



Distinct YFV Lineages Co-circulated in the Central-Western and Southeastern Brazilian Regions From 2015 to 2018

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The current outbreak of yellow fever virus (YFV) that is afflicting Brazil since the end of 2016 probably originated from a re-introduction of YFV from endemic areas into the non-endemic Southeastern Brazil. However, the lack of genomic sequences from endemic regions hinders the tracking of YFV's dissemination routes. We assessed the origin and spread of the ongoing YFV Brazilian outbreak analyzing a new set of YFV strains infecting humans, non-human primates (NHPs) and mosquitoes sampled across five Brazilian states from endemic and non-endemic regions between 2015 and 2018. We found two YFV sub-clade 1E lineages circulating in NHP from Goiás state (GO), resulting from independent viral introductions into the Araguaia tributary river basin: while one strain from 2017 clustered intermingled with Venezuelan YFV strains from 2000, the other YFV strains sampled in 2015 and 2017 clustered with sequences of the current YFV outbreak in the Brazilian Southeastern region (named YFV_{2015–2018} lineage), displaying the same molecular signature associated to the current YFV outbreak. After its introduction in GO at around mid-2014, the YFV_{2015–2018} lineage followed two paths of dissemination outside GO, originating two major YFV sub-lineages: (1) the YFV_{MG/ES/RJ} sub-lineage spread sequentially from the eastern area of Minas Gerais state to Espírito Santo and then to Rio de Janeiro states, following the Southeast Atlantic basin; (2) the YFV_{MG/SP} sub-lineage spread from the southwestern area of Minas Gerais to the metropolitan

region of São Paulo state, following the Paraná basin. These results indicate the ongoing YFV outbreak in Southeastern Brazil originated from a dissemination event from GO almost 2 years before its recognition at the end of 2016. From GO this lineage was introduced in Minas Gerais state at least two times, originating two sub-lineages that followed different routes toward densely populated areas. The spread of YFV outside endemic regions for at least 4 years stresses the imperative importance of the continuous monitoring of YFV to aid decision-making for effective control policies aiming the increase of vaccination coverage to avoid the YFV transmission in densely populated urban centers.

Keywords: yellow fever virus, Brazilian outbreak, lineages, amino acid changes, phylogeography

INTRODUCTION

In Brazil, the yellow fever virus (YFV) have been sporadically detected in human and non-human primates (NHPs) populations from the enzootic/endemic Northern (Amazon) and epidemic Central-Western regions during the second half of the 20th century (Carrington and Auguste, 2013; Monath and Vasconcelos, 2015). Since the early 2000s, the virus has progressively expanded to the Southeastern and Southern Brazilian regions and in December 2016 began the largest epizootic/epidemic of sylvatic YF registered in the country over the last 50 years (Vasconcelos, 2010; Possas et al., 2018a,b). Between December 2016 and June 2018, a total of 2,139 YF human cases were confirmed in all Southeastern Brazilian states of Minas Gerais ($n = 997$), São Paulo ($n = 577$), Rio de Janeiro ($n = 307$) and Espírito Santo ($n = 258$) with 735 deaths (case-fatality, 34%). Moreover, in 2019 YFV transmission continues in São Paulo and is emerging in the north of Paraná state from South region of Brazil (**Supplementary Figure 1**; Secretaria de Vigilância em Saúde, 2019).

Sequential YF outbreaks reported in the Southeastern and Southern Brazilian regions in 2000–2001, 2008–2009 and 2016–2018 were more likely caused by single independent events of re-introduction of YFV strains from endemic areas (Mir et al., 2017). A recent study speculated that the YFV strain causing the current outbreak would have been originated in the Brazilian Central-West region. This conjecture was based on the date of the most recent common ancestor of the 2016–2018 Brazilian YFV, which was estimated in a period (July 2014 to January 2016) when YFV circulation was reported in the state of Goiás (Central-Western region; Rezende et al., 2018). However, the precise routes of dissemination of YFV strains from endemic to non-endemic areas observed in Brazil in the last 15–20 years are difficult to elucidate because of the scarcity of sequences sampled from endemic regions in that period.

The spatiotemporal dynamics of dissemination of the 2016–2018 Brazilian YFV lineage within the Southeastern region also remained unclear. The first study considering full-genome YFV sequences based on samples from Espírito Santo and Rio de Janeiro states from 2017 placed the origin of the Southeastern outbreak in Espírito Santo in April 2016 (July 2015 to October 2016) and supported a rapid southward viral dissemination in direction to the great metropolitan area of Rio

de Janeiro (Gomez et al., 2018). A second study also comprising full-genome YFV sequences sampled from Minas Gerais, Espírito Santo and Rio de Janeiro in 2017 supports that the outbreak arose in Minas Gerais in July 2016 (March to November 2016) and was then southerly disseminated toward Espírito Santo and Rio de Janeiro (Faria et al., 2018). A third study, based on partial genome sequences, showed that YFV strains isolated in the state of São Paulo in 2016 branched in basal position relatively to those isolated in Minas Gerais and Espírito Santo at 2017–2018 and traced the origin of current outbreak to July 2015 (July 2014 to January 2016), thus suggesting that the 2016–2018 YFV Brazilian lineage may have circulated in São Paulo state before spread to Minas Gerais and Espírito Santo (Rezende et al., 2018).

Therefore, to define in greater detail the geographic origin and subsequent dissemination routes of the 2016–2018 Brazilian YFV clade, we generated and analyzed 13 YFV new genomes obtained from humans, NHPs and mosquitoes, between 2015 and 2018, from all Southeast Brazilian states (Minas Gerais, Espírito Santo, Rio de Janeiro e São Paulo) and from Central-West region (Goiás). Interestingly, we identified two YFV lineages circulating in Central-West (Goiás) during 2015–2017, one (GO27/2015 and GO21/2017) displaying the nine amino acid signature characteristic of the 2016–2018 YFV Southeastern outbreak (Bonaldo et al., 2017) and the other (GO05/2017) isolated 2 year later that displayed an amino acid pattern typical of older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010 (De Souza et al., 2010). Besides, these 13 new YFV complete coding region sequences (CDS) were combined with previously described YFV CDS from Brazil ($n = 67$), Venezuela ($n = 5$) and Trinidad and Tobago ($n = 1$) and then subjected to phylogeographic analyses to get a better picture of the ongoing YFV epidemic in Brazil.

MATERIALS AND METHODS

YFV Samples

Viral samples from 13 infected hosts from distinct biomes and river basins in the states of Goiás ($n = 3$), Central-Western region, and Rio de Janeiro ($n = 2$), Minas Gerais ($n = 3$), Espírito Santo

($n = 3$) and São Paulo ($n = 2$), Southeastern region of Brazil, were analyzed (Table 1). Serum samples of human cases, liver samples of human and NHP, and homogenates of entire bodies of pooled adult female mosquitoes were collected and processed as previously described (Ferreira-De-Brito et al., 2016; Bonaldo et al., 2017; Gomez et al., 2018; Abreu et al., 2019). The YFV isolates ABR1005 and ABR1009 were obtained from the sera of a 64 and 30-year-old male patients, respectively. The ABR1005 patient was hospitalized 4 days after onset of symptoms and died due to multiple organ failure. The ABR1009 patient was hospitalized 4 days after onset of symptoms but fully recovered from the infection.

YFV Genome Detection and Nucleotide Sequencing

The sera from the individuals from São Paulo state (ABR1005 and ABR1009) and an NHP liver homogenate sample from Goiás (GO27) were employed to obtain first-passage YFV isolates by infection of monolayer cell cultures of the C6/36 clone of *Aedes albopictus*. Viral RNA was obtained from cell cultures or directly from samples as described elsewhere (Bonaldo et al., 2017). The set of primers utilized in PCR and sequencing procedures followed a previous report (Gomez et al., 2018). Nucleotide sequences were determined by capillary electrophoresis at the sequencing facility of Fiocruz-RJ (RPT01A – Sequenciamento de DNA – RJ). The sequences were assembled with SeqMan Pro version 8.1.5 (DNASTAR, Madison, WI, United States). The Molecular Evolutionary Genetics Analysis 7.0 program (Kumar et al., 2016) was adopted to explore the amino acid differences as well as to calculate nucleotide and amino acid distances.

Evolutionary and Phylogeographic Analyses

Complete CDS (10,239 nt in length) of the 13 newly generated YFV genomes were combined with CDS of YFV American sequences available in GenBank¹ according to the following inclusion criteria: (1) link to a publication, (2) coverage of at least 99% of the viral CDS, and (3) known date and country of collection. The sequences were aligned with MAFFT (Katoh and Standley, 2013) and viral phylogenies were reconstructed by maximum likelihood (ML) analysis implemented in PhyML (Guindon et al., 2010) applying the best substitution model selected by jModelTest v1.6 (Darriba et al., 2012). The temporal signal of different combinations of sequences representing the South American genotypes I and II (SA-I+II), the South American genotype I (SA-I), and the Modern lineage of SA-I (Mir et al., 2017) were examined with TempEst v1.5.1 (Rambaut et al., 2016). The dataset with the best temporal structure (SA-I) was chosen for the subsequent time tree reconstructions by Bayesian method (Supplementary Table 1).

The time scale of the SA-I and 2016–2018 Brazilian YFV datasets were estimated using the Markov chain Monte

Carlo algorithms implemented in the BEAST v1.8.4 package (Drummond and Rambaut, 2007; Drummond et al., 2012) with BEAGLE (Ayres et al., 2012) to improve running time. The evolutionary process was estimated using the best-fit nucleotide substitution model (GTR+I+ Γ 4), a relaxed uncorrelated lognormal molecular clock model (Drummond et al., 2006) and the non-parametric Bayesian Skyline coalescent tree prior (Drummond et al., 2005). A CTMC rate reference prior (Ferreira and Suchard, 2008) and a normal prior (mean = 4.5×10^{-4} substitution/site/year, standard deviation = 1.0×10^{-4}) in the evolutionary rate were used for the analysis of the SA-I and 2016–2018 Brazilian YFV datasets, respectively.

The reconstruction of migration events throughout the phylogeny for the 2016–2018 Brazilian YFV lineage also employed the BEAST package using discrete and continuous models. The discrete phylogeographic analysis was performed using reversible (symmetric) and nonreversible (asymmetric) discrete phylogeographic models (Lemey et al., 2009), assigning discrete traits for each sequence representing the Brazilian state of isolation, except for MG, in which further geographic subdivision were employed. The spatiotemporal reconstruction in continuous space utilizing the geographic coordinates (latitude and longitude) of each YFV isolate was estimated with a homogenous Brownian diffusion (BD) model and the heterogeneous Cauchy, Gamma and Lognormal relaxed random walk (RRW) models (Lemey et al., 2010). Comparisons among the different discrete and continuous phylogeographic models were performed using the log marginal likelihood estimation (MLE) based on path sampling (PS) and stepping-stone sampling (SS) methods (Baele et al., 2012). Bayesian analyses were run for 10^8 generations and convergence (effective sample size > 200) was inspected using TRACER v1.7 (Rambaut et al., 2018) after discarding 10% burn-in. The maximum clade credibility (MCC) trees were summarized using TreeAnnotator v1.8.4 (Drummond et al., 2012) and visualized with FigTree v1.4.4.² The viral spatio-temporal diffusion was analyzed and visualized in SPREAD (Bielejec et al., 2011) and further projected in maps generated with QGIS software³ using public access data collected from the Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística [IBGE], 2019) and National Water Agency (Agência Nacional de Águas [ANA], 2019).

RESULTS

Identification of Two YFV Lineages Circulating in the State of Goiás During 2015–2017

Initially, we sequenced the complete genomes of YFV obtained at two Brazilian biomes to infer the origin and dissemination routes of YFV during the 2016–2018 outbreak in the country. Ten YFV strains were sampled from human, NHP and mosquitoes in all

¹www.ncbi.nlm.nih.gov

²<http://tree.bio.ed.ac.uk>

³<http://qgis.org>

TABLE 1 | YFV samples from Brazil sampled in 2015, 2017, and 2018.

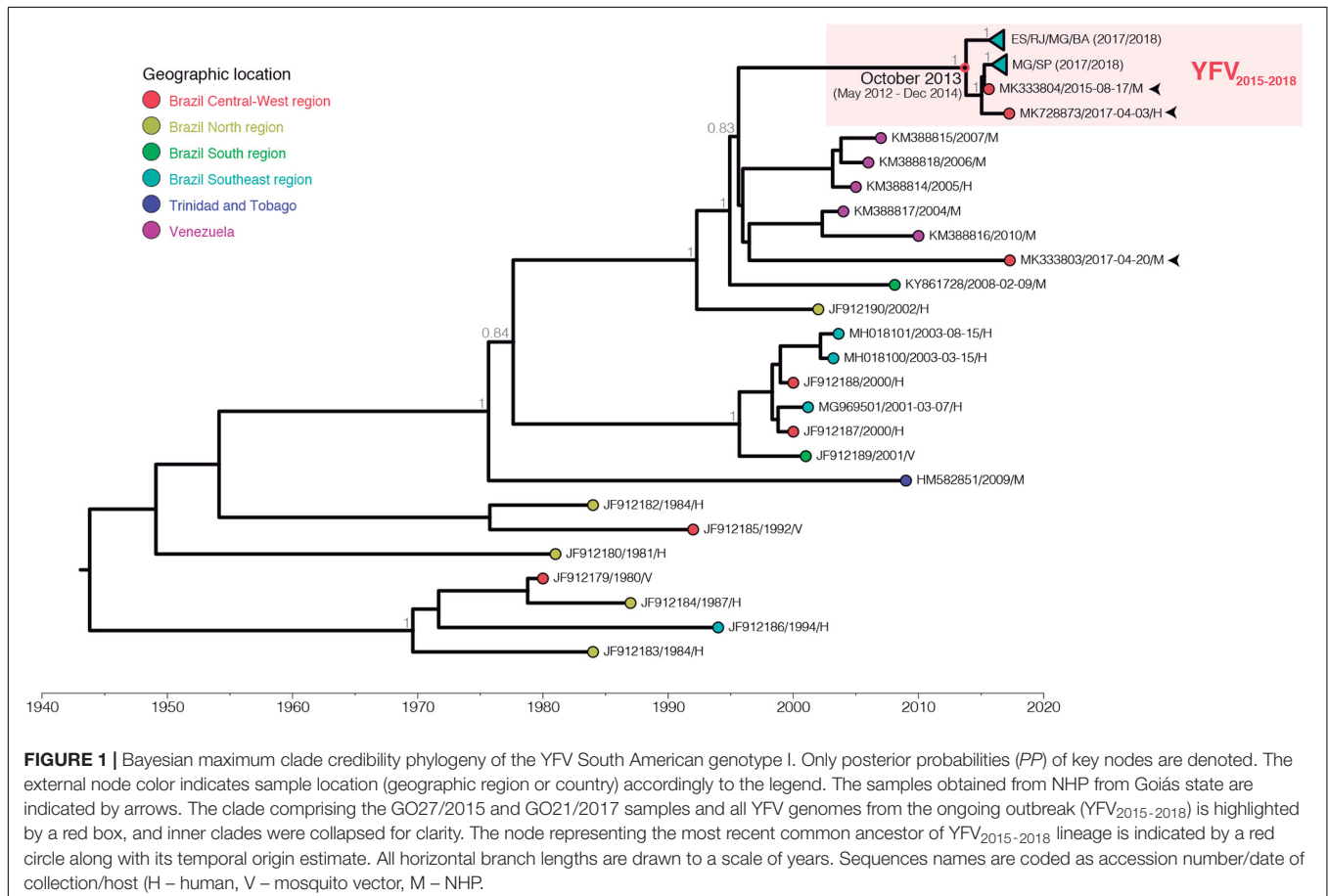
State (Region)	Biome	Primary river basin (Tributary basin)	Locality	Geographic coordinate	Date of collection	Sample ID	Host	Genbank accession number
Goiás (CW)	Cerrado	Tocantins-Araguaia (Araguaia)	Novo Brasil	16°02'12"S	17/08/2015	GO27	<i>Sapajus libidinosus</i> (capuchin-monkey)	MK333804
				50°43'24"W	20/04/2017	GO05	<i>Alouatta caraya</i> (howler-monkey)	MK333803
				13°40'39"S	03/04/2017	GO21	<i>Homo sapiens</i>	MK728873
Minas Gerais (SE)	Inland Atlantic Forest	Tocantins-Araguaia (Araguaia)	Belmiro Braga	16°36'19"S	18/01/2018	MG3121	<i>Haemagogus. Leucocealeus</i> (3 mosquitoes)	MK333807
				51°05'03"W	29/01/2018	MG3155	<i>Haemagogus janthinomys</i> (5 mosquitoes)	MK333808
				22°00'56"S	27/01/2018	JF2793	<i>Haemagogus janthinomys</i> (3 mosquitoes)	MK333806
Espírito Santo (SE)	Coastal Atlantic Forest	Southeast Atlantic (Itapemirim conjugated)	Juiz de Fora	43°22'19"W	20/02/2017	ES03	<i>Alouatta clamitans</i> (howler-monkey)	MK333800
				22°04'50"S	05/04/2017	ES2750	<i>Haemagogus. leucocealeus</i> (1 mosquito)	MK333802
				43°33'33"W	05/04/2017	ES2682	<i>Haemagogus janthinomys</i> (2 mosquitoes)	MK333801
Rio de Janeiro (SE)	Inland Atlantic Forest	Southeast Atlantic (Paraíba do Sul)	Valença	21°45'14"S	19/01/2018	VL2926	<i>Haemagogus janthinomys</i> (1 mosquito)	MK333809
				43°19'42"W	07/02/2018	IG3036	<i>Sabethes chloropterus</i> (1 mosquito)	MK333805
				20°16'52"S	08/01/2018	ABR1005	<i>Homo sapiens</i>	MK333798
São Paulo (SE)	Coastal Atlantic Forest	Sea Island	Ilha Grande	40°49'39"W	08/01/2018	ABR1009	<i>Homo sapiens</i>	MK333799
				20°05'12"S				
				40°31'07"W				
São Paulo (SE)	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	20°04'51"S				
				40°35'48"W				
				22°10'26"S				
São Paulo (SE)	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	43°43'25"W				
				23°11'01"S				
				44°11'56"W				
São Paulo (SE)	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	23°23'S				
				46°34'W				
				23°23'S				
São Paulo (SE)	Inland Atlantic Forest	Paraná (Tietê)	Metropolitan São Paulo	46°34'W				
				23°23'S				
				46°34'W				

CW, Central-West region; SE, Southeast region.

Southeastern Brazilian states: Minas Gerais, Espírito Santo, Rio de Janeiro and São Paulo, located in the Atlantic Forest biome and where massive epizootics and human cases occurred. Three other YFV genomes were recovered from human and NHP sampled in Goiás, a state of the Brazilian Central-Western region whose predominant vegetation is the *Cerrado*, a savanna-like biome occupying the territory between the Amazon and Atlantic rain forest of the Southeast region (Table 1). Both YFV strains from Goiás were sampled from sites located in the Tocantins-Araguaia river basin. The ML and Bayesian phylogenetic analyses placed the newly generated YFV genomes inside the sub-clade 1E (De Souza et al., 2010) of the Modern lineage of SA-I (Mir et al., 2017), with high support [aLRT/posterior probability (PP) = 1] (Figure 1 and Supplementary Figure 2). Two viral samples from Goiás (GO27/2015 and GO21/2017) infecting, respectively a capuchin-monkey from Novo Brasil on August 2015 and a person from Amarinópolis on April 2017, clustered in a highly supported (PP = 1) clade (YFV_{2015–2018}) with all Brazilian YFV sequences from the Southeastern region associated with the current outbreak. By contrast, the other strain from Goiás (GO05/2017) that infected a howler-monkey from Nova Crixás on April 2017, was intermingled among Venezuelan YFV genomic sequences from the 2000s. The mean evolutionary rate for the YFV SA-I was estimated at 4.3×10^{-4} substitution/site/year (Supplementary Table 2), fully

consistent with that previously reported (Nunes et al., 2012; Gomez et al., 2018), while the time of the most recent common ancestor (T_{MRC}A) of the YFV_{2015–2018} clade was estimated on October 2013 [95% Bayesian credible interval (BCI): May 2012 to December 2014].

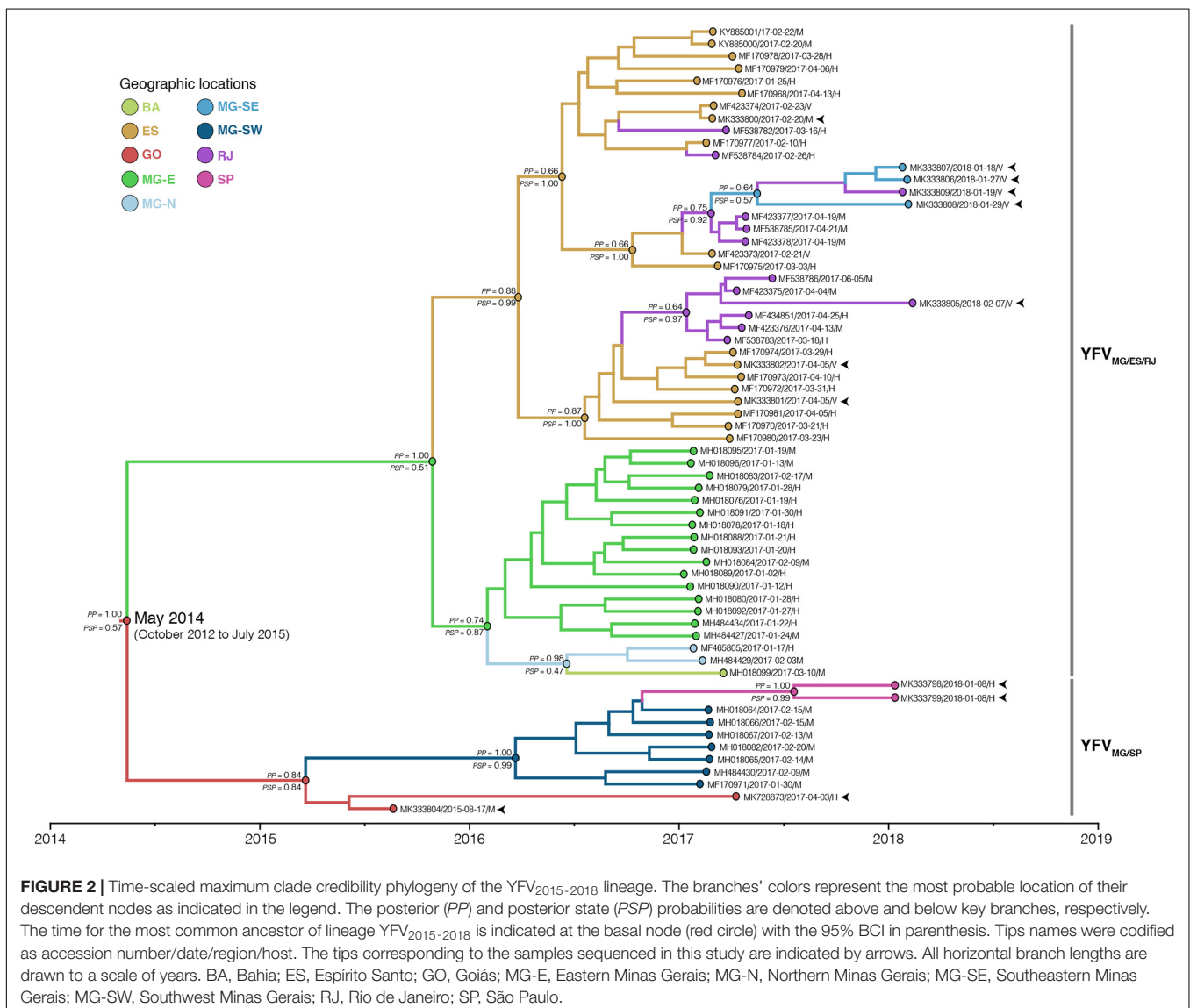
Analysis of amino acid signatures showed the frequent presence of the nine unique amino acid substitutions previously described (Bonaldo et al., 2017; Gomez et al., 2018) in almost all new YFV Brazilian genomes from the Southeastern region and in one sample from Goiás (GO27/2015; Supplementary Table 3 and Supplementary Figure 3). Distinctly, the other YFV strain from Goiás (GO05/2017), exhibited an amino acid pattern more similar to older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010 (Supplementary Table 3 and Supplementary Figure 3). These results clearly support the circulation of at least two YFV sub-clade 1E lineages in the *Cerrado* between 2015 and 2017 that probably resulted from independent viral introductions in the Araguaia tributary basin of the Tocantins-Araguaia primary watershed. Interestingly, the third sample from Goiás state (GO21/2017) displayed eight out of nine aminoacidic set of alterations (Supplementary Table 3). Moreover, these results revealed that the molecular signature previously associated with the 2016–2017 YFV Southeastern Brazilian strains was already present in a YFV strain isolated in the *Cerrado* biome in 2015.



The YFV_{2015–2018} Lineage Likely Arose in Goiás in 2014 and Was Disseminated to the Southeastern Region Following Two Major Routes

To determine with more precision the geographic origin and dissemination routes of the YFV_{2015–2018} lineage, we first applied discrete Bayesian symmetric and asymmetric phylogeographic models. The Bayes Factor test showed a strong support in favor of the discrete asymmetric phylogeographic model (**Supplementary Table 4**), presented in **Figure 2**. According to this analysis, the YFV_{2015–2018} lineage likely originated in the state of Goiás [posterior state probability (*PSP*) = 0.57] in May 2014 (95% BCI: October 2012 to July 2015). From there, it followed two paths of dissemination toward the state of Minas Gerais, originating two major YFV sub-lineages in the Southeastern region here called YFV_{MG/ES/RJ} and YFV_{MG/SP}.

The YFV_{MG/ES/RJ} sub-lineage probably reached initially the eastern region of Minas Gerais (MG-E, *PSP* = 0.51) on October 2015 (95% BCI: February 2015 to April 2016), from where it most likely spread to Espírito Santo state (*PSP* = 0.99) on March 2016 (95% BCI: September 2015 to August 2016) and to the northern area of Minas Gerais (MG-N, *PSP* = 0.47) on July 2016 (95% BCI: January 2016 to October 2016). From MG-N, the YFV spread to the south of the state of Bahia, in the Northeastern Brazilian region. From ES, the YFV was introduced in Rio de Janeiro at least four times, generating two successful intrastate transmission chains that advanced southwards: (1) one chain followed the northern side of the Serra do Mar along the Paraíba do Sul tributary basin (**Supplementary Figure 4**), bypassing the Rio de Janeiro metropolitan region toward the south (Valença municipality) and affecting the southeastern region of Minas Gerais (MG-SE, *PSP* = 0.57); (2) the other transmission chain spread through the coastal area South of Serra do Mar (Macaé



tributary basin), crossing the Rio de Janeiro state metropolitan region and reaching an island located in the southern region of Rio de Janeiro (Ilha Grande).

The YFV_{MG/SP} sub-lineage most likely spread from Goiás into the southwestern area of Minas Gerais (MG-SW, *PSP* = 0.99) on March 2016 (95% BCI: September 2015 to August 2016) and then disseminated from MG-SW to the metropolitan region of the state of São Paulo. Remarkably, four YFV genomes from MG-E and one from MG-SW displayed differences in the molecular amino acid signature (D15762E; R2607Q). Nevertheless, the variations were not fixed in 2018 YFV samples as identified in the position I2176V clustering in YFV_{MG/ES/RJ}, which were collected in Paraíba tributary basin in 2017 and 2018, suggesting the maintenance of the YFV polymorphism in this region. Overall, these results expanded the geographical and temporal edge of the YFV_{2015–2018} lineage and further revealed a very low degree of phylogenetic intermixing of YFV_{2015–2018} strains from different Brazilian states during viral dissemination in the Southeastern region.

The YFV_{2015–2018} Lineage Was Disseminated Following Primary River Basins in the Southeastern Region

To get some insight regarding the spatiotemporal dynamics of dissemination of the YFV_{2015–2018} lineage, we applied different

continuous phylogeographical models, assuming homogeneous (BD) and heterogeneous (RRW) dispersion rates among lineages. The RRW model with Lognormal distribution was strongly supported as the fittest diffusion model (**Supplementary Table 5**), indicating significant variation in the diffusion rate among the branches. The phylogeographic continuous model (**Figure 3**) changed slightly the epicenter of the YFV_{2015–2018} lineage but supports the existence of two main routes of dissemination within the Brazilian Southeastern region and few viral migrations between different states, consistent with the discrete phylogeographic reconstruction. This analysis also supports that the YFV was disseminated following major river basins.

According to the continuous model, the origin of the YFV_{2015–2018} lineage would be the central region of Minas Gerais, within the São Francisco watershed, from where it would have independently spread to the west, east and south, reaching the Tocantins-Araguaia basin in the Goiás in 2015, the Southeast Atlantic hydrographic region in Minas Gerais at the end of 2015, and the Paraná hydrographic region of that state in the middle of 2016, respectively. From the eastern of Minas Gerais, the virus moved southward following the Southeast Atlantic watersheds distributed among the states of Minas Gerais, Espírito Santo, and Rio de Janeiro (an area covered by the Atlantic forest biome) and northward, returning to the São Francisco river basin and south of Bahia.

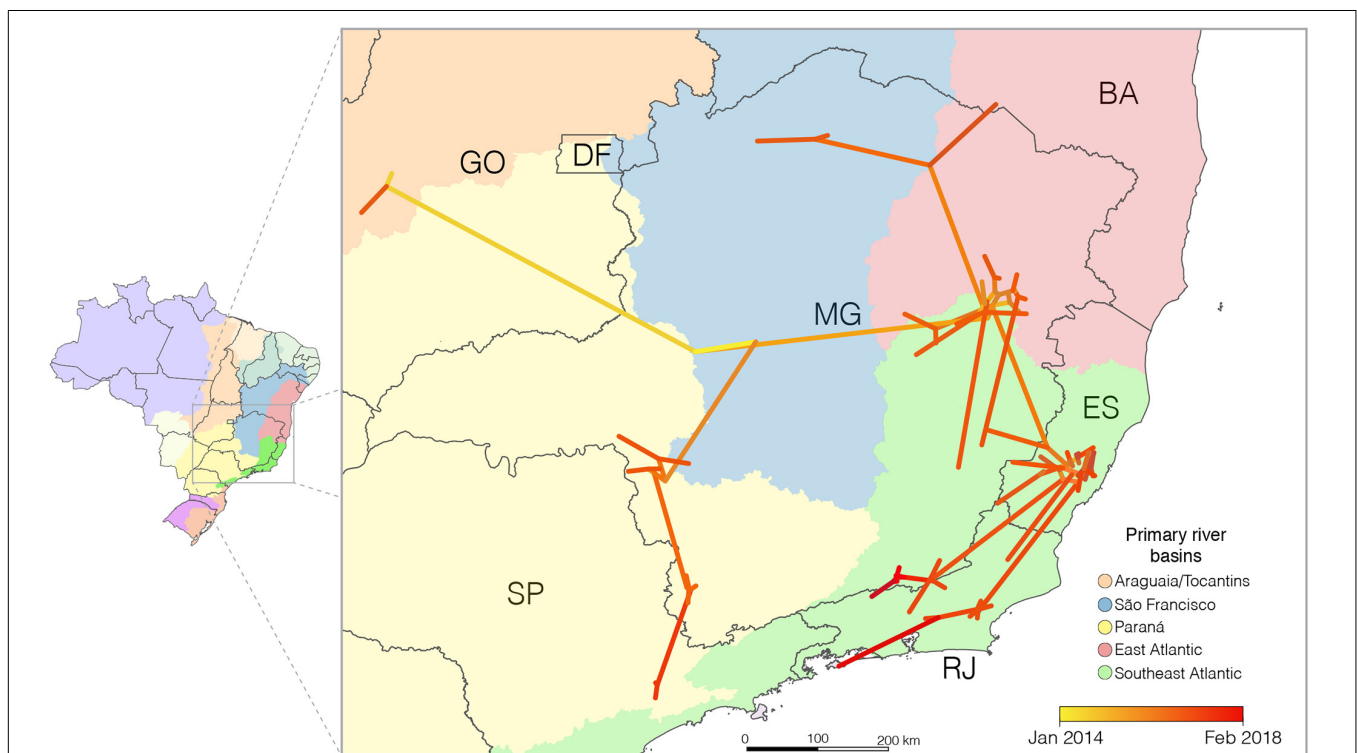


FIGURE 3 | Reconstructed spatiotemporal diffusion of the YFV_{2015–2018} lineage. Phylogeny branches were arranged in space according to the internal nodes locations inferred by the continuous phylogeographic model. Branches were colored according to time as indicated by the legend. The dark gray lines represent the Brazilian states boundaries while the colored areas the different primary river basins; additional details on tributary basins are available from Instituto Brasileiro de Geografia e Estatística [IBGE] (2019). Brazilian states: DF, Distrito Federal; ES, Espírito Santo; GO, Goiás; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo.

Simultaneously, the YFV lineage showed southerly dissemination from the southwestern region of Minas Gerais toward the São Paulo state, following the Paraná basin. We estimate that YFV lineages moved, on average, 0.5 km/day (95% BCI: 0.4 to 0.7 km/day).

DISCUSSION

The current re-emergence of YFV in the Southeastern Brazilian region resulted in the largest outbreak of sylvatic YF observed in South America in the last decades. The transmission has been expanding southward in Brazil reaching sites considered YFV-free areas for 80 years, and therefore with scanty YFV vaccination coverage. As a result, 38 cases and nine deaths have been reported in January 2019, around 1,160 km from the first signal of increased incidence of YF in the Southeast (north-western region of Minas Gerais) in late 2016 (Secretaria de Vigilância em Saúde, 2019).

Previous studies pointed out that the ongoing YFV outbreak in the Brazilian Southeastern region resulted from a single introduction event of a YFV Modern lineage strain from an endemic area (Mir et al., 2017; Faria et al., 2018; Gomez et al., 2018; Rezende et al., 2018). However, the precise route of viral dissemination was not achieved due to the scarcity of Brazilian YFV sequences sampled from endemic regions over the last years. Here, the analysis of two YFV samples from Goiás (Central-Western region) obtained in 2015 and 2017 revealed that they are phylogenetically related with and carry the same amino acid signature of the YFV strains causing the current outbreak in the Southeastern region. Moreover, the discrete phylogeographic analysis showed that the YFV causing the current Brazilian outbreak probably originated in Goiás at around mid-2014, a result congruent with epidemiological reports on human and NHP infections (**Supplementary Figure 1**). Altogether, our data stressed the origin of the current YFV outbreak in the Central-western region and expanded the estimated T_{MRCA} of the ongoing YFV outbreak to almost 2 years before it gained epidemiological visibility in the end of 2016 (Secretaria de Vigilância em Saúde, 2017). Interestingly, we also identified for the first time, two YFV lineages circulating in Goiás during 2015–2017, one of which followed two independent paths of dissemination toward Minas Gerais state, originating two major YFV sub-lineages in the Southeast region responsible for the severe ongoing outbreak. Of note, the molecular clock dating approach used to infer the YFV epidemic dynamics had limitations and would be undoubtedly benefited if more genome sequences (sampled from humans and reservoir populations) were available and if multiple scales of YFV evolution (between- and within-host) were considered (Frost et al., 2015).

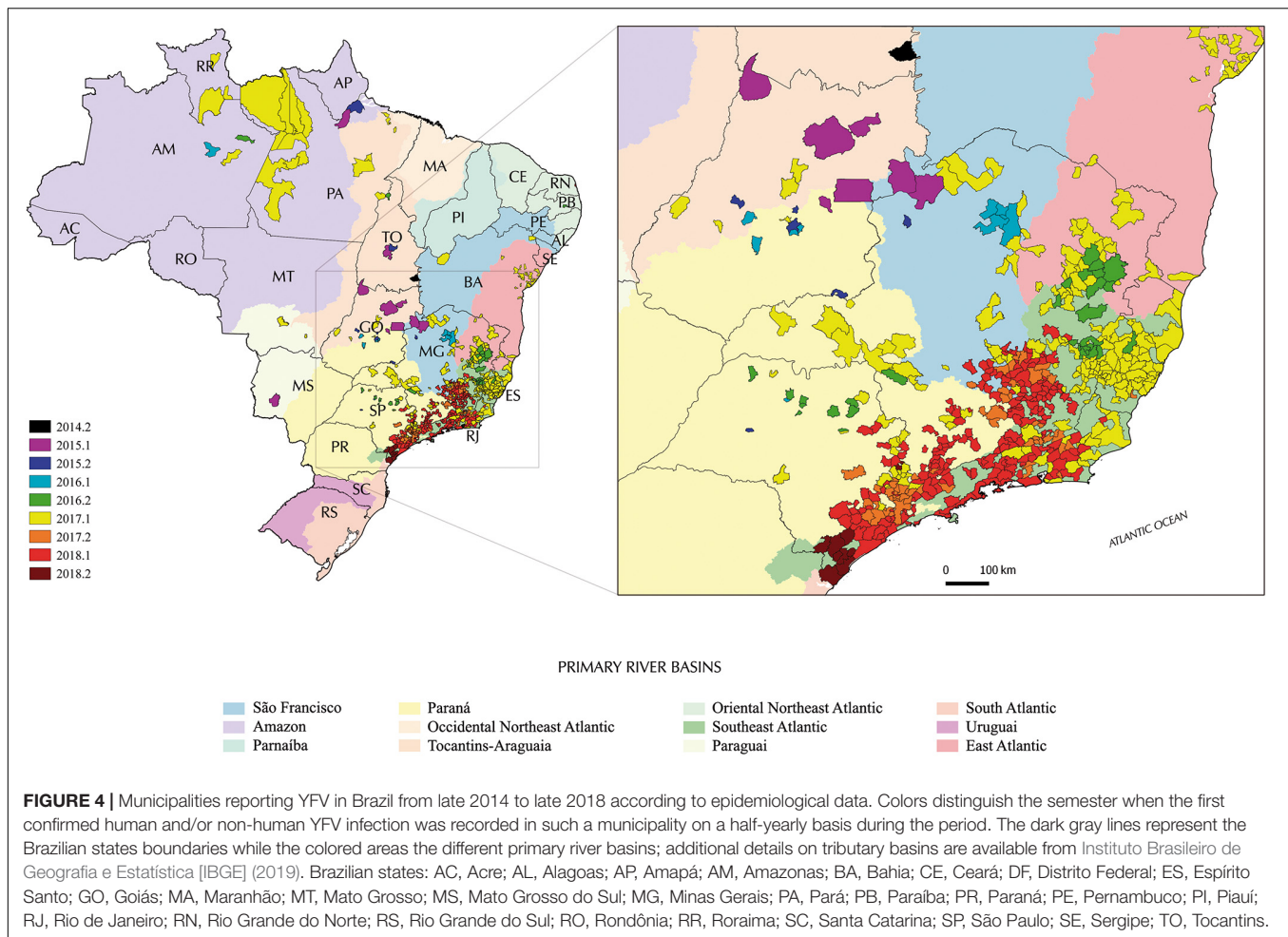
According to the spatiotemporal epidemiologic reports of YFV infections in both humans and NHPs in the national surveillance system, after 5 years of no records of YFV cases outside the Amazon, 31 NHPs died between May and August 2014 in seven municipalities of the Tocantins state, in the Tocantins-Araguaia basin. The only corpse found still adequate

to diagnosis was positive to YFV (Secretaria de Vigilância em Saúde, 2014). The Tocantins-Araguaia basin drains the territory of Tocantins and the great northern part of the neighbor state of Goiás into de Amazon river, and the gallery forests along its tributaries consist of a large network of corridors between the Amazon and *Cerrado* biomes. Thus, soon in early 2015, besides in the Tocantins, YFV infections were detected in Goiás, and the virus spilled over from the Tocantins-Araguaia into the São Francisco and Paraná basins, with reports scattered in northwestern Minas Gerais and southern Goiás and the Federal District (Brasília; **Figure 4**).

Brazilian Health authorities consider this YFV reemergence as the start of the north-southeast virus spreading wave that has not stopped yet (Secretaria de Vigilância em Saúde, 2015, 2017, 2019). Accordingly, in late 2015 and early 2016, the circulation of YFV was detected in sites of Minas Gerais and Goiás states, but still essentially limited to the *Cerrado* biome in the three above mentioned river basins. Intriguingly, in late 2016, human cases and epizooties due to YFV were recorded simultaneously and independently in the northeast Minas Gerais as well as in southeastern Minas Gerais/northern São Paulo, where the virus has then gained the Atlantic forest ecosystem. In fact, the outbreak was officially recognized only when the epidemiological records rapidly peaked and as that the virus reached the Southeast Atlantic river basin in Espírito Santo and Rio de Janeiro, as well as continued to spread in all primary watershed of São Paulo and Minas Gerais throughout 2017. One of the causes of this delay was the introduction of Chikungunya (2014) and Zika (2015) virus in Brazil and the resulting epidemics, which probably reduced the sensitivity of surveillance and interfered with the visibility of the YFV reemergence. In 2017, scattered records of YFV circulation were also made in the states of Bahia, Goiás, Mato Grosso and even in the Amazon.

In early 2018, epidemiological data suggested that transmission was mostly concentrated in the Southeast Atlantic basin in the states of Rio de Janeiro, Minas Gerais and São Paulo and eastern Paraná basin (**Figure 4**). Then, the place and time of origin for the current YFV Brazilian outbreak here estimated fully agree with the above mentioned officially confirmed infections in NHP and humans reported by the Brazilian Ministry of Health (Secretaria de Vigilância em Saúde, 2015). Indeed, the Novo Brasil municipality in Goiás, from where the NHP YFV sample analyzed here was taken, is located about 100 km from the banks of the Araguaia river. The earlier detection of YFV in NHP from the Tocantins indicates that the state of Goiás probably acted as a staging post-during the dissemination of the YFV_{2015–2018} lineage from the North (Amazon basin) to the Southeast Brazilian regions. In this sense, the Tocantins-Araguaia watersheds may have played a major role in YFV dissemination as it extends from more than 2,500 km following two river axes (Araguaia and the Tocantins), offering a contiguous connection between the *Cerrado* (southward, Goiás and Tocantins states) and Amazon (northward, Pará state) biomes (Agência Nacional de Águas [ANA], 2019).

Both discrete and continuous phylogeographic models combined with the epidemiological records support of the YFV_{2015–2018} lineage moved toward densely populated



Southeastern urban regions with low YFV vaccine coverages following major routes along different primary river basins. The phylogeographic analyses pointed out that the YFV_{2015–2018} lineage probably arrived in the Southeast region via the São Francisco watershed located in Minas Gerais and then moved to the Southeast Atlantic watersheds in the east and the Paraná hydrographic region in the southwest. The viral lineage that moved following the Southeast Atlantic watersheds reached the eastern and northern areas of Minas Gerais state, as well as the south of Bahia, Espírito Santo and Rio de Janeiro states. The viral lineage that followed the Paraná hydrographic region spread to the Southwest of Minas Gerais and São Paulo states. A previous study proposed that the YFV_{2015–2018} lineage was introduced in the southeastern region through São Paulo and then moved to other Southeastern states (Rezende et al., 2018). However, this conclusion is hampered as they used only partial genomes (with a very low number of nucleotide substitutions supporting the phylogenetic relationships) and did not conduct a formal phylogeographic analysis. Although Faria et al. (2018) already described that the YFV_{2015–2018} lineage displayed southward and eastward expansion from its inferred origin in Minas Gerais, our results consist of the first description of concurrent dispersion of the YFV_{2015–2018} lineage following

two independent routes that seems to be linked to the main hydrographical basins.

Our phylogeographic analyses also support that the rapid spread of the YFV_{2015–2018} lineage in the Southeastern region seems to have resulted from a few successful viral dissemination events between states. Most YFV transmission in Espírito Santo was probably originated from a single successful transmission from Minas Gerais, while most viral transmissions in Rio de Janeiro seems to have resulted from two independent introductions from Espírito Santo that subsequently spread along the coastal and northern sides of the Serra do Mar mountain system, as previously described (Gomez et al., 2018). We found that both transmission chains previously detected in Rio de Janeiro state continued to expand to beyond the metropolitan region, reaching municipalities close to the border with the state of São Paulo during 2018. The two YFV 2018 genomes from São Paulo analyzed here are the first described of the current YFV outbreak from that state and were the result of independent dissemination from the southwestern region of Minas Gerais, but more sequences from São Paulo are necessary to understand the epidemic dynamic in this state. It is unclear if most YFV infections in São Paulo resulted from a single or a few founder viral strains that spread from the southwest of Minas Gerais along

the Paraná hydrographic basin, or if other viral strains may have also been disseminated from Rio de Janeiro along the Southeast Atlantic watersheds. The recent detection of YFV in NHP from the coastal area of Paraná state in 2019 (Secretaria de Vigilância em Saúde, 2019) indicates continuous dissemination of YFV into the Southern region probably following the Paraná and/or the Southeast Atlantic hydrographic basins.

We estimated that the YFV_{2015–2018} lineage moved on average 0.5 km/day and similar results were obtained when the YFV_{2015–2018}'s outgroup sequences GO27/2015 and GO21/2017 were removed from the analysis. This velocity is lower than the estimates described by Faria et al. (2018) and Gomez et al. (2018) that also analyzed the dispersion of the ongoing YFV outbreak in Brazil and found dispersion rates of 4.2 and 3.4 km/day, respectively. However, those studies analyzed sequences sampled between January–April of 2017, corresponding to the wet and warmer season, when there is an increase in the density of vectors (Alencar et al., 2018) facilitating the transmission. The primary vectors in the current outbreak in Southeast Brazil are the mosquitoes *Haemagogus leucocelaenus* and *Haemagogus janthinomys* (Abreu et al., 2019), which can disperse large distances in short time (Causey et al., 1950). Our conservative velocity of YFV dispersion fully agrees with the observed distances traveled by mosquitoes, but also with howler monkeys in Southeastern Brazil (Jung et al., 2015) and also agrees with an epidemiological model based on dates and place of reported monkey deaths, which estimated YFV displacement speeds of 2.7 km/day in the warmer months and 0.5 km/day in the coldest months (Fioravanti, 2018). Thus, the YFV dispersion velocity estimated in this study would correspond to a median value between these two speeds. Besides the role of NHPs and mosquitoes, the hypothetical potential of viremic humans in the spread of sylvatic yellow fever has yet to be confirmed (Vasconcelos, 2010; Possas et al., 2018a).

Surprisingly, we found two YFV sub-clade 1E lineages circulating in the Araguaia tributary basin, indicating at least two independent introductions of YFV in that region probably from the enzootic/endemic Amazon biome in a narrow time frame. While sample GO27/2015 isolated in 2015 displays the nine unique amino acid signatures characteristic of the 2016–2018 YFV Southeastern outbreak, sample GO05/2017 isolated 2 years later at the same watershed exhibited an amino acid pattern typical of older YFV sub-clade 1E strains sampled in Brazil and Venezuela between 2000 and 2010. According to our analysis, only YFV strains related to the GO27/2015 and GO21/2017 were able to further disseminate from Goiás toward the Southeast region. We can speculate that the different pattern of molecular signatures present in the two YFV strains from Goiás modulate the spread of each viral lineage since some of them were located in key viral enzymes (Gomez et al., 2018). Alternatively, the GO05/2017 lineage may have been introduced from the Amazon into Goiás at a later time, and its dissemination toward the Southeast was hampered due to the reduction or even exhaustion of susceptible NHP hosts caused by the previous passage of the lineage that originated the YFV_{2015–2018} clade. Consistent with this last hypothesis, a recent ecological study concludes that dissemination of YFV in

South America is not random, but it is influenced by key geo-environmental factors like diversity and number of susceptible NHP hosts (Hamrick et al., 2017). Curiously, epidemiological records showed YFV transmission in several sites in the Amazon in early 2017, including in the Tocantins-Araguaia basin in Pará state (Figure 4).

In summary, we showed that at least two different YFV lineages circulated in the *Cerrado* biome (Araguaia tributary basin) in a narrow time frame. One of these lineages further spread out the *Cerrado* biome in Goiás to the Atlantic forest biome in the Southeastern Brazilian region, originating the current Brazilian outbreak (YFV_{2015–2018} lineage) at around mid-2014. The ongoing YFV outbreak in Brazil disseminated in the Southeast region following two independent routes that seem to be linked to the Paraná and Southeast Atlantic hydrographic basins, comprising densely populated regions. The spread of the YFV outside the Amazon and *Cerrado* biomes following primary hydrographic watersheds comprising large metropolis stresses the imperative importance of the continuous monitoring of YFV coupled with in-depth phylogeographic analysis to aid decision-making Health authorities for effective prophylactic and control policies aiming the increase of vaccination coverage to avoid the YFV transmission in densely populated urban centers.

DATA AVAILABILITY

All new YFV genomes generated for this study were submitted to GenBank and their accession numbers are included in the manuscript. The information of all YFV genomes used in this study is provided in the **Supplementary Table 1**.

ETHICS STATEMENT

The YFV isolates ABR1005 and ABR1009 were obtained from two patients following a study protocol approved by the institutional review boards at the Hospital das Clínicas (School of Medicine, University of São Paulo) and the Infectiology Institute Emilio Ribas (CAAE: 59542216.3.1001.0068). The analysis of human samples was carried out in accordance with the recommendations of the Ethics Committees for human research at Instituto Oswaldo Cruz (CAAE 69206217.8.0000.5248), which exempted the need of a specific written informed consent from patients or their legal representatives. Capture of NHPs and mosquitoes, as well as management of NHP samples, were carried out in accordance with the Brazilian environmental authorities (SISBIO-MMA licenses 54707-6 and 52472-2, and INEA licenses 012/2016, 019/2018) and Ethics Committee of Animal use at Instituto Oswaldo Cruz (CEUA license L037/2016).

AUTHOR CONTRIBUTIONS

ED, GB, MB, and RL-d-O conceived the study. FdA and MN carried out the collection of biological specimens. EK provided the samples. IB and MN identified the mosquito species. AF-d-B, FdA, IB, LdS, MdC, and RdM carried out viral RNA

extraction from the biological specimens and the diagnosis by RT-PCR. LdS and LR conducted the inoculation of biological specimens in cell culture. AdS, IR, and MG performed rapid viral RNA extraction and genome sequencing. AdS, MB, MG, and NF analyzed the genome sequences. ED and GB performed phylogenetic and phylogeographic analysis. ED, GB, MB, MG, NF, and RL-d-O prepared figures, tables, and supplementary material. ED, FdA, GB, IR, MB, MG, RL-d-O, EK, and AV prepared the manuscript. AR, DR, and FdA gathered, systematized, and illustrated epidemiological records. All authors critically read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.01079/full#supplementary-material>

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