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Mutation hotspots and spatiotemporal distribution of SARS-CoV-2 lineages in Brazil, February 2020-2021

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**A R T I C L E   I N F O**

Keywords:
- COVID-19
- Severe acute respiratory syndrome coronavirus 2
- Infectious diseases
- High-Throughput Nucleotide Sequencing
- Molecular Epidemiology
- Phylogeography

**A B S T R A C T**

The COVID-19 pandemic has already reached more than 110 million people and is associated with 2.5 million deaths worldwide. Brazil is the third worst-hit country, with approximately 10.2 million cases and 250 thousand deaths. International efforts have been established to share information about **Severe acute respiratory syndrome coronavirus 2** (SARS-CoV-2) epidemiology and evolution to support the development of effective strategies for public health and disease management. We aimed to analyze the high-quality genome sequences from Brazil from February 2020-2021 to identify mutation hotspots, geographical and temporal distribution of SARS-CoV-2 lineages by using phylogenetics and phylodynamics analyses. We describe heterogeneous sequencing efforts, the progression of the different lineages along time, evaluating mutational spectra and frequency oscillations derived from the prevalence of specific lineages across different Brazilian regions. We found at least seven major (1–7) and two minor clades related to the six most prevalent lineages in the country and described its spatial distribution and dynamics. The emergence and recent frequency shift of lineages (P.1 and P.2) carrying mutations of concern in the spike protein (e.g., E484K, N501Y) draws attention due to their association with immune evasion and enhanced receptor binding affinity. Improvements in genomic surveillance are of paramount importance and should be extended in Brazil to better inform policy makers about better decisions to fight the COVID-19 pandemic.

**1. Introduction**

After its initial emergence in China in late 2019 (Huang et al., 2020), **Severe Acute Respiratory Syndrome 2 virus** (SARS-CoV-2) has spread rapidly around the world causing the COVID-19 pandemic (World Health Organization, 2020). New epicenters of the disease have been established throughout 2020, mainly in Europe, USA, and South America (Pei et al., 2020; Ruiu, 2020). As of February 22, 2021, more than 110 million cases and approximately 2.5 million deaths worldwide have been confirmed (Johns Hopkins Coronavirus Resource Center, 2021). Several countries are currently experiencing second waves of infections, decreasing optimism regarding a brief solution to the pandemic.

Since the sequencing of the first SARS-CoV-2 genome (Zhou et al., 2020), international efforts have been established through data sharing in the Global initiative on sharing all influenza data (GISAID) database (https://www.gisaid.org/, Shu and McCauley 2017). These sequencing and metadata information were made public and have enabled the study of the viral spread pattern through space and time. However, sequencing facilities and research investments are very heterogeneous across the world. Asian, European, North American and Oceanian countries have contributed with more data proportionally to the number of cases (Furuse, 2021), while African and South American genomic surveillance have been more limited. Disparities are even deeper on an individual country basis. For instance, as of February 2021, while the United States was Sequencing efforts were extended in Brazil to better inform policy makers about better decisions to fight the COVID-19 pandemic.
Kingdom (UK) leads sequencing efforts (5.85% of cases sequenced), Brazil has sequenced only 0.03% of all cases, despite being the third worst-hit country, with approximately 10.2 million cases and 250 thousand deaths. Currently (mid-July 2021), the UK has expanded sequencing capacity (11.1%), as modestly occurred in Brazil (0.12%). In South America, Chile (0.30%), Ecuador (0.22%), and Uruguay (0.15%) lead sequencing rates, while the remaining countries (except Brazil) that reported > 100,000 cases have < 0.1% sequenced cases (https://www.gisaid.org/, Johns Hopkins Coronavirus Resource Center, 2021).

Many studies were performed to characterize early viral introductions and the transmission dynamics in several countries (e.g. China Lu et al., 2020, USA Deng et al. 2020, Mauarano et al. 2020 and Worobey et al. 2020, Australia Seemann et al. 2020, Italy Bartolini et al. 2020, United Kingdom da Silva Filipe et al. 2021 and du Plessis et al. 2021). In Brazil, SARS-CoV-2 arrived officially on February 25, 2020, in a returning traveller from Italy, and early efforts were made both at the national (Candido et al., 2020a) and regional levels (Paiva et al., 2020; Xavier et al., 2020) to further characterize the viral introduction and spread. B.1 and derived lineages were prevalent in the country at the beginning of the pandemic and significant movements between state borders after international travel restrictions have been demonstrated (Candido et al., 2020a). Unfortunately, little is known about the viral evolution in the entire Brazilian territory after these earliest studies.

More recently, new SARS-CoV-2 lineages have emerged and are considered as “Variants of Concern” (VOC), mainly those carrying mutations in the spike (S) glycoprotein due to its role in binding to the human Angiotensin-converting enzyme 2 receptor (hACE2). Up to July 2021, there are four VOCs described worldwide, namely: B.1.1.7, B.1.351, P.1, and B.1.617.2. More recently, the World Health Organization (WHO) assigned easy to remember labels based on Greek letters, mainly to avoid discriminatory associations related to places of first detection. Therefore, the four VOCs are named, respectively, Alpha, Beta, Gamma, and Delta following the WHO-nomenclature. The former (B.1.1.7) emerged in England in mid-September 2020 and it is characterized by 14 lineage-specific amino acid substitutions, especially N501Y (a key contact residue interacting with hACE2) and P681H (one of four amino acids comprising the insertion that creates a novel furin cleavage site between S1 and S2) (Rambaut et al., 2020b). The second (B.1.351) emerged in South Africa in October 2020 and harbor a constellation of mutations in the Receptor Binding Domain (RBD) (especially K417N, E484K, and N501Y) (Tegall et al., 2021a). The third is P.1, derived from B.1.1.28, a widespread lineage from Brazil. It was reported in returning travelers from Manaus (Amazonas, Brazil), after arriving in Japan. It has the same three mutations (except for K417T instead of K417N) in RBD as B.1.351, but it arose independently (Faria et al., 2021). More recently, the B.1.617.2 lineage, first detected in India, has also been characterized as a VOC, primarily for carrying a constellation of mutations in the spike protein (especially L452R and P681R) (Peacock et al., 2021), its wide spread worldwide even out-performing other VOCS (Mullen et al., 2021), and reduced antibody sensitivity in vaccinated individuals (Planas et al., 2021). Importantly, B.1.351 and P.1 carry the E484K mutation associated with escape from neutralizing antibodies (Baum et al., 2020; Greaney et al., 2020 and Weisblum et al., 2020). Recently, a E484K harboring virus was identified in a reinfected patient (Nonaka et al., 2021) from Brazil, confirming the ability to evade naturally developed antibodies from previous infection as well. Moreover, all three VOC lineages harbor N501Y mutation, already associated with enhanced receptor binding affinity (Starr et al., 2020), which could lead to increased infectiousness.

It is believed that after more than a year of its emergence, some mutations of SARS-CoV-2 (e.g. L452R, E484K, N501Y, P681H/R) have been positively selected, since they may confer adaptive advantages leading to convergent evolution in different lineages spreading across multiple countries (Martin et al., 2021). Despite the low sequencing rate, a deeper analysis of mutations and lineages throughout the Brazilian states would allow a better understanding of viral diversity and spread patterns inside the country. Thus, we aimed to identify mutation hotspots, geographical and temporal distribution of SARS-CoV-2 lineages in the Brazilian territory by using phylogenetics and phylodynamics analyses from high-quality SARS-CoV-2 genome sequences.

2. Materials and methods

2.1. SARS-CoV-2 genomes and epidemiological data retrieval

Complete SARS-CoV-2 genomes (> 29,000 bp) and the associated metadata were obtained from the GISAID database. Considering 2,751 available sequences from Brazil submitted until February 16, 2021, 2,732 were retrieved applying filters for human host and complete collection date. Number of cases per state per day and across Brazil were downloaded from https://covid19br.wcota.me/en/ (Cota, 2020) on the same date. This initiative aggregates data from the Brazilian Ministry of Health and epidemiological bulletins of each federative unit. Geographical maps and general purpose plots were generated using R v3.6.1 (R Core Team, 2020), and the ggplot2 v3.3.2 (Wickham, 2009), geobr v.1.4 (Pereira et al., 2019), and sf v0.9.8 (Pebesma, 2018) packages.

2.2. Mutation analysis

The GenBank RefSeq sequence NC_045512.2 from Wuhan (China) was used as the reference for our analysis. Single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) were assessed by using snippy variant calling pipeline v4.6.0 (https://github.com/tseemann/snippy), which uses FreeBayes v1.3.2 (Garrison and Marth, 2012) as variant caller and snpEff v5.0 (Cingolani et al., 2012) to annotate and predict the effects of variants on genes and proteins. Mutations and lineages were concatenated with associated metadata and counted by Brazilian states using custom Python and R scripts. Histogram of SNPs were generated after running MAFFT v7.471 (Katoh and Standley, 2013) alignment using msasats.py script, plotAlignment and plotSNPHist functions (Du Plessis, 2020).

2.3. Phylogenetics analysis

All available SARS-CoV-2 genomes (537,360 sequences) were retrieved from GISAID on February 16, 2021. These sequences were then subjected to analysis inside NextStrain ncov pipeline (https://github.com/nextstrain/ncov) (Hadfield et al., 2018) through a Brazilian-focused subsampling scheme using time- and worldwide-representative contextual samples.

In this workflow, sequences were filtered out based on high
were visualized using FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/) and ggtree R package v2.0.4 (Yu et al., 2017). We identified global lineages using the dynamic nomenclature (Rambaut et al., 2020a) implemented in Pangolin v2.2.2 (https://github.com/cov-lineages/pangolin).

2.4. Bayesian phylogeographic and phylodynamic analysis

All major clades associated with massive community transmission in Brazil (clades 3–6) as defined by the previous maximum likelihood analysis were included in this analysis. Clade 7 was discarded due to the low correlation of genetic distances and sampling dates (R² = 0.211 × 10⁻², correlation coefficient = 0.1488). Additionally, most of its sequences ranged from 2020.8 and 2021.1 and had high divergence, suggesting important undersampling for this clade (represented by sequences from the P2 lineage). The spatiotemporal diffusion of these important circulating lineages through Brazil were separately estimated for each clade using a Bayesian Markov Chain Monte Carlo (MCMC) approach as implemented in BEAST v1.10.4 (Suchard et al., 2018), using the BEAGLE library v3 (Ayres et al., 2012) to enhance computational time. Time-scaled Bayesian trees were estimated in BEAST using: HKY+Γ nucleotide substitution model (Hasegawa et al., 1985), a strict molecular clock model with a Continuous Time Markov Chain (CTMC) (Ferreira and Suchard, 2008) prior (mean rate = 8 × 10⁻⁴), and a parametric Exponential Growth coalescent tree prior (Griffiths and Tavare, 1994). Although it is not the most appropriate model to reconstruct the demographic and evolutionary processes, it was used given the difficulty of convergence in more complex models due to the lack of sufficient evolutionary information contained in the data. For clade 4, we used a non-parametric Bayesian Skygrid prior with 10 parameters (Gill et al., 2013).

Viral migrations across time were reconstructed using a Brownian random walk continuous phylogeographic model (Lemey et al., 2010; Pybus et al., 2012) to generate a posterior distribution of 1,000 trees. We assigned sampling coordinates using the centroid point of each municipality (when available) and the centroid of the Brazilian state’s capital (when the municipality was unavailable) using a random jitter window size of 0.01 to add a small amount of noise to duplicated sampling coordinates assigned to the tips of the tree.

Two MCMC chains were run for > 100 million generations and convergence of the MCMC chains was inspected using Tracer v1.7.1 (Rambaut et al., 2018). After removal of 10% burn-in, log and tree files were combined using LogCombiner v1.10.4 (Suchard et al., 2018). Maximum clade credibility (MCC) trees were generated using TreeAnnotator v1.10.4 (Suchard et al., 2018). The seraphim B package (Deliour et al., 2016) and SPREAD3 (Bielejec et al., 2016, p. 3) were used to extract and map the spatiotemporal information embedded in the MCC trees. Finally, these trees were visualized using the ggtree R package v2.0.4 (Yu et al., 2017).

3. Results

3.1. Distribution of Brazilian sequences through time and space

Sequencing efforts from Brazil were concentrated mainly in the first epidemic wave (March to April, 2020) (Fig. 1A and 1B). In March, 503 genomes (8.64% of the confirmed cases) and in April, 942 sequences (1.16% of the cases) were sampled. All following months had a sequencing rate below 1% (Table S1). All Brazilian states sequenced less than 0.1% of the confirmed cases through the first year of the pandemic (Fig. 1C). From the Southeast region, the states of Rio de Janeiro (0.09%) and São Paulo (0.06%) have led the country’s sequencing initiatives, followed by Rio Grande do Sul (0.045%) from the South region, Amazonas (0.041%) from the North region and Pernambuco (0.040%) from the Northeast region. The Centre-West was the region with the lowest sequencing rate (Fig. 1C, Table S2).

The Southeast region contributed with 1,704 genomes (62.53%), followed by Northeast (n = 359; 13.17%), South (n = 319; 11.71%), North (n = 310; 11.38%), and Centre-West (1.21%) regions (Fig. 1D, Table S3). In total, 59 different lineages were detected in Brazil, and the states sequencing more genomes (São Paulo, Rio de Janeiro and Rio Grande do Sul) detected higher numbers of circulating lineages (33, 17 and 16, respectively) (Fig. 1E, Table S3). Importantly, all states that sequenced at least 100 genomes identified ≥ 10 lineages (Table S3).

3.2. High frequency mutations

A total of 3,919 mutations were detected across the 2,731 Brazilian genomes and only 354 (12.96%) occurred in >5 sequences, 44 (1.61%) in >50 genomes, and 38 (1.39%) in >100 sequences (Fig. 2, Table 1). Twenty-five (65.79%) of these 38 mutations were non-synonymous. Of these, 11 (44.0%) were in the spike protein, 5 (20.0%) in the nucleocapsid protein, and 5 (20.0%) in the ORF1ab polyprotein (Table 1).

Three mutations were found in > 95% of the genomes: A23403G (S: D614G), C14408T (ORF1ab:L4715), and C3037T (ORF1ab:F924), which are signatures of the B.1 and derived lineages that spread early in the pandemic. The adjacent replacement GGG28881AAC (NG:RG203-204KR) was found in 85.17%, representing a clear signature of B.1 lineage. The defining-mutations of the Brazilian most widespread lineages (B.1.1.28 and B.1.1.33) were also found in high abundance. The G25088T (SV:1176F) replacement from B.1.1.28 occurred in 47.56% of all sequences, while T29148C (N:I292T) and T27299C (ORF6:I33T) from B.1.1.33 in ~32.5%. The G23012A (S:E484K) mutation in the Receptor Binding Domain (RBD) of spike that recently emerged independently in three Brazilian lineages (B.1.1.33, P.1 and P.2) is already among the most frequent detected up to February, 2021 (11.42%). The viruses containing the E484K mutation have been spreading mostly between mid-2020 up to early-2021. Additionally, the multiple lineage-defining mutations found in emergent P.1 and P.2 lineages from Brazil were observed in > 100 and > 200 genomes, respectively (Fig. 2, Table 1).

3.3. Lineage dynamics of Brazilian epidemic

In March 2020, the majority of Brazilian sequences belonged to 3 lineages: B.1 (n = 101; 20.08%), B.1.1.28 (n = 156; 31.01%) and B.1.1.33 (n = 131; 26.04%). The first was probably introduced in Brazil through multiple imports from other continents, and the others probably emerged from community transmission inside the country (Candido et al., 2020a). Between April and August, > 75% of all sequences per month were classified as B.1.1.28 or B.1.1.33. During October and November, the B.1.1.28 derived lineage P.2 was the most prevalent (n = 32; 37.65% and n = 92; 40.71%), while B.1.1.28 and B.1.1.33
represented together < 50% of the genomes. From December 2020 onward, the VOC P.1 emerged in Manaus and has been established, together with P.2, as the most prevalent lineages represented by sequencing data (Fig. 3A).

Regarding distribution between the five different Brazilian regions, the Southeast and Southern regions sequenced a larger proportion of B.1.1.28 and B.1.1.33 viruses. The Northeast apparently has a slightly different dynamics, since B.1.1.74 \((n = 138; 38.44\%)\) is the most prevalent lineage followed by B.1.1.33 \((n = 91; 25.35\%)\). In the Northern region, P.2 is already the second most prevalent lineage \((n = 81; 26.13\%)\) (Fig. 3B and 3C), but this may be related to the low quantity of sequences from the beginning of the pandemic, and the higher surveillance in the region at present due to enhanced sequencing efforts after the emergence of P.1. In the Centre-West, the extremely low sequencing rate prevents us from making any assumptions about the genetic diversity of the circulating lineages (Fig. 3B).
Statewide view shows the wide distribution of lineages B.1.1.28, B.1.1.33 and P.2 across almost all Brazilian states. Although the P.1 lineage has been represented by genomes from only 7 states (AM, PA, RS, RO, RR, SC, and SP) up to February 16, 2021, other states have already reported its detection after this date. States reporting a higher proportion of B.1 lineage (CE, GO, MG, SC, and SE) have concentrated their sequencing efforts mainly in the early phase of the pandemic. B.1.1.74 is overrepresented (> 50%) in two states from the Northeast (PE and PB), suggesting a more geographically restricted distribution. Additionally, the contribution of other lineages that are not among the 10 most frequent in the country are apparently limited (Fig. 3 C and Fig. S1).

The median time between the first and last detection of the 18 lineages represented by more than five Brazilian genomes was 179.0 days (~6 months). However, the standard deviation was high (119.58 days). Eleven of the 18 lineages were sampled in different states between the first and last detection, suggesting its spread through the Brazilian territory (Table S4). B and B.1 lineages have presented a relatively low time of spread, while several B.1.1 derived lineages probably have spreaded for a longer period (e. g, B.1.1.143, B.1.1.28, B.1.1.314, B.1.1.33, B.1.1.74, B.1.1.94) (Fig. S2). B.1.1.7 (Alpha) was firstly detected in the country in mid-December (Claro et al., 2021), while P.1 was found in November in Manaus (Amazonas, Brazil) and Japan (Faria et al., 2021). P.2 was discovered in mid-April 2020, but has been increasing in frequency since August 2020 (Fig. 3A, Fig. S2).

3.4. Phylogenetic analysis of SARS-CoV-2 genomes from Brazil

We obtained 10,573 time-, geographical- and genetic-representative genomes to proceed with phylogenetic inferences. Of these, 1,135 were from Africa, 1,963 were from Asia, 3,248 from Europe, 612 from North America, 285 from Oceania, and 3,330 from South America. Among the latter, 2,350 were from Brazil.

The maximum likelihood tree showed the evolutionary diversity of SARS-CoV-2 sequences across Brazilian territory (Fig. 4A). Almost all sequences from Brazil harbor the S:D614G and ORF1b:P314L, which were imported from other continents to Brazil (mainly to Southeastern states) in the early epidemic wave of COVID-19. After the importation of B.1.1 lineages characterized by N:R203K and N:G204R mutations, community transmission massively occurred and gave rise to B.1.1.28 (S:V1176F) and B.1.1.33 lineages (ORF6:I33T and N:I292T), widely distributed in Brazilian regions along the first year of the pandemic (Fig. 4 A and 4 B). B.1.1.28 has diversified into two lineages, P.1 (20J/501Y.V3) and P.2, which are already widely represented by many sequences and distributed across all regions. The larger branch length leading to P.1 lineage draws attention and its emergence is probably driven by an accelerated molecular evolution (Fig. 4B and 5A).

We estimated an evolutionary rate of 7.76 \( \times 10^{-4} \) substitutions per site per year (23.22 mutations per year) for the Brazilian-focused sub-sampling (Fig. 5A). Since the end of 2020, two evolutionary patterns are observed in the root-to-tip regression of sampling dates. Despite the
majority of sequences following the expected substitution rate for SARS-CoV-2, we observe a rise in P.1 (20J/501Y.V3) and B.1.1.7 (20I/501Y.V1) abundance, both characterized by an abnormal clock rate (Fig. 5A) and a constellation of mutations in the spike protein. Time-resolved maximum likelihood tree highlighted the importation of lineages in the early phase of the pandemic and its rapid diversification through community transmission inside the country, leading to the spread of two main lineages (B.1.1.28 and B.1.1.33) within and between state borders (Fig. 5B) and a more restricted circulation of B.1.1.74 lineage in the Northeast. A time-resolved phylogeny considering only Brazilian sequences reinforces the nationwide distribution of multiple SARS-CoV-2 lineages and clades (Fig. 5C and Fig. S3).

Table 1

| REF | Genomic position | ALT | Effect | Amino acid | Gene | Product | Number of sequences | % in Brazilian genomes | Major associated lineages |
|-----|------------------|-----|--------|------------|------|---------|---------------------|------------------------|--------------------------|
| A   | 23403            | G   | Missense | D614G      | S    | Surface glycoprotein | 2651                | 97.07                   | B.1 and derived          |
| C   | 14408            | T   | Synonymous | L4715L      | ORF1ab | RdRp         | 2648                | 96.96                   | B.1 and derived          |
| C   | 3037             | T   | Synonymous | F924F       | ORF1ab | nsP3         | 2635                | 96.48                   | B.1 and derived          |
| C   | 241              | T   | Intergenic | -           | -    | N           | 2374                | 86.93                   | B.1 and derived          |
| GGG | 28881            | AAC | Missense | RG203-204KR | N    | phosphoprotein      | 2326                | 85.17                   | B.1.1 and derived        |
| G   | 25088            | T   | Missense | V1716F      | S    | Surface glycoprotein | 1299                | 47.56                   | B.1.1.28, B.1.1.143,     |
| T   | 29148            | C   | Missense | L292T       | N    | N           | 888                 | 32.52                   | B.1.1.33, B.1.1.314, N.1, N.4 |
| T   | 27299            | C   | Missense | I33T        | ORF6  | N            | 885                 | 32.52                   | B.1.1.33, B.1.1.314      |
| T   | 12053            | T   | Missense | L3930F      | ORF1ab | nsP7         | 761                 | 27.87                   | B.1.1.28, B.1.1.74,      |
| G   | 23012            | A   | Missense | E484K       | S    | Surface glycoprotein | 312                 | 11.42                   | B.1.1.33, P.1, P.2       |
| T   | 26149            | C   | Missense | S2353P      | ORF3a | ORF3a protein   | 249                 | 9.12                    | B.1.1.28, P.1            |
| A   | 6319             | G   | Synonymous | P2018P      | ORF1ab | nsP3         | 244                 | 8.93                    | B.1.1.33, P.1            |
| C   | 28253            | T   | Synonymous | F120F       | ORF8  | ORF8 protein   | 222                 | 8.13                    | B.1.1.28, B.1.1.33, P.2  |
| G   | 28975            | T   | Missense | MG34I       | N    | N            | 217                 | 7.95                    | B.1.1.28, P.2            |
| G   | 28628            | T   | Missense | A119S       | N    | N            | 211                 | 7.73                    | P.2                      |
| C   | 11824            | T   | Missense | L3653I      | ORF1ab | nsP6         | 207                 | 7.58                    | P.2                      |
| T   | 10667            | G   | Missense | L3468V      | ORF1ab | 3C-like proteinase | 206                | 7.54                    | P.2                      |
| A   | 12964            | G   | Synonymous | G4233G      | ORF1ab | nsP9         | 176                 | 6.44                    | P.2                      |
| A   | 6613             | G   | Synonymous | V2116V      | ORF1ab | nsP3         | 137                 | 5.02                    | B.1.1.28, P.1            |
| GTCGGTTTT | 11287        | G   | Deletion | 3675-3677   | ORF1ab | nsP6         | 130                 | 4.76                    | B.1.1.7, P.1             |
| C   | 29754            | T   | Intergenic | -           | -    | N            | 117                 | 4.28                    | P.2                      |
| AGTAGGG | 28877       | TCTAAC | Missense | RG203-204KR | N    | phosphoprotein      | 116                 | 4.25                    | B.1.1.28, P.1            |
| C   | 21614            | T   | Missense | L188F       | S    | Surface glycoprotein | 116                 | 4.25                    | B.1.1.28, P.1            |
| G   | 17259            | T   | Missense | S5665I      | ORF1ab | Helicase       | 116                 | 4.25                    | P.1                      |
| A   | 23063            | T   | Missense | N501Y       | S    | Surface glycoprotein | 115                 | 4.21                    | B.1.1.7, P.1             |
| C   | 21638            | T   | Missense | P26S        | S    | Surface glycoprotein | 113                 | 4.14                    | P.1                      |
| T   | 733              | C   | Synonymous | D156D       | ORF1ab | Leader protein  | 111                 | 4.06                    | P.1                      |
| C   | 13860            | T   | Missense | T4532I      | ORF1ab | RdRp         | 111                 | 4.06                    | P.1                      |
| C   | 24642            | T   | Missense | T1027I      | S    | Surface glycoprotein | 111                 | 4.06                    | P.1                      |
| C   | 12778            | T   | Synonymous | Y4171Y      | ORF1ab | nsP9         | 111                 | 4.06                    | P.1                      |
| C   | 21621            | A   | Missense | T20N        | S    | Surface glycoprotein | 111                 | 4.06                    | P.1                      |
| C   | 28512            | G   | Missense | P80R        | N    | phosphoprotein      | 110                 | 4.03                    | P.1                      |
| A   | 5648             | C   | Missense | K1795Q      | ORF1ab | nsP3         | 109                 | 3.99                    | P.1                      |
| C   | 23525            | T   | Missense | H655Y       | S    | N            | 109                 | 3.99                    | P.1                      |
| G   | 28167            | A   | Missense | E92K        | ORF8  | N            | 108                 | 3.95                    | P.1                      |
| G   | 21974            | T   | Missense | D138Y       | S    | N            | 107                 | 3.92                    | B.1.1.33, P.1            |
| C   | 22132            | T   | Missense | R190S       | S    | N            | 105                 | 3.84                    | P.1                      |
| C   | 2749             | T   | Synonymous | D828D      | ORF1ab | nsP5         | 105                 | 3.84                    | P.1                      |

Ref: Reference nucleotide(s); ALT: Replaced nucleotide(s); UTR = Untranslated region; ORF= Open reading frame; S: Spike; N: Nucleocapsid; nsP: nonstructural protein; RdRp: RNA-dependent RNA polymerase.
and Northern regions (Fig. 6C). Clade 3 is represented by 804 genomes, mostly from B.1.1.33 lineage. It reached an unprecedented dissemination through Brazil since March 2020, whose introductions in different regions and states were mostly driven by the Southeast followed by massive community transmission and the establishment of local clusters (Fig. 6D). Clade 4 (n = 107) is characterized by B.1.1.74 sequences and it is more geographically restricted to the Northeast, especially Pernambuco (n = 41) and Paraiba (n = 23). However, it accounts for occasional introductions to Southern and Southeastern regions (Fig. 6E). Clade 5 harbors B.1.1.28 (Clade 5.1) and P.1 (Clade 5.2) sequences. B.1.1.28 sequences are mostly widespread in Northern and Southeastern regions (Fig. 6F), and P.1 genomes (n = 96) are mostly found in Northern Brazil, especially Amazonas (n = 50) and Rondônia (n = 15), and Southeast (São Paulo; n = 17) (Fig. 6H). Clade 6 is also represented by genomes that fall into the B.1.1.28 lineage (n = 529). However, it is more widespread in the Southeast (n = 436), especially in São Paulo (n = 412) and
in the Southern region \((n = 72)\), especially Rio Grande do Sul \((n = 65)\) (Fig. 6G). Clade 7 is highly supported by sequences of P.2 lineage \((n = 204)\). This clade is also widely distributed throughout Brazilian regions, especially the Southeast \((n = 83)\), South \((n = 63)\), and North \((n = 42)\), even giving rise to new local clusters in the end of 2020 and early-2021 (Fig. 6H).

### 3.5. Phylogeographic and phylodynamic patterns of SARS-CoV-2 spread in Brazil

Considering the four major clades mostly represented by Brazilian sequences and with sufficient temporal signal (clades 3 to 6), we reconstructed their spatiotemporal diffusion in the Brazilian territory.

Clade 3 (lineage B.1.1.33) presented a median evolutionary rate of \(5.11 \times 10^{-4}\) (95% Highest Posterior Density (HPD): \(4.68 \times 10^{-4}\) to \(5.57 \times 10^{-4}\)). The phylogeographic reconstruction suggests multiple introductions of this clade in different Brazilian regions followed by massive intrastate spread and few transmissions to surrounding states (Fig. 7A, Fig. S5A, Video S1). It can be illustrated in the MCC tree, which shows five subclades, two mostly represented by sequences from the Southeast, one from the South, one from the Northeast and the other from the North (Fig. SSA).

Clade 4 (lineage B.1.1.74) probably emerged in the Southeast and rapidly spread to Northeast and Southern regions, dispersing progressively from state to state in these regions. A new introduction into the Northeast occurred around September 2020, seeding transmission events to neighboring Northern states (Fig. 7B, Fig. S5B, Video S2). This clade had a median evolutionary rate of \(6.66 \times 10^{-4}\) (95% HPD: \(5.49 \times 10^{-4}\) to \(7.79 \times 10^{-4}\)) and is more geographically restricted to the Brazilian states in the eastern coast, especially states from the Northeast.

Clade 5 (lineages B.1.1.28 and P.1) had a median evolutionary rate of \(4.92 \times 10^{-4}\) (95% HPD: \(3.94 \times 10^{-4}\) to \(5.82 \times 10^{-4}\)). This estimate led to an older dating (2018.63, 95% HPD: 2018.03 to 2019.14) of the most recent common ancestor (TMRCA) and instabilities in the tree rooting. Thus, time- or rate-related inferences for this clade should be considered with extreme caution. A hallmark of this clade is its rapid diffusion to all five Brazilian regions (until around May 2020), followed by massive local transmission within the same state or neighboring states in the
Fig. 5. Molecular clock estimates of SARS-CoV-2 genomes from Brazil. (A) Root-to-tip regression of genetic distances (in number of mutations) against sampling dates filtered by Brazilian sequences. States belonging to each specific Brazilian region are colored using similar colors (Centre-West: yellow; North: red; Northeast: pink and gray; South: blue; Southeast: green). Nextstrain clades are represented in key branches. (B) Time-resolved Maximum Likelihood phylogenetics tree of the 10,573 worldwide genomes included colored by Brazilian states. (C) Maximum likelihood phylogenetics tree considering only the dynamics inside Brazil. In (B) and (C), state colorings follow the same scheme as (A), except for Northeast, where purple and pink colors define sequences from this region. Tree topology remains the same for these two trees, but node ordering is slightly different.
same region, with higher prevalence in Amazonas (North) and São Paulo (Southeast) (Fig. 7C, Fig. S5C, Video S3).

Clade 6 (B.1.1.28) had a median evolutionary rate of $6.06 \times 10^{-4}$ (95% HPD: $5.46 \times 10^{-4}$ to $6.37 \times 10^{-4}$). Each of the three subclades within this clade are homogeneously distributed in states from the same region (Fig. SSD) and phylogeographic estimates highlighted early introductions in the Southeast, Southern and Northeast regions seeding mostly intra and interstate spread, mainly in the Southeast and Southern Brazil over 2020 (Fig. 7D, Video S4).

4. Discussion

Viral sequencing is essential to track viral evolution and spread patterns. Despite the initial efforts to obtain a representative genomic dataset of the Brazilian first epidemic wave to better characterize viral introductions and early spread (Candido et al., 2020a), the initiatives across different regions have been limited and non-uniform after on. Interestingly, the previously described clades (Candido et al., 2020a) have spread and diversified through the country. Clade 1 (after named B.1.1.28) was mostly restricted to the Southeast (São Paulo) and Clade 2 (after named B.1.1.33) was already present in 16 states in this early phase. In this work, we showed the emergence of at least four clades derived from B.1.1.28. The first was widely distributed in Brazilian regions (Clade 5.1) and evolved to P.1 lineage (Clade 5.2). The second (Minor clade 5.3) and third (Clade 6) are most widespread in the Southeast, accounting for a few introductions in other regions. The fourth gave rise to P.2 lineage (Clade 7), which is distributed in all Brazilian regions. B.1.1.33 continues to be composed of a larger clade (Clade 3) with a wide distribution among all Brazilian regions.

The B.1.1.33 lineage was studied in further detail using 190 genomes from 13 Brazilian states, showing its variable abundance in different states (ranging from 2% in Pernambuco to 80% in Rio de Janeiro), and
its moderate prevalence in South American countries (5–18%). Surprisingly, this lineage was firstly detected in early-March in other American countries (e.g., Argentina, Canada, and USA), and additional analysis suggest that an intermediate lineage (B.1.1.33-like) most probably arose in Europe and was later disseminated to Brazil, where its spread gave origin to lineage B.1.1.33 (Resende et al., 2021) and possibly seeded secondary outbreaks in Argentina and Uruguay (Mir et al., 2021; Resende et al., 2021).

The states of Pernambuco (Northeast) and Minas Gerais (Southeast) presented more restricted viral dynamics. In Pernambuco, 88% of 101 early sequences were classified as lineage B.1.1 and six local B.1.1 clades were seeded through both national and international traveling (Paiva et al., 2020). This finding is consistent with the prevalence of B.1.1.74 lineage found in the Northeast, especially in Pernambuco. In Minas Gerais, 92.5% of the 40 genomes from March 2020 belong to the B lineage (mostly B.1.1) and epidemiological analysis revealed that the distribution of cases and deaths was more spatially uniform, while in other Southeastern states it was more centralized around capital cities (Xavier et al., 2020).

Studies from Rio de Janeiro (Southeast) and Rio Grande do Sul (South) identified B.1.1.28 and B.1.1.33 in higher proportion from April to December 2020 (Franceschi et al., 2021; Francisco Jr et al., 2021; Voloch et al., 2021). The emergence of a B.1.1.28-derived lineage carrying the E484K mutation (later named P.2) was dated in July 2020, however it began to appear more frequently and almost simultaneously in October 2020 in the Rio de Janeiro state (Voloch et al., 2021) and in the small municipality of Esteio, Rio Grande do Sul (Franceschi et al., 2021), suggesting its wide distribution in the Southern and Southeastern regions of Brazil and uncertainty regarding its origin. This assumption and the frequency increase of B.1.1.28 and derived lineages were
chronically infected with SARS-CoV-2 and treated with convalescent plasma have been linked to viral breakthroughs caused by mutant viruses (Kemp et al., 2021). Whichever phenomena allow for faster viral evolution, they probably have to permit multiple substitutions to occur almost simultaneously, since our molecular clock analysis shows constant and relatively slow rates of mutation accumulation, except for VOC viruses.

Although the proximal origins of the most important VOCs remain to be determined, some conclusions about their nature could be already drawn. First, the mutations shared between P.1 and B.1.1.351 seem to be associated with a rapid increase in cases even in locations where previous attack rates were thought to be very high (Buss et al., 2020). Lineage P.1, which emerged from the Brazilian state of Amazonas between November and December 2020, has accumulated a high number of non-synonymous mutations and is now dispersed across novel Brazilian regions, representing one of the most frequent lineages up to February 2021 (Faria et al., 2021; Naveca et al., 2021). Second, the fact that the set of mutations shared by P.1, B.1.1.7 and B.1.351 seem to have arisen independently, as previously demonstrated with emergence of E484K in others Brazilian lineages (P.2, B.1.1.28 and B.1.1.33), is suggestive of convergent molecular evolution (Faria et al., 2021; Ferrarzce et al., 2021).

Our study shows that the Brazilian territory was affected by at least 59 different lineages during the first year COVID-19 pandemic. This is not completely unexpected, considering the size of the country and its touristic and economical relevance. South Africa, the original source of SARS-CoV-2, had many sub-populations, indicating that the sequence was already diverse before the pandemic. Almost all Brazilian sequences harbor S:D614G, a hallmark of the ancestral B.1 lineage. Although this mutation is outside the RBD, it is speculated that it abolishes the hydrogen bond between the 614 position in S1 and a threonine residue located at S2 from the neighbour protomer. In consequence, RBD would be locked in its activated conformation and decreased post-vaccination neutralizing activity (Li et al., 2021; Nelson et al., 2021).

Our phylogeographic reconstruction demonstrated the widespread dissemination of multiple SARS-CoV-2 lineages inside Brazil and the major role of intrastate diffusion of the most prevalent lineages. The evolutionary rate estimates for clade-specific MCC trees (especially clades 3, 5, and 6) were significantly smaller than previous findings (8 to 9 × 10^{-4} subst/site/year) (Rambaut, 2020; Su et al., 2020). These differences have contributed to an older dating of the MRCA, since the accumulation of more mutations is expected to occur in a wider timeframe. While these older MRCA datings are probably not realistic in the context of the COVID-19 pandemic, they highlight issues with the data collection process. Potential explanations for this behavior are: related samples having the same age (phylodynamic clustering), along-lineage rate variation and non-random sampling (Tong et al., 2018).

The dispersion model used is well suited for spread over land, in opposition to long distances traveled by plane, a common practice in the
Brazilian territory due to its continental size. The major consequence is that inferences of the nodes locations near to the root of the tree must be analyzed very cautiously. For example, in Fig. 7C the green ellipses in the northwest-southeast direction are a consequence of sparse sampling and nodes representing travel events between the North and Southeast. Therefore, the strong uncertainty in these estimates (large ellipses) reflects limitations of both the data and the employed model. Despite its limitations, more local dispersal routes represented here closer to terminal nodes were well captured by the phylogeographic model, which was the main objective of this study since there was a greater contribution of intra- and interstate diffusion after the pandemic was established in the country.

Unfortunately, incomplete and erratic sequencing efforts have limited a better SARS-CoV-2 characterization in Brazil, since genomes are not equally distributed in geographical or temporal scales due to episodic sampling efforts prompted by resource availability. This is reflected by a very small fraction of SARS-CoV-2 cases being sequenced. Unequal temporal distribution implies that some of the conclusions are disproportionately affected by events in heavily (March-May 2020) and poorly (June 2020 onward) sampled periods. Therefore, different lineage age distributions could be an artefact of distinct sequencing coverage among states and across different time frames. Importantly, as Brazil is currently the new epicenter of the pandemic and identified variants of concern and interest, the country initiatives doubled the quantity of genomes deposited in GISAID (5,468 in April 8th vs 2,751 used in this analysis until February 16). Only 420 of these were collected between mid-February and early-April 2021, showing important delays between sample collection and sequencing or sample storage for long periods before sequencing and submission (e.g., waiting for reagents and/or research investments). Therefore, a significant amount of sequences from 2020 were deposited lately and were not included in this analysis. By mid-July, 23,351 genomes from Brazil are available, of which 17,894 (76.63%) have collection dates in 2021 and almost almost half are from the state of São Paulo (https://www.gisaid.org/). These data demonstrate the absence of countrywide temporally and spatially representative datasets even with increased investment and sequencing capacity.

Notably, the majority of the sequences analyzed here come from the Southeastern region of Brazil. While this is in fact an economic and travel hub for the country, accounting for >70% of the international passengers arriving in Brazil in the beginning of the pandemic (Candido et al., 2020b), inferences regarding this region can be inflated in relation to undersampled regions. However, this is, to the best of our knowledge, the first attempt to characterize sequencing efforts, SARS-CoV-2 mutations, phylogenetics, phylogeography and phyldynamics in the entire Brazil after the study that characterized the first epidemic wave in Brazil using 490 representative genomes (Candido et al., 2020a). In the near future, it will be important to describe and track the spread of the P.1 and P.2 lineages, which have already shown to be replacing the other lineages identified in Brazil (https://outbreak.info/situation-reports).

5. Conclusions

In summary, by systematic analysis of viral genomes distributed across Brazil over time, we were able to confirm the early introductions of multiple lineages, its rapid diversification to constitute new lineages, probable convergent evolution of important mutations (e.g., E484K, N501Y), and the emergence of P.1, arguably one of the most potentially convergent lineages identified worldwide up to February 2021. The occurrence of this lineage and the emergence of novel variants (e.g., B.1.617.2) could jeopardize the efficacy of vaccines and immunotherapies and may lead the health care system to overload. We concluded that enhanced genomic surveillance is, therefore, of paramount importance and should be extended as soon as possible as a means to better inform policy makers and enable precise evidence-based decisions to fight the COVID-19 pandemic.

Supplementary files

Supplementary File 1. GISAID acknowledgement table of worldwide SARS-CoV-2 genomes used in this study.

Table S1. Comparison of genomes sampled from Brazil and confirmed cases per month from March 2020 to mid-February 2021.

Table S2. Comparison of genomes sampled from each Brazilian state and confirmed cases from March 2020 to mid-February 2021. Data are ordered from the higher sequencing rate (genomes per case) to lower.

Table S3. Number of genomes and lineages sampled from each Brazilian state from March 2020 to mid-February 2021. Data are ordered from the higher number of genomes to lower.

Table S4. First and last detection of SARS-CoV-2 lineages represented by > 5 Brazilian genomes, including information on state of detection and minimum time of spread according to available data.

Fig. S1. Distribution of the 10 most prevalent SARS-CoV-2 lineages across all Brazilian states considering the absolute number of sequences per state.

Fig. S2. Time between first and last detection of the 18 lineages observed in more than 5 Brazilian genomes.

Fig. S3. Time-resolved Maximum likelihood phylogenetics tree dropping sequences from other countries and highlighting the most frequent Brazilian lineages.

Fig. S4. Zoom-in on clades corresponding to the two minor clades from B.1.1.74 and B.1.1.28 Brazilian lineages not represented in Fig. 6. (A) Time-resolved ML tree of Brazilian sequences colored by PANGO lineages. Letters around clades are augmented in respective figures. (B) Clade 4.2 is represented by 27 sequences (B.1.1.74 lineage). (C) Clade 5.3 harbors 162 sequences from B.1.1.28 and B.1.1.94. In (B) and (C), states belonging to each specific Brazilian region are colored using similar colors (Centre-West: yellow; North: red; Northeast: purple and pink; South: blue; Southeast: green).

Fig. S5. Maximum clade credibility trees of major Brazilian clades with sufficient temporal signal. (A) Clade 3 (lineage B.1.1.33), (B) Clade 4 (B.1.1.74), (C) Clade 5 (B.1.1.28 and P.1) and (D) Clade 6 (B.1.1.28). In (A), (B), (C), and (D), states belonging to each specific Brazilian region are colored using similar colors (Centre-West: yellow; North: red; Northeast: purple and pink; South: blue; Southeast: green).

Video S1. Animation of the phylogeographic reconstruction of Clade 3. Node polygons and lines represent posterior probabilities (orange to red scale). Colors on the map represent the five Brazilian regions (South, Southeast, Centre-West, Northeast and North).

Video S2. Animation of the phylogeographic reconstruction of Clade 4. Node polygons and lines represent posterior probabilities (orange to red scale). Colors on the map represent the five Brazilian regions (South, Southeast, Centre-West, Northeast and North).

Video S3. Animation of the phylogeographic reconstruction of Clade 5. Node polygons and lines represent posterior probabilities (orange to red scale). Colors on the map represent the five Brazilian regions (South, Southeast, Centre-West, Northeast and North).

Video S4. Animation of the phylogeographic reconstruction of Clade 6. Node polygons and lines represent posterior probabilities (orange to red scale). Colors on the map represent the five Brazilian regions (South, Southeast, Centre-West, Northeast and North).

Funding

This study was supported by the Brazilian National Research Council (CNPq) and Amazon Web Services (AWS) joint grant 032/2019 (Grant No. 440084/2020-2). Scholarships and Fellowships were supplied by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and Universidade Federal de Cien-
cias da Saúde de Porto Alegre (UFCSPA). The funders had no role in the study design, data generation and analysis, decision to publish or the preparation of the manuscript.
Availability of data and material

A full table acknowledging the authors and corresponding labs submitting sequencing data used in this study can be found in Supplementary File 1. Additional information used and/or analysed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Vinícius Bonetti Franceschi: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

Patricia Aline Grohs Ferrarezenze: Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Ricardo Ariel Zimmerman: Investigation, Writing – original draft, Writing – review & editing.

Claudia Elizabeth Thompson: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgment

We thank the administrators of the GISAID database and research groups across the world (especially Brazilians) for supporting the rapid and transparent sharing of genomic data during the COVID-19 pandemic. We also thank the Mayor’s Office, Health Department and São Camilo Hospital (Esteio, RS, Brazil), Leonardo Duarte Pascoal and Ana Regina Boll for their work in combating COVID-19 and for supporting the work developed by our research group.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2021.198532.

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16

V.B. Franceschi et al.