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Vector surveillance during a major chikungunya outbreak in northwestern São Paulo state, Brazil

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Brazil constitutes a hotspot for arbovirus emergence, posing persistent public health challenges. In São José do Rio Preto (SJdRP), dengue has historically predominated, whereas only 62 chikungunya virus (CHIKV) cases were reported between 2015 and 2023, suggesting cryptic circulation. However, from 2024 onwards, CHIKV notifications began to increase across São Paulo State. Because arboviral febrile illnesses frequently overlap clinically, early detection necessitates robust surveillance systems. To address this, mosquito collections were conducted over 15 months in SJdRP and screened for CHIKV RNA. Positive specimens were further analyzed through virus isolation and whole-genome sequencing. Overall, 5.6% of specimens tested positive, with prevalence rising from December 2023 and peaking in May 2024, coinciding with human cases. Phylogenetic reconstruction indicated multiple introductions, primarily from southeastern Brazil, driving viral establishment. These events likely facilitated transmission within a dense, susceptible vector population, contributing to extensive spread and culminating in a major CHIKV outbreak in northwestern São Paulo.

Chikungunya virus (CHIKV) (family *Togaviridae*, genus *Alphavirus*) is one of the main arthropod-borne viruses transmitted by *Aedes aegypti* and *Ae. albopictus*[1]. It causes a substantial impact on global public health, especially in tropical and subtropical areas where competent vectors are widely distributed[2].

The presence of CHIKV in the Americas was first detected in 2013[3], and the introduction of the Asian and East/Central/South-African (ECSA) genotypes was subsequently reported in the North and Northeast regions of Brazil, respectively[4]. The ECSA genotype was first identified in September 2014 in the state of Bahia, and it rapidly spread throughout the country, causing annual epidemic waves of chikungunya fever (CHIKF), particularly in the Northeast and Southeast regions[4]. Between 2017 and 2024, Brazil reported 950,551 confirmed cases, of which 218,840 (23%) were registered during January–December 2024[5]. Within Brazil, an alarming number of confirmed cases are appearing in the states of Southeast region, which accounted for 74.3% of all CHIKF cases reported in 2024[5]. The rise of CHIKV infections in Southeast Brazil is driven by multiple factors that include (1) high infestation rates of *Ae. aegypti* and *Ae. albopictus*, especially in São Paulo state[6]; (2) large, populous metropolitan areas with inadequate sanitation that allows *Aedes* mosquitoes to proliferate; (3) climate change (since the rise in average temperatures during drier and cooler seasons creates favorable conditions for established mosquito populations to breed for longer periods of time)[7]; and (4) a population susceptible to CHIKV infection (e.g., individuals who had not been previously exposed to CHIKV infections), as indicated by the low anti-CHIKV IgG seroprevalence reported in some major cities in São Paulo state[8,9]. This is a concerning scenario, as CHIKV currently co-circulates in several Southeastern cities

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with other high-incidence arboviruses, such as dengue virus (DENV) serotypes 1, 2, and 3[10–13]. Moreover, the increased prevalence of CHIKF in DENV-endemic areas presents a challenge for differential diagnosis, since the symptoms of these acute febrile diseases overlap. This situation highlights the need for continuous monitoring of circulating arboviruses, since CHIKV can cause long-lasting sequelae such as debilitating arthritis and arthralgia or more severe outcomes like neurological disorders[14]. Additionally, real-time data on areas with endemic or epidemic CHIKV transmission is crucial to guide health authorities in effectively prioritizing vaccine distribution. This is particularly relevant given that, on April 14, 2025, the Brazilian Ministry of Health approved the use of the first CHIKV vaccine (Ixchiq) in the country. The vaccine is produced by the Butantan Institute in partnership with the French pharmaceutical company Valneva[15–17]. At present, the Ministry of Health is working on incorporating it into the Brazilian public health system.

A recent study conducted in São José do Rio Preto (SJdRP), a medium-sized city in northwestern São Paulo state that is classified as hyperendemic for DENV, revealed low circulation of CHIKV[8], exhibiting only 62 confirmed cases from January 2025 to October 2023[18]. This study warned of potential outbreaks of this disease in the future, due to the presence of competent vectors and a substantial immunologically naïve CHIKV population[8]. Along similar lines, considering the recent rise in CHIKF cases in São Paulo state[5], in this study, we integrated entomological, epidemiological, and genomic data to investigate CHIKV circulation in SJdRP during the period immediately preceding and during the first epidemic in northwestern São Paulo, and to trace the spatiotemporal dynamics of the virus in the region.

Results

Prevalence of CHIKV in Brazil between 2017 and 2024

Several waves of infection have been observed since the introduction of CHIKV into Brazil (Fig. 1A). According to the SINAN[5], from the Brazilian Ministry of Health, a total of 950,551 confirmed cases of CHIKF were reported in all Brazilian states from January 2017 to December 2024 (Supplementary Table 2). During this same period, eight peaks of infections were identified across the country, occurring predominantly in April of each year. The Northeast and Southeast regions of the country accounted for most of these reported cases (Fig. 1A, Supplementary Table 3), but the incidence rates per 100,000 inhabitants clearly show that in 2017 the highest incidence of CHIKF was observed in the North and Northeast, particularly in the states of Roraima and Ceará, which reported 723 and 1196 cases per 100,000 inhabitants, respectively (Fig. 1B and Supplementary Table 4). In 2018, the states with the highest incidence shifted to Pará, Mato Grosso, Rio de Janeiro, and Minas Gerais, in the North, Midwest, and Southeast regions of the country, respectively (Fig. 1B and Supplementary Table 4). During 2020 and 2021, the highest incidence of CHIKF cases was concentrated in the Northeast, but from 2022, an increase was also observed in the Southeast as well as certain states in the Midwest and North (Fig. 1B and Supplementary Table 4).

Among the Southeastern states, Minas Gerais has been the most severely impacted by CHIKV infections (Fig. 1C and Supplementary Table 5), with the number of reported cases rising from 2022 to 218,844 confirmed infections in 2024. Similarly, the number of confirmed CHIKV cases in São Paulo increased from 2022, although to a lesser extent compared with other Southeastern states (Fig. 1C, Supplementary Fig. 2 and Supplementary Table 5). Notably, the low number of CHIKV cases in São Paulo is

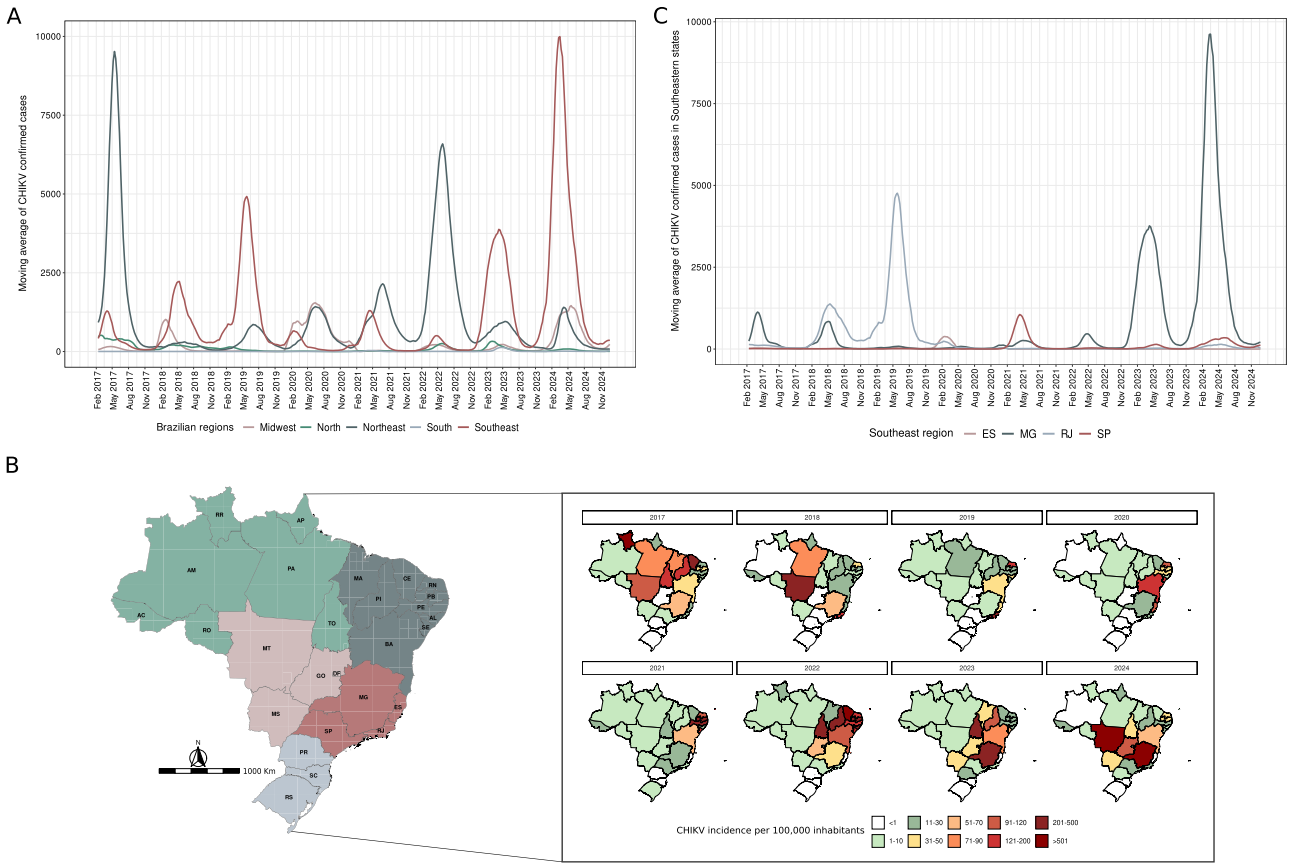

Fig. 1 | Laboratory confirmed cases of chikungunya virus (CHIKV) in Brazil. **A** Moving average of confirmed CHIKV infections across all five Brazilian regions from 2017 to 2024, highlighting several epidemic waves. **B** A map illustrating the division of Brazilian regions and their respective states, along with the incidence rate of CHIKV infections per 100,000 inhabitants in all Brazilian states from 2017 to 2024. The color scale represents variations in the incidence rates. **C** Moving average of confirmed CHIKV infections reported in the Southeastern states of Brazil from 2017 to 2024. Shapefiles were obtained from the Brazilian government through IBGE on its official website, available at: https://www.ibge.gov.br/geociencias/organizacao-do-territorio/malhas-territoriais/15774-malhas.html.

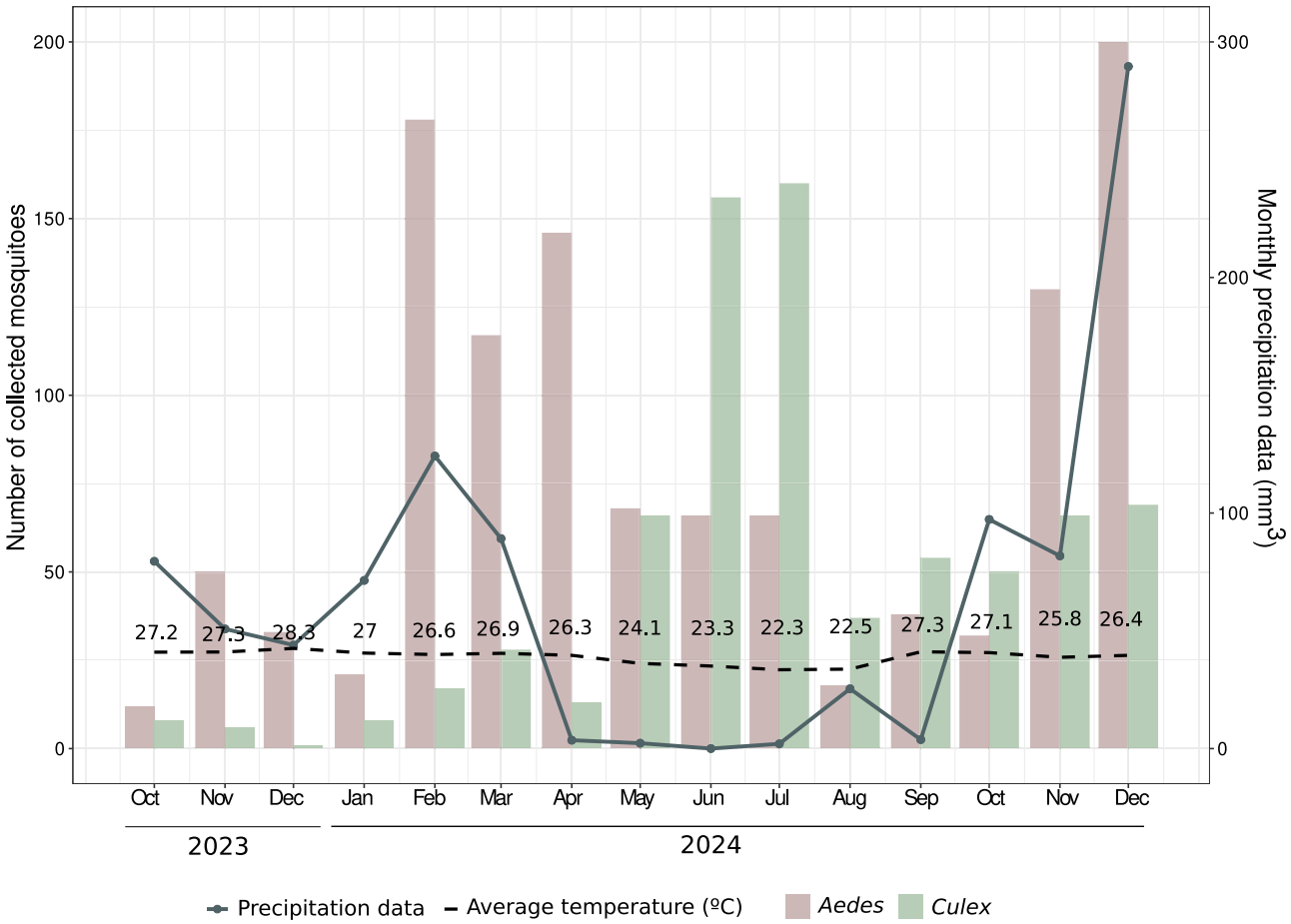

Fig. 2 | Vector surveillance. Monthly number of mosquitoes collected by genus from October 2023 to December 2024. The dotted line represents the average monthly temperature, while the solid line indicates the recorded precipitation for each month of the study period.

reflected in several cities, including SJdRP in the northwestern region of the state, supporting the observation of consistently low case numbers from 2015 to October 2023[18].

Entomological surveillance as a tool for early detection of arboviruses

Considering the increase in CHIKF cases in the state of São Paulo, which could potentially lead to outbreaks in cities already severely affected by other arboviral diseases like dengue fever, a mosquito surveillance was established in SJdRP from October 2023. The city is considered hyperendemic for DENV, with a concerning number of confirmed cases and three different serotypes (DENV-1-3) co-circulating in late 2023 and 2024[12]. In early 2023, some CHIKV infections were reported in the city, with 17 confirmed cases from January to September 2023[18].

Between October 2023 and December 2024, our entomological surveillance system collected and identified a total of 1914 mosquito specimens, comprising *Ae.* ($n = 1164$; 60.8%), *Ae. albopictus* ($n = 11$; 0.6%), and *Culex* spp. ($n = 739$; 38.6%). Specimens of both *Aedes* and *Culex* were collected continuously throughout the study period (Fig. 2, and Supplementary Table 6). Overall, a significantly higher number of *Aedes* specimens were collected compared to *Culex* ($\chi^2 = 99.319$, df = 1, $p < 2.2 \times 10^{-16}$). *Aedes* mosquitoes were more abundant between November 2023 and April 2024, with peaks in February, March, and April. In contrast, *Culex* mosquitoes were predominantly collected from June to August 2024 (Fig. 2 and Supplementary Table 6). The abundance of *Aedes* mosquitoes was influenced by abiotic factors such as precipitation and temperature. Increases in rainfall were followed by a rise in the number of *Aedes* specimens collected (Fig. 2), suggesting that climatic conditions played a key role in mosquito population dynamics during the surveillance period.

All collected mosquito specimens were subjected to molecular testing to detect CHIKV RNA. Our results showed that a total of 107 individuals (5.6%) tested positive by RT-qPCR. Among these, 35 were *Ae. aegypti* females (32.7%), 30 *Ae. aegypti* males (28%), two *Ae. albopictus* females (1.9%), 28 *Culex* spp. females (26.2%), and 12 *Culex* spp. males (11.2%). Interestingly, only seven collected females that tested positive for CHIKV RNA were engorged (one *Ae. aegypti* and six *Culex* sp.) (Supplementary Table 7). When considering only female specimens as recognized as CHIKV vectors (*Ae. aegypti* and *Ae. albopictus*), 37 out of 582 (6.3%) individuals tested positive for CHIKV RNA. After molecular screening, all CHIKV-positive mosquito macerates were subjected to virus isolation. We successfully isolated CHIKV in Vero cells after four consecutive passages from 33/79 samples (41.8%) (Supplementary Table 7), corresponding to an overall infection rate of 1,7% ($n = 33/1914$). Among the positive isolates, 36.4% ($n = 12/33$) originated from *Ae. aegypti* females, 36.4% ($n = 12/33$) from *Ae. aegypti* males, 3% ($n = 1/33$) from an *Ae. albopictus* female, 12.1% ($n = 4/33$) from *Culex* spp. females, and 12.1% ($n = 4/33$) from *Culex* spp. males. When considering only *Aedes* females, an infection rate of 2.2% ($n = 13/582$) was observed.

The highest proportion of CHIKV RNA-positive mosquitoes was recorded in May 2024, followed by January and August of the same year (Fig. 3A). When considering only female *Ae. aegypti* and *Ae. albopictus* specimens, the recognized CHIKV vectors, the highest detection rate was observed in August 2024, with 36.4% of tested females positive for CHIKV RNA ($n = 4/11$) (Supplementary Table 8).

Fig. 3 | Entomological surveillance and spatial-temporal distribution of CHIKV. A Monthly proportion of mosquito samples testing positive for CHIKV RNA from October 2023 to December 2024. **B** Spatiotemporal distribution of laboratory-confirmed chikungunya cases among residents of São José do Rio Preto (SJdRP), overlaid with the monthly number of *Aedes* and mosquitoes testing positive for CHIKV RNA. Geographic boundaries used in the spatial analysis were obtained from official shapefiles provided by the Brazilian Institute of Geography and Statistics (IBGE), available at: https://www.ibge.gov.br/geociencias/organizacao-do-territorio/malhas-territoriais/15774-malhas.html.

Interestingly, CHIKV RNA was also detected in male mosquitoes in nearly every month sampled (Supplementary Table 8), suggesting widespread viral dissemination throughout the natural mosquito population. This persistent detection in males may be indicative of vertical or sexual transmission mechanisms. Supporting this hypothesis, a male *Culex* sp. tested positive for CHIKV RNA in October 2023, a month in which no human CHIKF cases were reported in SJdRP (Supplementary Table 8). Furthermore, our spatiotemporal analysis revealed a clear association between the increasing number of CHIKF cases and the concurrent rise in CHIKV-positive mosquitoes, across both sexes and genera (*Aedes* and *Culex*) (Fig. 3B). These findings underscores the widespread presence of the virus within the mosquito population across

all sampled areas, including in periods preceding the detection of human cases.

Next, we applied Pearson's product-moment correlation to evaluate the association between the number of *Aedes* and *Culex* males and females testing positive for CHIKV RNA and the confirmed human CHIKV cases in SJdRP. The analysis revealed a statistically significant positive correlation (Pearson's $r = 0.528$, $p = 0.042$, 95% CI: 0.022–0.818), indicating that increases in CHIKV detection in field-collected mosquitoes are associated with subsequent rises in human CHIKV cases (Fig. 4A and Supplementary Table 9). Furthermore, focusing exclusively on female *Aedes* mosquitoes (the recognized primary vector of CHIKV) we performed a correlation analysis to examine the relationship between the number of CHIKV-positive *Ae. aegypti* and *Ae. albopictus* females and confirmed human cases with a one-month lag. This analysis demonstrated a statistically significant positive correlation (Pearson's $r = 0.719$, p value = 0.003, 95% CI = 0.306–0.904), supporting the potential predictive value of CHIKV RNA detection in mosquitoes for subsequent human incidence (Fig. 4B and Supplementary Table 9).

Resurgence of CHIKV in northwestern São Paulo state

Using next-generation sequencing of CHIKV-positive samples, we successfully recovered the complete CHIKV genome from 66 mosquito specimens (22 *Ae. aegypti* females, 19 *Ae. aegypti* males, one *Ae. albopictus* female, 16 *Culex* spp. females, and eight *Culex* spp. males) as well as from all the human samples (Supplementary Table 7).

The phylogenetic dataset comprises Brazilian sequences collected between 2014 and 2025 (Supplementary Table 10). Temporal analysis via root-to-tip genetic distance regression revealed that the dataset exhibits a suitable temporal signal ($R^2 = 0.53$ and correlation coefficient = 0.73) that permits reconstruction of a time-scaled phylogenetic tree (Fig. 5). Our maximum likelihood phylogenetic tree indicated that all sequences from this study were grouped along with sequences from the Southeast, South and North regions of Brazil. The CHIKV genomes derived from human and mosquito samples were predominantly divided into two distinct groups, exhibiting high branch support (Fig. 5). One group comprised exclusively human sequences from several locations including SJdRP, cities within the RHD-XV region, and municipalities in Minas Gerais bordering the state of São Paulo. The second clade consisted of CHIKV sequences from different mosquito hosts (*Ae. aegypti*, *Ae. albopictus* and *Culex* spp.) as well as human samples, primarily collected from SJdRP and with less representation from RHD-XV municipalities. Importantly, RHD-XV sequences within both clades were interspersed with those from SJdRP, suggesting significant viral exchange between these locations (Fig. 5).

Notably, no distinct clusters were formed according to host: sequences derived from *Aedes* and *Culex* samples were grouped together with high branch support, indicating considerable genetic similarity. Our root-to-tip genetic distance analysis supported these findings, demonstrating that these sequences were clustered with high similarity (Fig. 5). Further analyses revealed that all sequences from SJdRP (collected in this study and also retrieved from the GISAID database) were closely related to CHIKV genomes from São Paulo and Minas Gerais states in Brazil's Southeast region as well as genomes from Tocantins in the North, all collected between 2022 and 2025. Interestingly, examination of the nodes in the ML tree that correspond to the most recent common ancestor (TMRCA) of the sequences obtained in this study shows that they date back to late 2022 and early 2023 (Fig. 5), correlating with the incidence rates of CHIKF cases reported in 2022 and 2023, particularly highlighting the states of São Paulo and Minas Gerais (Fig. 1B). This suggests that both states may have contributed to the recent introduction and increased circulation of CHIKV in the state of São Paulo.

Our analysis of migration patterns revealed a substantial degree of viral exchange, particularly between SJdRP and other municipalities within the RHD-XV region (Fig. 6 and Supplementary Table 11), supporting the findings from our phylogenetic reconstruction. Additionally, our results indicate that the Southeastern states have served as a major source of CHIKV exportation to other regions of Brazil. This pattern may be associated with the recent wave of infections affecting the Southeast, as well as the large number of viral genome sequences from São Paulo and Minas Gerais included in our dataset, especially from 2022 onward (Fig. 6 and Supplementary Tables 10).

By annotating both internal nodes and terminal tips of the phylogenetic tree with geographic information, we were able to trace CHIKV importation events into the RHD-XV region and specifically into SJdRP (Supplementary Table 11). Our data show that, in early 2023, CHIKV was imported into municipalities of the RHD-XV region from both the Midwest and Southeast regions. In contrast, importation into SJdRP occurred primarily from neighboring municipalities within the RHD-XV. Starting in December 2023, a marked increase in importation events was observed in both SJdRP and other municipalities within RHD-XV (Supplementary Table 11). This intensification of viral movement coincided with the onset of the CHIKV epidemic in the region, corroborated by a sharp rise in the number of infected mosquitoes collected during this period. Interestingly, SJdRP also acted as a source of CHIKV exportation, not only to other cities within the RHD-XV region but also to more distant locations, including the Southeast (specifically Minas Gerais), the South (Paraná), and the North (Tocantins) regions (Supplementary Table 11). These findings highlight the role of SJdRP in the dissemination of CHIKV across different regions of Brazil.

Discussion

Arboviral diseases pose a significant challenge to public health, particularly in tropical countries with conditions that favor the proliferation and spread of vectors. Brazil is currently endemic for several arboviruses; in 2024 alone, epidemics of DENV (serotypes 1-3), Oropouche, and CHIKV have been reported[7,10,12,19]. Of these viruses, CHIKV is particularly concerning due to its potential to severely impact quality of life in infected individuals, causing debilitating and chronic symptoms[20]. Accurate differential diagnosis is crucial for better treatment of long-term conditions. For this reason, continuous surveillance of acute febrile illness combined with entomological monitoring are important tools for early detection of new viral introductions that could lead to higher numbers of cases.

In this study, we combined epidemiological, entomological, and genomic data to better understand the spatiotemporal dynamics and transmission patterns of CHIKV in Northwestern São Paulo state. We found that annual epidemic waves have been occurring since the introduction of this virus into Brazil. Although during 2017–2021 the incidence rate was notably higher in Northeastern states, an increase in cases has been reported from 2022 onward in various regions of Brazil (North, Midwest, and especially the Southeast). These observations are consistent with several studies revealing similar epidemiological patterns across different Brazilian regions[19,21–23]. In line with our results, transmission hotspots have been identified in Pará and Tocantins in the North, and in Rio de Janeiro and eastern Minas Gerais in the Southeast region[19].

Overall, São Paulo (in the Southeast) has experienced fewer cases of CHIKF compared to other states, which is confirmed by low anti-CHIKV IgG seroprevalence in major cities such as SJdRP and Ribeirão Preto[8,9]. However, our findings indicate that since February 2023 the number of cases in São Paulo has been rising. This was the reason why we selected SJdRP, a city without previous CHIKV outbreaks and only cryptic circulation of this virus[8], as a site for active entomological surveillance to detect introductions or highly active arboviruses.

From October 2023 to December 2024, *Aedes* and *Culex* mosquitoes were collected, revealing a combined CHIKV RNA detection rate of 5.6% as determined by RT-qPCR. This rate exceeds previously reported values for field-captured mosquito populations in SJdRP[8] and in other regions of Brazil[24,25]. However, it is important to note that high detection or infection rates in field-captured mosquitoes have also been reported for other arboviruses, such as Zika virus[26,27] and DENV[28,29], especially during epidemic periods. These conditions are similar to the timeframe of our study and are consistent with our observations. Our findings showed that CHIKV RNA detection began to increase in January 2024, reaching a peak in May of the

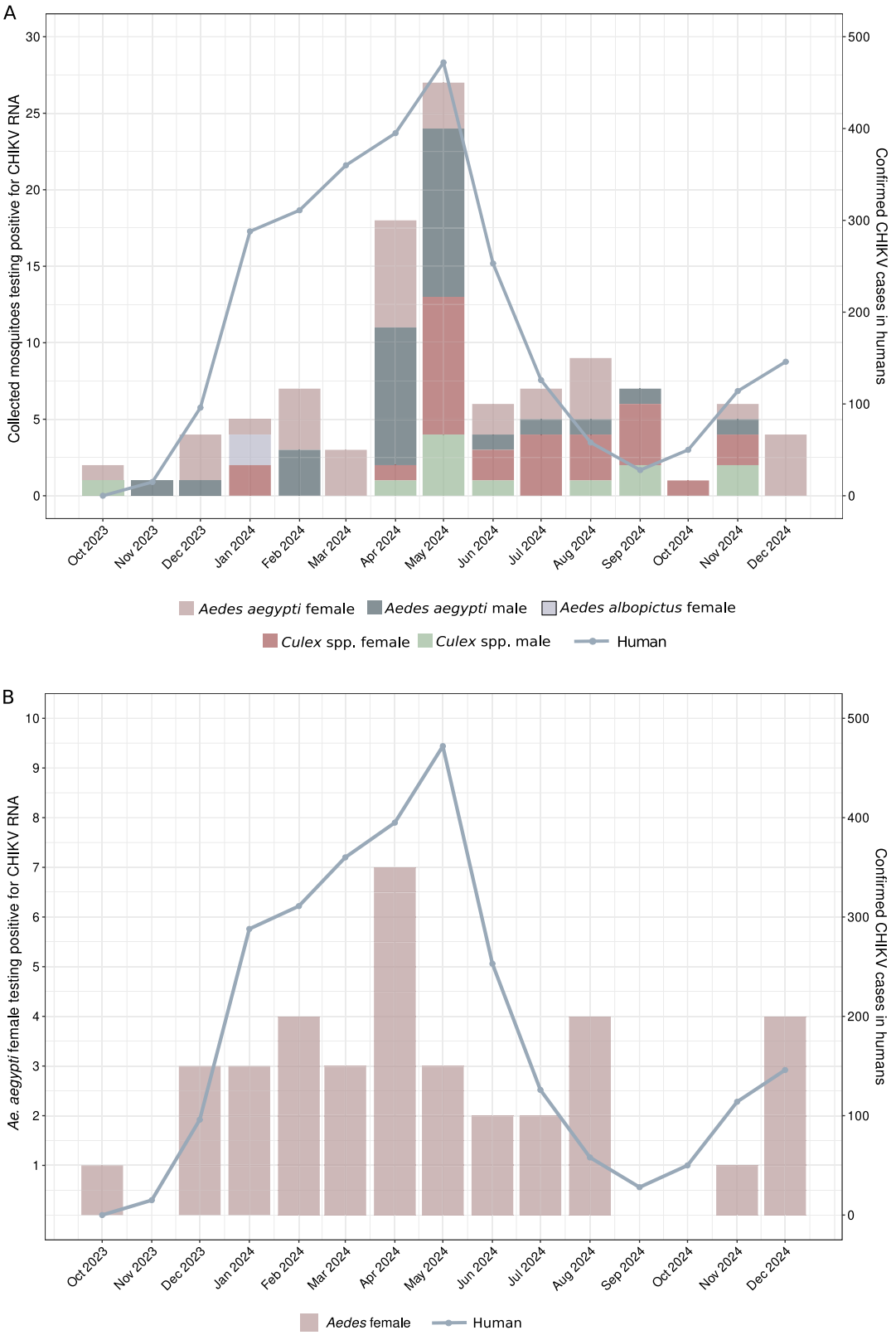

Fig. 4 | Monthly distribution of CHIKV-positive mosquitoes and confirmed human cases. A Number of *Ae. aegypti*, *Ae. albopictus*, and *Culex* spp. mosquitoes (male and female) testing positive for CHIKV RNA per month (left-*y* axis), overlaid with the number of laboratory-confirmed human CHIKV cases (blue line) (right-*y* axis). **B** Number of CHIKV-positive *Aedes* females (*Ae. aegypti* and *Ae. albopictus*) per month (left-*y* axis) plotted alongside confirmed human cases in the corresponding month (blue line) (right-*y* axis).

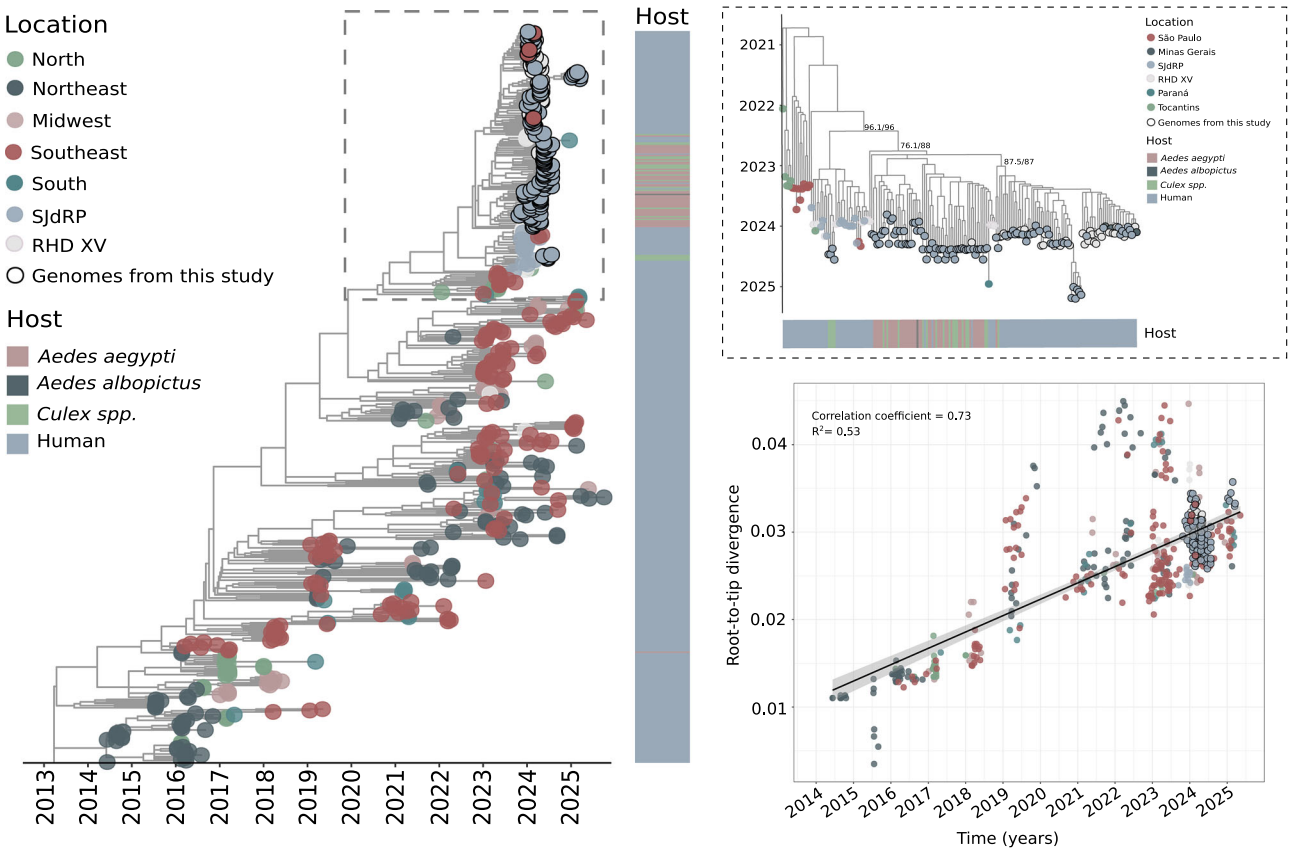

Fig. 5 | Maximum likelihood tree for CHIKV based on complete genome sequences from SJdRP, other cities within the RHD-XV and all Brazilian regions. Time-stamped phylogenetic tree reconstructed using 526 Brazilian complete genomes of ECSA genotype (141 from this study) from all Brazilian regions (Supplementary Table 10), highlighting the clade formed by CHIKV sequences obtained from *Aedes*, *Culex* and human samples generated in this study, which are grouped with sequences from Minas Gerais, São Paulo, Paraná and Tocantins states. Linear regression of root-to-tip genetic distance of CHIKV versus sampling date. Colors represent different Brazilian regions or locations. The bar next to the phylogeny represents different hosts. Genome sequences from mosquitoes and human samples obtained in this study are highlighted with black borders.

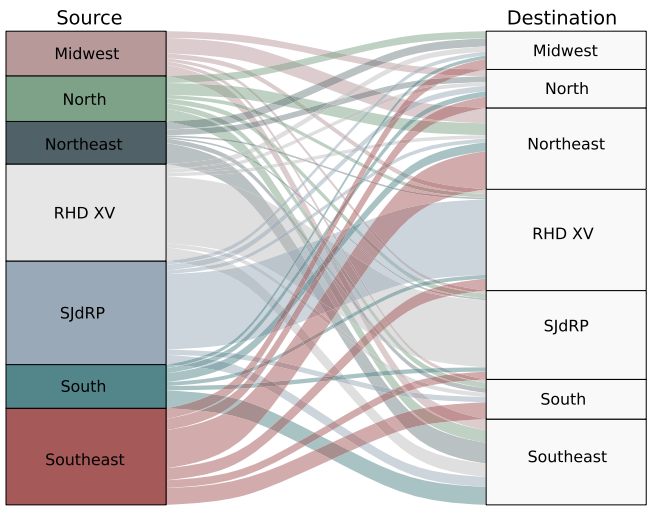

Fig. 6 | Chikungunya virus exchange across the region. Pattern of CHIKV importation and exportation events among SJdRP, RHD-XV municipalities and all Brazilian regions (North, Northeast, Midwest, Southeast, and South).

same year. The majority of CHIKV-positive specimens were *Ae. aegypti* females, consistent with their recognized role as primary vectors. These results align with the observed rise in confirmed CHIKF cases among SJdRP residents, supporting a positive relationship between the abundance of infected vectors and human incidence. This correlation reinforces the importance of entomological surveillance as an early indicator of arbovirus transmission dynamics in endemic areas.

Our study indicates that CHIKV infections exhibit a well-established seasonal pattern similar to dengue epidemics, which are strongly associated with the rainy season in Brazil when higher vector population densities are observed[21,23,30]. This pattern became more pronounced with the decline observed in the number of *Aedes* mosquitoes collected and a reduction in human cases starting in June 2024. One factor that may contribute to reduced CHIKV circulation is the decline in average rainfall observed during late autumn and early winter in the Southern Hemisphere. Lower precipitation levels can significantly reduce the availability of breeding sites, thereby influencing the seasonality and abundance of mosquito populations, as previously demonstrated in several studies[20,21,31]. This pattern is consistent with the monthly precipitation data for SJdRP, which show a marked decrease in rainfall during this period[32], potentially contributing to a reduction in vector density and viral transmission. Additionally, previous studies conducted in Brazil have documented seasonal fluctuations in mosquito populations, with *Culex* species peaking between August and October, while *Aedes* populations increase during warmer and wetter months[31,33,34]. Such ecological dynamics, in combination with continuous vector-control measures, particularly the elimination of breeding sites, may have contributed to the reduced circulation of CHIKV observed between June and October 2024.

We also detected a substantial number of male *Aedes* and *Culex* mosquitoes carrying CHIKV RNA, suggesting the occurrence of vertical and/or sexual transmission, which may contribute to viral persistence during unfavorable or inter-epidemic periods, as some studies have

reported[23–25]. Another noteworthy finding is both male and female *Culex* mosquitoes carrying CHIKV, despite this genus not being recognized as a competent vector. Currently, *Ae. aegypti* is regarded as the principal urban vector of CHIKV[20], whereas *Ae. albopictus* plays a major role in rural and temperate regions[20,35,36]. Moreover, other sylvatic *Aedes* species have also been implicated in CHIKV transmission[37,38]. In contrast, although CHIKV has occasionally been detected in *Culex* mosquitoes, particularly during outbreaks[24,39,40], and some have hypothesized a potential role in sustaining transmission[41] evidence for their vector competence remains inconclusive. To date, no species within the genus *Culex* have been conclusively established as competent vectors for CHIKV transmission[42–44].

Furthermore, the presence of CHIKV in naturally infected male mosquitoes in October 2023, even though zero human cases of CHIKF were reported that month, suggests that the virus was already circulating widely in the city well before cases began to climb in November 2023. This observation indicates potential underreporting of CHIKF cases throughout 2023. These findings are supported by data from the SJdRP municipal health department indicating that the majority of confirmed arboviral cases in 2023 and 2024 are attributed to DENV, which contrasts with our results[18]. According to the Brazilian Ministry of Health's Epidemiological Bulletin[11] there is a noted discrepancy between reported cases of dengue and chikungunya across several Brazilian states: dengue is the predominant diagnosis. This highlights the critical need for differential diagnosis using laboratory assays like RT-qPCR to identify additional cases of CHIKF and suggests that CHIKV may be underreported in certain regions.

Our spatiotemporal analysis of CHIKV in northwestern São Paulo state revealed multiple introduction events, primarily originating from the Southeast (including Minas Gerais and other cities in São Paulo state) and the Midwest. These introductions were crucial for the establishment and dissemination of the virus and marked the first outbreak of CHIKV in this area. Factors contributing to this initial epidemic include a high density of competent vectors and a substantial susceptible population for CHIKV infections[6,8,45]. Similarly, Souza et al.[30] identified the introduction of a new lineage of the CHIKV-ECSA variant, closely related to CHIKV sequences circulating in São Paulo during 2021–2022, as the cause of CHIKV recurrence in Ceará and Tocantins (Northeast and North regions, respectively) in 2022. This resurgence predominantly affected cities that had reported few or no CHIKV cases in previous epidemic waves. Furthermore, while the Northeast region of Brazil has been implicated as the original source of CHIKV in the country[19,46], the recent establishment of CHIKV in Southeast Brazil has significantly facilitated exchange of this virus throughout the country, as our migration pattern analysis shows.

Our results also indicate that the introduction of CHIKV in the Northwest region of São Paulo likely occurred in late 2022, and that the virus circulated for several months prior to the increase in human cases observed in November 2023. This observation supports our findings of both male and female mosquitoes carrying CHIKV and underscores the urgent need to implement an entomological surveillance program for early detection of circulating arboviruses.

Overall, our findings indicate widespread viral dissemination within the natural mosquito population, potentially sustained through vertical or sexual transmission. Such mechanisms may contribute to viral persistence during unfavorable environmental conditions or inter-epidemic periods. From January 2024 onward, we observed a progressive monthly increase in CHIKV RNA detection in *Ae. aegypti*, closely mirroring the rise in reported human CHIKF cases in the municipality. The re-emergence of CHIKV in northwestern São Paulo state appears to have been driven by multiple introduction events, which facilitated viral establishment in a highly abundant vector population and accelerated its rapid spread, leading to a major CHIKV outbreak in the region. Considering the co-circulation of multiple arboviruses across several municipalities in São Paulo State, our findings underscore the critical role of entomological surveillance in such regions. While entomological surveillance alone cannot prevent outbreaks, its integration with clinical and laboratory monitoring provides essential insights into viral dynamics, facilitating early detection, informing public health planning, and enabling timely, targeted interventions.

Methods

Study area

SJdRP is a city in northwestern São Paulo state with an estimated 480,393 inhabitants, 94% in urban areas and 6% in rural settings[47]; average annual temperature and rainfall are 27 °C and 139 mm, respectively[32]. It is the largest municipality in the northwestern region of the state and the headquarters for the XV Regional Health District (RHD-XV), which comprises 101 adjacent municipalities. The city is considered hyperendemic for dengue fever, as successive epidemics have been recorded in recent years[12,48].

Recruitment and collection points

Because of this hyperendemic status, in SJdRP we established a sentinel program for early detection of emergent or highly active arboviruses within vectors in urban areas. First, we conducted an epidemiological survey in partnership with the city department of health to determine the neighborhoods with the most reported arbovirus cases and/or vector infestations over the past 5 years. From this analysis, six neighborhoods were selected based on the high numbers of arboviral disease cases reported in recent years (Supplementary Table 1). These neighborhoods, organized into a north–south and east–west grid within the municipality, were primarily composed of residential houses. We avoided installing traps in the central area of SJdRP, where there is a high density of buildings and commercial establishments (Supplementary Fig. 1). For collection points, we prioritized locations that shielded traps from direct sunlight and rainfall near areas with accumulated trash or recyclable materials and vacant lots with vegetation. Furthermore, whenever possible, we ensured that the selected collection points were at least 100–300 meters apart, as this range has been shown to fall within the average flight distance of *Ae. aegypti*[49–52]. Because obtaining consent from residents was required for monthly visits, the number of collection points could not be standardized across the six selected neighborhoods, resulting in a total of 43 residences recruited as sampling sites (Supplementary Fig. 1). Fieldwork began in October 2023, and verbal consent was obtained from residents at each visit to authorize the placement of mosquito traps in peridomestic areas. Up to 43 BG-Sentinel traps (Biogents, Germany) were deployed each month, corresponding to one trap per residence; however, not all residences were sampled every month due to logistical constraints. Traps were placed in shaded locations at or near vegetation and maintained at the collection points for 24 hours. After this period, the specimens were transferred to appropriate containers and transported to the Laboratório de Pesquisas em Virologia (LPV) at the Faculdade de Medicina de São José do Rio Preto (FAMERP) for species-level identification. Only live mosquitoes were used for subsequent analyses to ensure the integrity of molecular assays and because identification required intact specimens. Mosquitoes were identified to the species level using standard taxonomic keys[53,54]; however, specimens of the genus *Culex* were classified only to the genus level (*Culex* spp.) due to the well-documented morphological similarity among species and the recognized difficulty of achieving reliable species-level identification without molecular confirmation[55–57]. After identification, the collected specimens were stored, individually, in 1.5 ml polypropylene tubes in a −80 °C freezer until subsequent analyses.

This study was approved by the ethics review board of the FAMERP, protocol CAAE 79090324.8.0000.5415, approved on April 30, 2024. All data were analyzed anonymously, ensuring total confidentiality for all participants.

Geoprocessing

Epidemiological data for confirmed cases of CHIKF during 2017–2024 for all Brazilian states, stratified by year and epidemiological week, were obtained from the Brazilian Ministry of Health[5] (Supplementary Tables 2 and 3). Chikungunya incidence was calculated per 100,000 inhabitants

based on the estimated populations of Brazilian states from 2017 to 2024 as reported by the Instituto Brasileiro de Geografia e Estatística (IBGE) in its Sistema IBGE de Recuperação Automática database[58] (Supplementary Table 4). Case numbers for CHIKF reported in SJdRP were obtained from the city health department[18]. The Brazilian government, through IBGE, provides a database on its official website, offering shapefiles of the country's regions and municipalities for free download[59]. Maps were created using R v.4.0.114 software[60], with the sf v. 1.0-16[61,62] and ggplot2[63] packages, using shapefiles from the Brazilian states and SJdRP. The leaflet package[64] (an open-source JavaScript library) was employed to create dynamic online maps illustrating the distribution of collection points (Supplementary Fig. 1) (https://github.com/Leaflet/Leaflet). Data on CHIKV-positive mosquitoes obtained in this study, along with chikungunya human cases, sourced from the Sistema de Informação de Agravos de Notificação (SINAN) from the Brazilian Ministry of Health[5], were used to create a map illustrating the spatiotemporal distribution of CHIKV detection cases in SJdRP.

Arbovirus detection

The adult mosquitoes collected each month were individually macerated in 400 µL of ice-cold 1X PBS with a homogenizer bead, using the L-BEADER mechanical cell disruptor (Loccus, Brazil) for three 30-s cycles at 3000 rpm. The samples were then centrifuged at 5340 rcf for 10 min at 4 °C and the mosquito macerates were used for viral RNA extraction, according to Machado et al.[65] and for virus isolation. Next, molecular analyses were conducted to detect the presence of CHIKV RNA. One-step real-time polymerase chain reaction (RT-qPCR) was performed using the GoTaq Probe 1-Step RT-qPCR system (Promega, Madison, USA) along with TaqMan fluorescent primers and probes specific to CHIKV obtained from Lanciotti et al.[66]. The reactions were conducted with a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). All analyses were performed in linear and multi-component mode, adjusting the baseline to the cycle threshold (Ct) of the negative control to eliminate possible reagent interference.

Cells

C6/36 cells are an epithelial cell line derived from *Ae. albopictus* mosquito larvae. These cells were cultured in Leibovitz's L-15 medium (Cultilab, BRL), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 50 µg/mL streptomycin. The cultures were maintained at 28 °C in a non-CO_2 incubator. Vero cells, a continuous cell line derived from the kidney epithelial cells of the African green monkey, were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 50 µg/mL streptomycin. These cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

Virus Isolation

A total of 200 µL of the mosquito macerate supernatant was filtered through a 0.22 µm syringe filter, and the filtrate was used for virus isolation. Monolayers of C6/36 and Vero cells, previously seeded in 24-well plates and grown to approximately 90% confluence, were inoculated with 180 µL of the filtered supernatant. Viral adsorption was performed for 120 minutes, with the plates agitated every 10 min. Following adsorption, the cell monolayers were overlaid with MEM containing 2% FBS (for Vero cells) or L-15 medium with 2% FBS (for C6/36 cells). Cultures were incubated and monitored daily for 96 h to evaluate cytopathic effects (CPE). After this period, the supernatant was collected and subjected to four consecutive passages in fresh C6/36 and Vero cells, following the same procedure. After the fourth passage, the supernatant was harvested and stored at −80 °C until RNA extraction and subsequent RT-qPCR analysis for viral detection.

Statistical analysis

Data analysis was performed using R v. 4.0.114[60] software applying standard statistical methodologies appropriate to the type and distribution of the variables under investigation. Monthly comparisons of the number of *Aedes* and *Culex* mosquitoes collected were conducted to detect potential differences in species abundance throughout the study period. For each month, Pearson's Chi-squared test for goodness-of-fit was employed when expected cell frequencies were ≥5. When statistically significant differences were observed, standardized residuals were examined to identify which species or months contributed most to the deviations. To evaluate the temporal association between entomological CHIKV detection and human CHIKF incidence, correlation analyses were performed. These included: (i) the total number of CHIKV RNA-positive mosquitoes (regardless of species or sex), and (ii) the number of CHIKV-positive *Aedes* females, compared with the number of laboratory-confirmed human CHIKV cases per month. Prior to correlation analysis, the distribution of each variable was assessed using the Shapiro–Wilk test. Pearson's correlation coefficient was applied when assumptions of normality and linearity were met. Potential time-lagged associations were also explored by correlating the number of CHIKV-infected mosquitoes in a given month with the number of human cases in the subsequent month (1-month lag). All statistical analyses were performed at a significance level of 5% ($p < 0.05$).

CHIKV whole-genome sequencing

After molecular screening for CHIKV in the mosquitoes, positive samples underwent whole-genome sequencing. Library construction and complete genome sequencing were performed using next-generation sequencing (NGS); cDNA synthesis, genome amplification, and library preparation were carried out according to the instructions provided for the Illumina CovidSeq Test (Illumina, San Diego, CA, USA) but adapted by replacing the SARS-CoV-2 primer pools with CHIKV-specific primer pools described by Quick et al.[67]. Library quantification was performed using the Qubit dsDNA HS Assay on a Qubit 2.0 device (Invitrogen, Waltham, MA, USA). Quality control for the libraries was verified with a TapeStation 4150 system and High Sensitivity D1000 ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing was done with a MiSeq Reagent kit v3 (2 × 150 cycles) (Illumina, San Diego, CA, USA), and the NextSeq PhiX Control kit was used as a normalization sample with the libraries sequenced on the MiSeq system (Illumina, San Diego, CA, USA).

Serum samples

In order to link detection of CHIKV-positive mosquitoes in SJdRP with human cases and better characterize the CHIKV genotype circulating in northwestern São Paulo state, we included 77 serum samples from symptomatic patients with a positive diagnosis of CHIKF. These human samples were collected at the Hospital de Base de São José do Rio Preto (HB). This hospital is one of the largest and most important health complexes in the municipality and serves as a reference health center for more than two million inhabitants across 102 municipalities within the 15th Regional Health Department (RHD XV), headquartered in SJdRP. The samples were obtained during January—May 2024 and January—March 2025 from individuals residing in 13 different municipalities within the RHD-XV and two cities in the adjacent state of Minas Gerais (Frutal and Fronteira) which border São Paulo and are less than 115 kilometers from SJdRP. These analyses utilized samples collected for routine diagnosis, and the need for informed consent was waived by the Ethics Committee of the FAMERP, protocol CAAE 02078812.8.0000.5415, approved on July 03, 2012, and amended with approval on May 11, 2016.

Total RNA extraction was performed on all serum samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Next, RT-qPCR to detect CHIKV RNA and whole-genome sequencing were performed as previously described.

Genome assembly and phylogenetic analyses

The quality of raw reads was assessed using the FastQC v. 0.11.4 program[68], and Cutadapt v. 4.6 software[69] was used to remove low-quality reads (Phred score >30) shorter than 50 base pairs, as well as duplicate sequences, adapters, and primers used during library construction. Clean reads were then mapped against the genomes of their respective hosts (GCA_002204515.1, GCA_001444175.1, and GCA_015732765.1,

available at: https://vectorbase.org/vectorbase/app/search/organism/GenomeDataTypes/result) using Bowtie2 v. 2.3.5.1[70]. Reads not mapped to the host genome were filtered using SAMtools v. 1.6[71] and mapped against the CHIKV reference genome (NC_004162.2) using BWA mem v. 0.7.17-r1188 software and SAMtools v. 1.6[71,72] for read sorting and indexing. After post-processing steps, the assembled genomes were recovered using iVar v. 1.3.1[73,74].

Next, all the generated consensus sequences were analyzed using the Genome Detective virus typing tool[75] for genotype classification. Phylogenetic analyses were also performed to confirm the genotype of the circulating virus and divergence from viruses sequenced from other Brazilian regions. To do so, the assembled genomes were aligned with a dataset containing CHIKV sequences available in the EpiArbo-GISAID database[76] and the GenBank NCBI database[77], using MAFFT v. 7.520[78] and edited with AliView v. 1.28[79]. Maximum likelihood (ML) trees were reconstructed using IQ-TREE v. 1.6.9 software[80], with the best nucleotide substitution model inferred according to the Bayesian information criterion by ModelFinder[81]. Branch reliability was tested using a combination of the ultrafast bootstrap approximation approach (UFBoot)[82] and SH-like approximate likelihood ratio test (SH-aLRT)[83] with 10,000 replicates each, respectively. Phylogenetic trees were visualized and edited using R v.4.0.114 software[60] and the ggtree package[84].

To investigate the temporal signal from the ML trees, we regressed root-to-tip genetic distances against sample collection dates using the TempEst tool v. 1.5.1[85], considering a correlation coefficient of >0.4 to accept temporal structure. Next, the generated phylogenies were subjected to TreeTime v. 0.9.3[86] to convert the raw ML trees into time-scaled trees, as described by Banho et al.[87]. Finally, we used the time-scaled tree topology to infer the number of viral exchange events between the five Brazilian regions, the cities within the RHD-XV and SJdRP using TreeTime mugration v. 0.9.3[86], and by mapping the locations to tips and internal nodes from the annotated tree topology we were able to estimate the number of virus importations and exportations among regions/cities.

Data availability

All the chikungunya genomes generated and analyzed in this study are available in the GISAID database, under accession numbers provided in the Supplementary Table 10. All data used for epidemiological and phylogenetic analyses are available in the Mendeley Data repository (https://data.mendeley.com/datasets/7yr7xtvp2k/1).

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Author contributions

C.A.B. and M.L.N. conceived and designed the study. C.A.B., M.C.P.P., O.B.N., G.P.M., M.V.M.F., A.P.L., R.M.T.M. and J.T.D. collected and identified mosquito samples. C.A.B., M.C.P.P., O.B.N., M.V.M.F. and A.P.L. processed and screened mosquito samples. M.L.B. collected and screened human samples. C.A.B., M.C.P.P., O.B.N., M.V.M.F. and A.P.L., curated the metadata and performed molecular screening in mosquito samples. K.L.L. performed CHIKV isolation from mosquito macerate samples. A.F.N. provided metadata from SJdRP. C.F.E. curated metadata for the human samples. L.S. standardized the arbovirus sequencing protocol. J.P.A.J. provided materials and equipment for the sequencing CHIKV-positive samples. C.A.B. and B.C.M. performed sequencing. C.A.B. performed epidemiological, geoprocessing and phylogenetic analyses. C.A.B. interpreted the data. C.A.B., M.C.P.P., B.C.M., N.V. and C.F.E. wrote the first draft of the manuscript. C.A.B., M.C.P.P., B.C.M., C.F.E., N.V. and M.L.N. edited and revised the manuscript. N.V. and M.L.N. provided the resources for the survey. All authors approved the final version of this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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