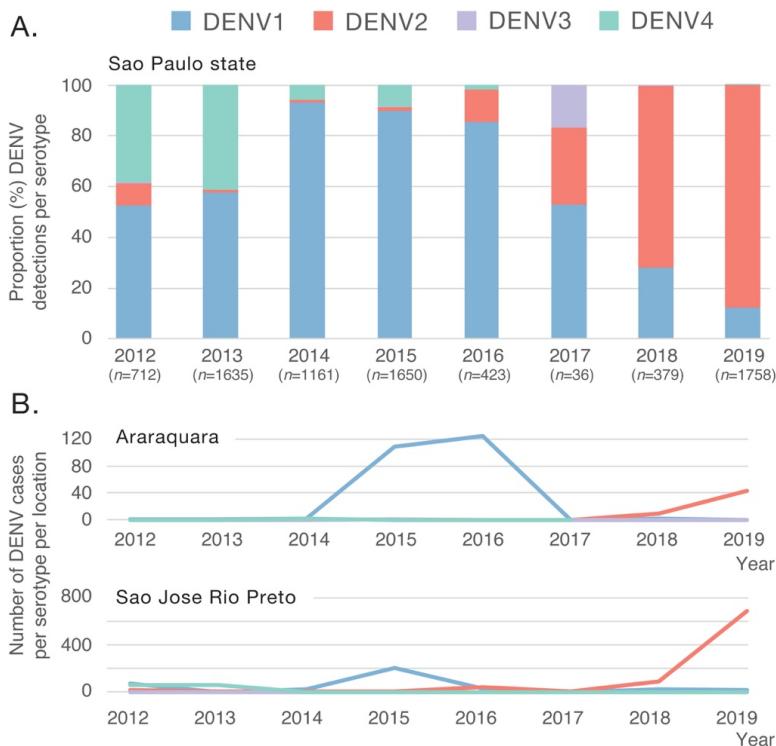
174

175 To investigate the origins of the newly generated genomes we downloaded all DENV2
176 nucleotide sequences longer than 1400 nucleotides (nt) from GenBank [16] that had a known
177 date (year, and month and day when available) and location (country, and city when
178 available; n=1630 as of 20 June 2019). We aligned these sequences using MAFFT automatic
179 settings [18] and manually edited them with AliView v1.19 [19]. We subsequently
180 constructed an initial maximum likelihood phylogeny to help identify the genotypes of strains
181 that have historically circulated (collected before 2019) and are currently circulating
182 (collected in 2019) in Brazil. For this genotype assessment, we constructed phylogenies using
183 FastTree v.2 with gamma-distributed among site rate heterogeneity and a general time
184 reversible nucleotide substitution model [20]. We observed that all sequences from the
185 Americas (including the newly generated sequences) grouped together in a well-supported
186 monophyletic clade. Therefore we next constructed a dataset comprising only sequences
187 collected in the Americas (n=670). To reduce sampling bias towards a high number of
188 samples from well-sampled countries, we removed duplicate sequences (same day and
189 location) from Nicaragua and Peru, yielding a final dataset of 436 genomes (including 66
190 genomes collected in Brazil between 1990 to 2013). Maximum likelihood phylogenies of the
191 American DENV2 genomes (n=670 and n=436) were generated using PhyML [20] available
192 through Seaview v.4.6.1, using gamma-distributed among site rate heterogeneity and a
193 general time reversible nucleotide substitution model [21]. Root-to-tip divergence and
194 temporal signal was evaluated using TempEst [21].

195 Georefenced and time-stamped phylogenies were constructed using a discrete
196 phylogeographic approach as previously described [19, 22]. In brief, countries were grouped
197 into four geographic regions consisting of Brazil (n=86), Central America and Mexico
198 (n=45), South America (n=132) and Caribbean (n=173). Inferred locations at each internal
199 node and corresponding dated phylogenies trees were estimated using BEAST1.10 [23].
200 MCMC convergence was inspected using Tracer.v1.7 and summary trees were generated
201 using TreeAnnotator [23].

203 **Results and Discussion**

204 Epidemiological information on the number of confirmed dengue cases with associated
205 serotype information shows that all four dengue serotypes co-circulate in São Paulo state
206 (**Figure 1A**). While in 2012 DENV1 (52%, n=373/712) and DENV4 (39%, n=274/712)
207 predominated, DENV 2 (9%, n=64/712) and DENV3 (0.1%, n=1/712) were also detected.
208 Since 2014 a notable increase in DENV2 cases is observed, with frequencies increasing
209 rapidly from 1.03% (n=12/1161) in 2014, to 1.39% (n=23/1650) in 2015, 12.53% (n=53/423)
210 in 2016, 30.56% (n=52/423) in 2017, 71.5 (n=271/379) in 2018 to 87.5% (n=1539/1758) in
211 the first semester of 2019 (**Figure 1A**).



212

213 **Figure 1.** Annual number of DENV cases by serotype reported to the Centro Vigilância
214 Epidemiológica (CVE), São Paulo state, Brazil, between January 2012 and June 2019. A.
215 Proportion (percentage) of dengue cases by serotype reported in São Paulo state (total
216 n=7754). B. Number of dengue cases by serotype in Araraquara (total n=294) and in São José
217 do Rio Preto (total n=1347).

218 We next report genomic epidemiological findings from our surveillance of two municipalities
219 in the São Paulo state, Araraquara (ARA) and São José do Rio Preto (SJRP), between early
220 June 2017 and the end of April 2019. In Araraquara, a total of 96% (n=52/54) dengue cases
221 notified to CVE were caused by DENV2 between 2017 and June 2019; in São José do Rio,
222 Preto, during the same period, 95% (n=779/821) were caused by this serotype (**Figure 1B**).
223 From each location, ten RT-qPCR positive samples (mean cycle threshold: 19.8, range: 16.4
224 to 25) were randomly selected for complete genome sequencing using a previously developed
225 amplicon-based approach for on-site sequencing using the minION platform [24, 25].

226
227 All sequenced samples were classified as DENV2 using an automated phylogenetic-based
228 serotyping tool [3]. To investigate the genotypic diversity and the origins of the ongoing
229 DENV2 outbreak in Brazil, we performed phylogenetic analysis of DENV2 using all publicly
230 available complete or partial DENV2 genome sequences (n= 1,630 as of 21 June 2019).

231
232 DENV2 is classified into six genotypes, named I-VI [3]. To date, 3 genetic lineages of
233 DENV2 genotype III (DENV2-III) have been reported in Brazil on the basis of phylogenetic
234 analysis of the relationships of partial and complete genomes of circulating strains. These
235 three circulating lineages have been named as lineages 1-3 [5] or BR1-BR3 [26]. Our
236 maximum likelihood (ML) phylogenetic analysis shows a separate introduction of DENV2,
237 hereafter named DENV2-III BR-4 (**Figure 2A**).
238

239 Our ML phylogenetic analysis further shows that virus genomes recovered from the 2019
240 cases all belong to genotype III (also known as American/Asian genotype). Notably, our
241 analysis strongly supports (approximate likelihood ratio test = 1.00) the clustering of the
242 2019 DENV2 cases from Brazil (18 of the 19 sequences collected in 2019) into a single

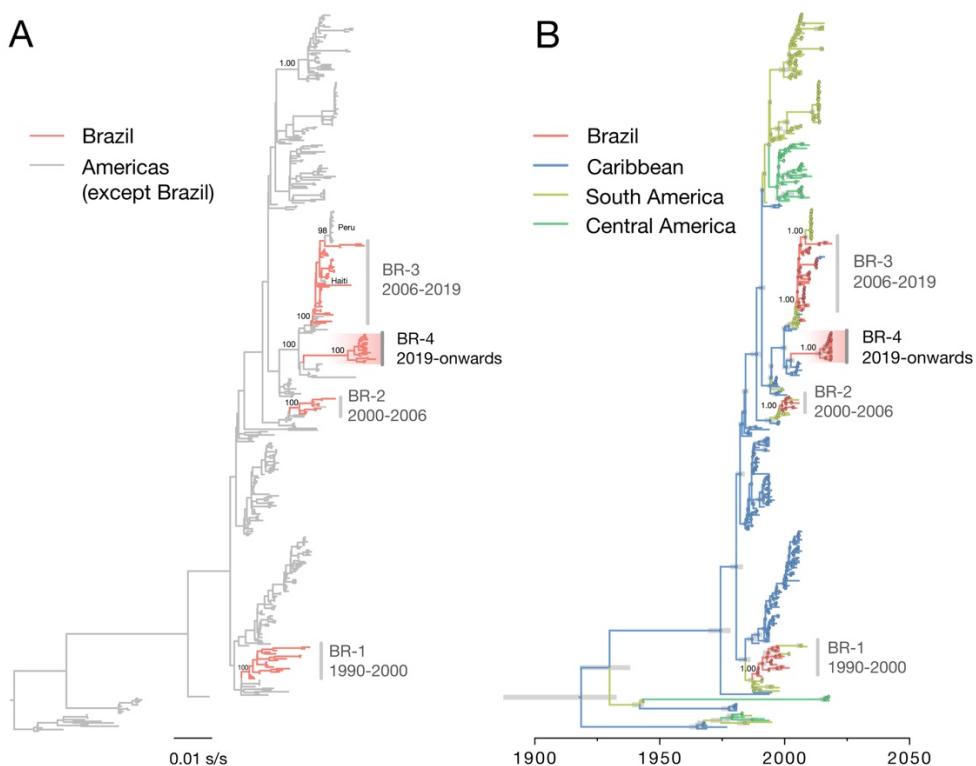
243 monophyletic group (named here as DENV2-III BR-4), which is the result of a new and
244 recent introduction of DENV2-III from outside of Brazil (**Figure 2A**). In addition, two
245 sequences from São José do Rio Preto, one sampled in June 2017 (ID: 126) and another in
246 January 2019 (ID: 140) grouped with the isolates from a previous clade circulating since
247 2006 in the Southeast Region (BR-3).

248
249 To investigate in more detail the origins of the new DENV2-III BR-4 circulating in Brazil in
250 2019, we conducted a statistical phylogeographic analysis of 436 DENV2 genome sequences
251 representing DENV2 genotypes circulating in the Americas. Our analysis reveals that the
252 DENV2-III BR-4 lineage was introduced in or around 2014 (95% Bayesian credible interval:
253 2012 to 2015) (**Figure 2B**). From then onwards, the proportion of DENV2 cases in São Paulo
254 state increased (**Figure 1A**). Estimation of the ancestral location of this lineage reveals that it
255 likely originated in the Caribbean region (posterior support = 1.00). However, we note that
256 the new lineage could have also been introduced from countries with tropical climates in
257 South America (e.g. French Guiana or Suriname) from where no recent virus genomic data
258 has been made available. Molecular clock analyses have shown that novel DENV lineages
259 have been introduced in Brazil every 7 to 10 years, after which they are replaced by a novel
260 lineage introduced from other locations. For example, DENV2-III BR-1 was introduced in
261 1990, DENV2-III BR-2 in 1998, DENV2-III BR-3 in 2005 [5]. Given that the former lineage
262 replacement in Brazil resulted from a strain introduced around 2005, our dating estimate for
263 the introduction of DENV2-III BR-4 and the noticeable increase in number of DENV2 cases
264 in São Paulo state are indicative of a new DENV2 lineage replacement in Brazil.

265
266 Our data reveals that two distinct virus lineages (DENV2-III BR-3 and DENV2-III BR-4) are
267 co-circulating in a single location (São José do Rio Preto) (**Figure 2**). Although we are

268 limited by the small sample size of the data analysed here, it is remarkable that the frequency
269 of DENV2-III 2019 strains belonging to BR-3 is 5.3% (n=1/19) and for BR-4 is 94.7%
270 (n=18/19). The upsurge in the number of dengue and dengue severe cases observed in São
271 Paulo state, combined with the simultaneous detection of these two lineages and the
272 increased frequency of BR-4 suggests that we are capturing a lineage replacement in real-
273 time.

274



275

276

277 **Figure 2. Evolutionary history of DENV2 in Brazil.** Maximum likelihood phylogeny (A)
278 and dated phylogeographic tree (B) of DENV serotype 2 ($n=436$) in the Americas. Tips (and
279 nodes leading to) Brazilian strains are shown in red. New clade comprising isolates from
280 2019 collected in Araraquara and São José Rio Preto (São Paulo state) are highlighted with a
281 red gradient. In panel B, 95% Bayesian credible intervals for node ages are shown for nodes
282 with posterior support above 0.95.

283 The surveillance lag between the detection and estimated date of introduction of the DENV2-
284 III BR-4 could result from a lack of genomic surveillance of dengue in a period when Brazil
285 was hit by explosive epidemics of Zika, chikungunya and yellow fever viruses [27, 28]. This
286 highlights the need of improved longitudinal surveillance using, for instance, sequence-
287 independent approaches for arbovirus detection. Such approaches will be particularly critical
288 in geographic regions with year-round DENV transmission in *Aedes* spp. mosquitoes, such as
289 locations with tropical and subtropical climates [2]. Integration of routine serological,
290 molecular, and genomic data along with improved digital surveillance [29], will help to
291 anticipate the arrival and establishment of new virus lineages and other pathogens in the
292 Americas.

294 **Data availability**

295 XML files and datasets analysed in this study are available in the GitHub repository
296 (<https://github.com/arbospread/DENV2>). New sequences have been deposited in GenBank
297 under accession numbers XXXXX.

298

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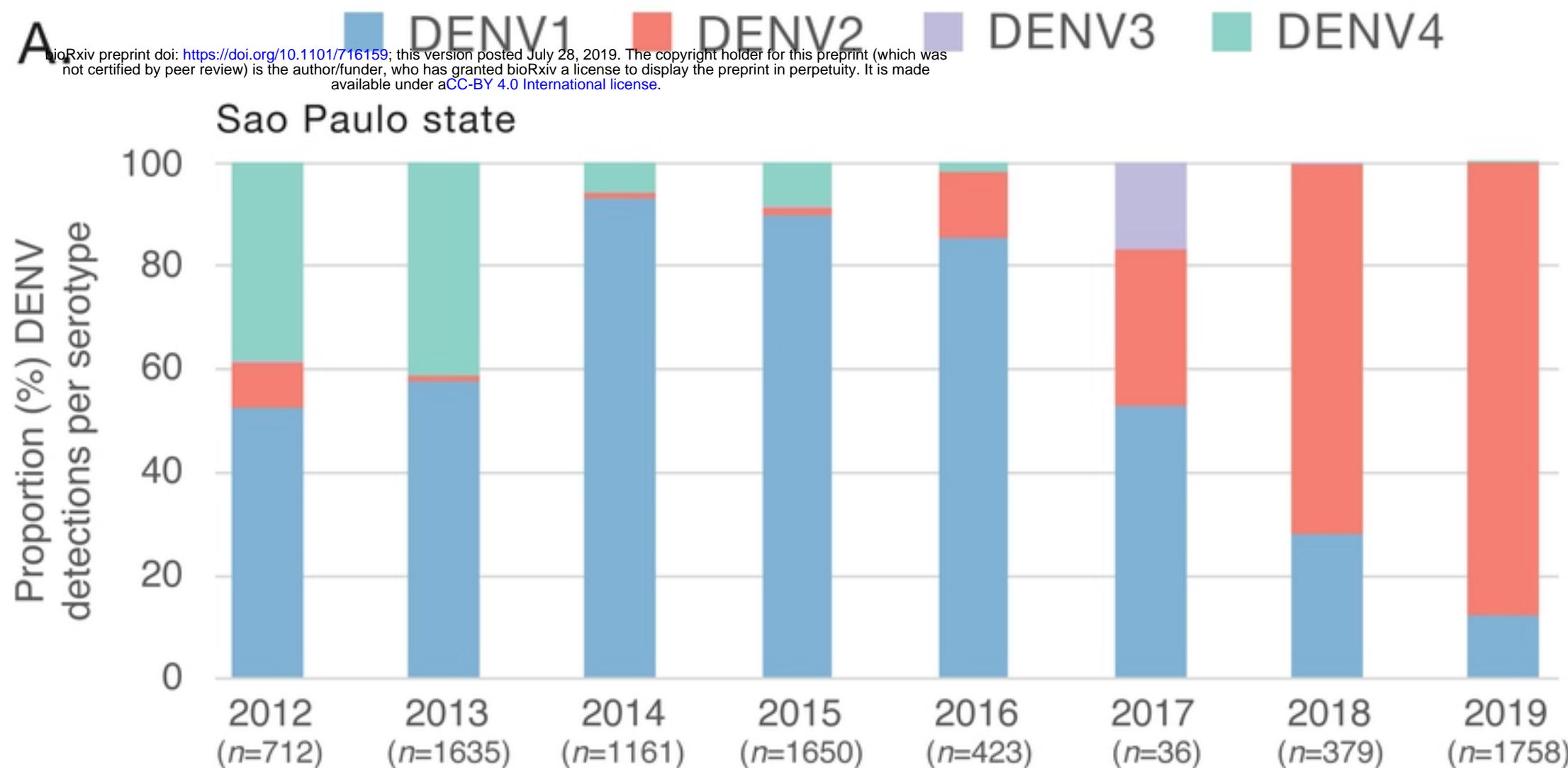
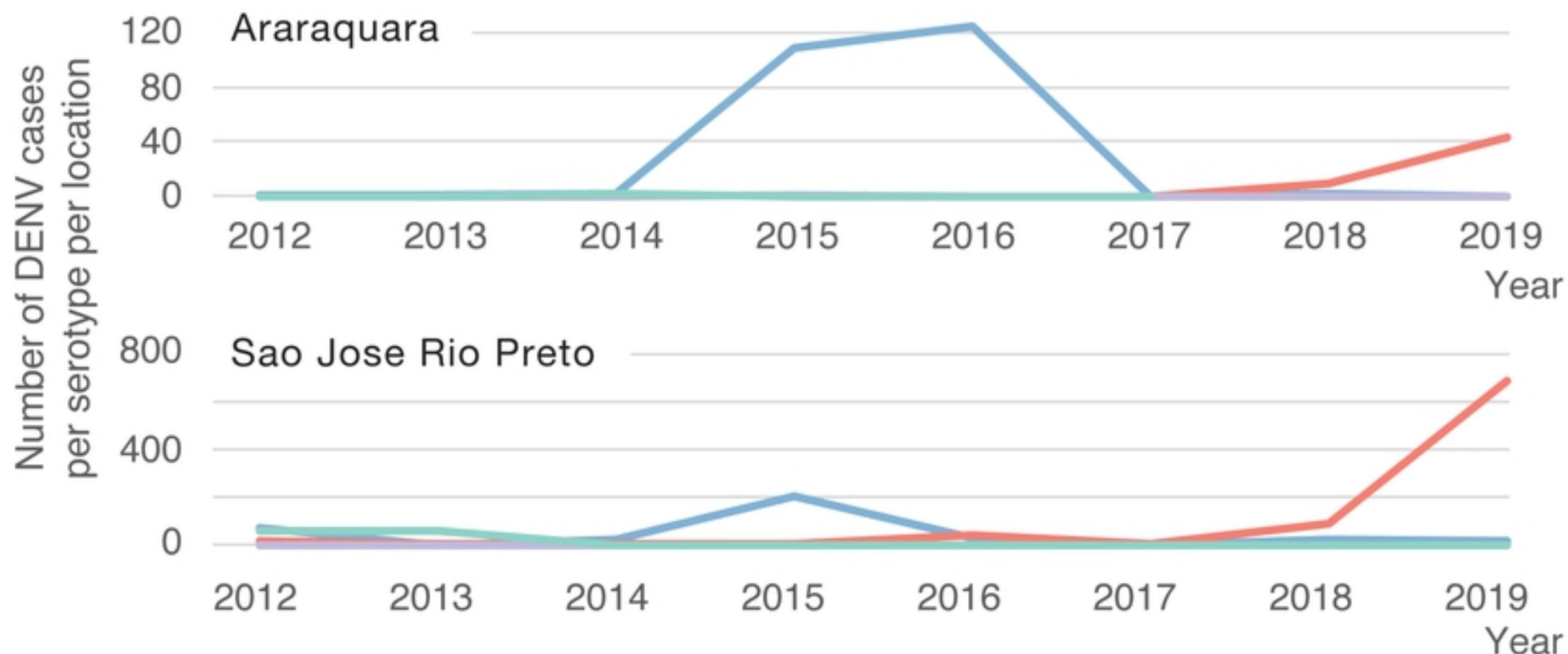
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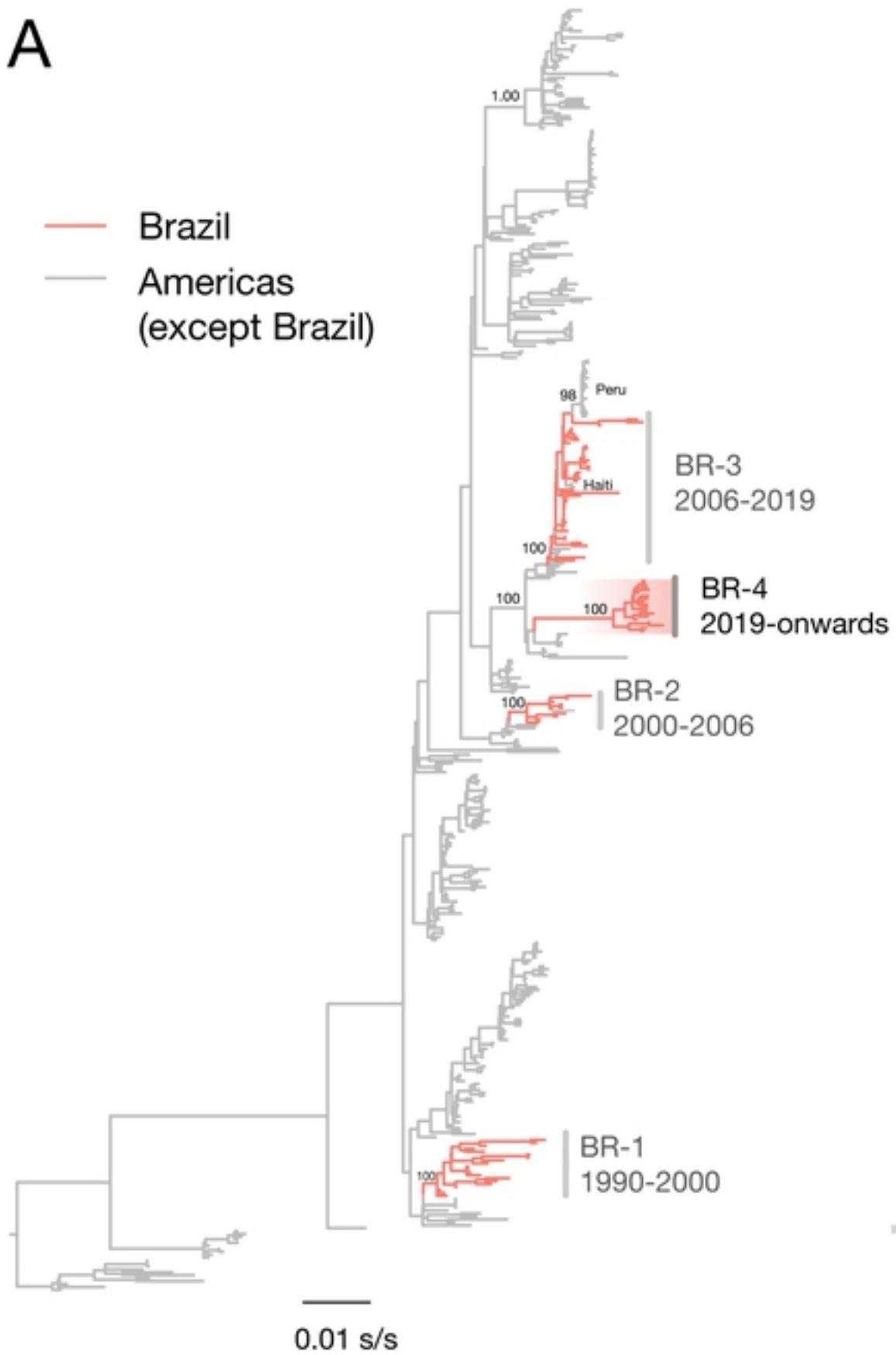
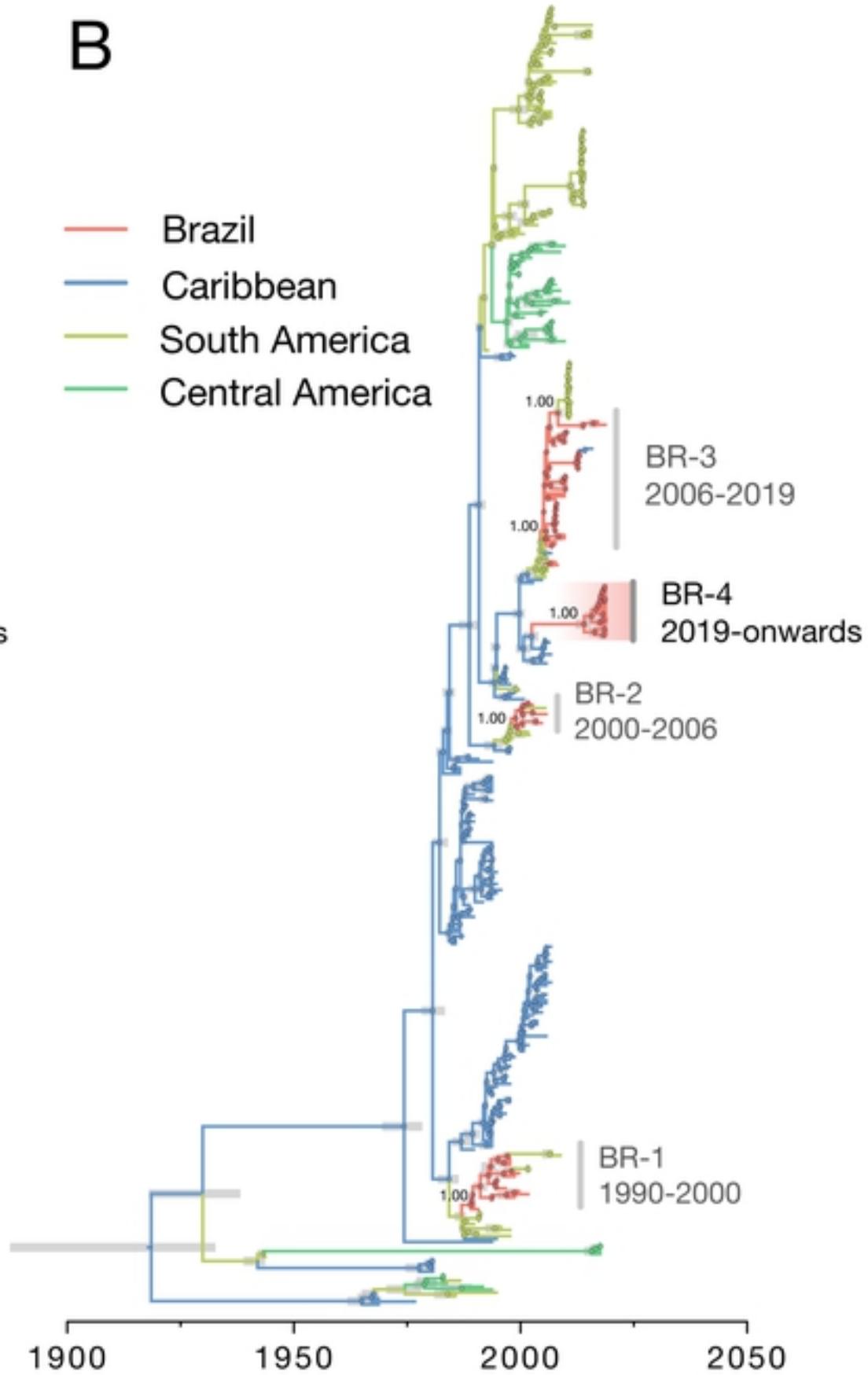
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A.**B.****Figure 1**

A**B****Figure 2**