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Genetic diversity and evolutionary dynamics of Senecavirus A in Brazil

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Abstract

Senecavirus A (SVA) is a picornavirus that was first isolated in the USA in 2002; however, there is evidence that the virus was circulating in swine herds since 1988. Despite frequent reports of vesicular disease outbreaks caused by SVA infection in swine in Brazil since 2014, there is limited data on the genetic diversity and evolution of the virus in the country. SVA was isolated from swine exhibiting vesicular lesions, with samples originating from farms or slaughterhouses across 57 municipalities in 8 Brazilian states between 2018 and 2022. We obtained 501 SVA genomes through Sanger and Oxford Nanopore sequencing. Phylogenetic analysis revealed that Brazilian SVA sequences are genetically distinct from sequences from other countries, including China, USA and Canada, and form a monophyletic cluster, indicating a common ancestor for the viruses currently circulating in Brazil. Furthermore, there are two main clusters with sequences from the Midwest and Southern regions, suggesting that SVA is evolving independently in the swine population of the country. Pairwise sequence comparisons allowed us to identify seven unique mutations with high frequency in the Brazilian SVA sequences. Notably, mutations were identified in specific regions of the capsid proteins that interact with the host cell receptor (ANTRX1) and in surface-exposed residues, suggesting potential evolutionary changes due to receptor interaction or immune pressure. Recombination analysis provided evidence of at least five recombination events among the Brazilian strains. These findings offer new insights into the evolution of SVA circulating in Brazil and into the global epidemiology/evolutionary dynamics of the virus.

DATA AVAILABILITY

SVA sequences generated by this work were deposited at GenBank (NCBI) under accession numbers (OR567038 to OR567087 and PQ117094 to PQ117544).

INTRODUCTION

Senecavirus A (SVA), also known as Seneca Valley virus, is an emerging viral pathogen causing vesicular disease (VD) in swine. SVA infection in pigs can result in a range of clinical signs that are indistinguishable from other VDs, including foot-and-mouth disease (FMD) [1]. This clinical similarity requires laboratory-based differential diagnosis to rule out other VD-causing agents, which leads to animal transportation restrictions and consequently increased production costs.

SVA belongs to the family *Picornaviridae*, genus *Senecavirus* [2]. The SVA genome is ~7.3 kb in length, consisting of a single-stranded positive-sense RNA (ssRNA) molecule and containing a single ORF that encodes a 2,181 amino acid polyprotein.

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Abbreviations: ATCC, American Type Culture Collection; FMD, foot-and-mouth disease; GO, Goiás; MAPA, Ministry of Agriculture and Livestock; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; PR, Paraná; RS, Rio Grande do Sul; SC, Santa Catarina; SH-aLRT, Shimodaira–Hasegawa approximate likelihood ratio test; SP, São Paulo; UFBoot, ultrafast bootstrap.
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Three supplementary tables are available with the online version of this article.

The ORF is flanked by a 5′ UTR containing a type IV internal ribosome entry site and a short 3′ UTR followed by a poly A tail. The polyprotein is cleaved into 12 proteins: leader (L)-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D [3]. The virus shares common structural characteristics with the members of the *Picornaviridae* family, including absence of envelope, particle size with ~30–32 nm in diameter and a capsid with 60 protomers of the structural proteins VP4, VP2, VP3 and VP1 [2]. The remaining eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C and 3D) play multiple roles in the viral life cycle, replication and evasion from host antiviral immune responses [4].

The virus was first identified as a contaminant in PER.C6 cell lines, which are transformed cells derived from human foetal retinoblasts [3]. The origin of the virus is thought to be linked to the use of contaminated porcine trypsin during culture of the PER.C6 cells [3]. Although SVA was found in samples dating back to 1988 in the USA, it was only in 2007 that the virus was associated with VD in pigs [5]. Reports of SVA VD have since been documented in many countries, including the USA, Canada, China, Colombia, Thailand, Vietnam, Mexico, India, Russia, England, Chile and Brazil [6–15]. VD outbreaks associated with SVA were first reported in Brazil during 2014 [14, 16] and were the first outbreaks identified outside of North America. In addition to VD, the virus has also been associated with sporadic cases of neonatal mortality in piglets [14]. Since its first detection in 2014, outbreaks of SVA have been frequently reported in the country [17].

Several studies have used genomic surveillance of field SVA isolates to investigate potential epidemiological links between SVA outbreaks and isolates in given geographic locations and to gain insights into the evolution of the virus globally [5, 18, 19]. The forces driving SVA evolution include the lack of 3′→5′ exonuclease proofreading activity of RdRp (RNA-dependent RNA), immune pressure and recombination events [20, 21]. An early study analysing the capsid VP1 protein from Brazilian SVA isolates suggested a common origin among the viruses circulating in the country and a high genetic divergence to viruses circulating in other countries [22]. Similarly, Joshi and collaborators [5] showed that SVA isolates cluster mostly according to their geographical origin with sequences obtained in different countries forming separate phylogenetic branches. These results indicate that the virus is probably evolving independently in swine populations of each endemic country.

Here, we analysed whole-genome sequences of 501 SVA isolates obtained from swine from Brazil between 2018 and 2022. We investigated evolutionary patterns and dynamics of SVA over the years and compared sequences and phylogeographic data to infer potential dispersal pathways within Brazil.

METHODS

Viral isolates

A total of 614 SVA previously isolated viruses, collected between 2018 and 2022 from pigs presenting VD, were provided by the Federal Laboratory for Agricultural Defense – LFDA/MG, the official veterinary laboratory from the Ministry of Agriculture and Livestock (MAPA). All samples had a negative diagnostic result for FMD virus (*Aphthovirus vesiculae*), vesicular stomatitis Alagoas virus (*Vesiculovirus alagoas*), Cocal virus (*Vesiculovirus cocal*), swine vesicular disease virus (*Enterovirus betacoxsackie*) and vesicular exanthema of swine virus (*Vesivirus exanthema*). The SVA isolates originated from 57 municipalities distributed in 8 different Brazilian states: Goiás (GO), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Paraná (PR), Rio Grande do Sul (RS), Santa Catarina (SC) and São Paulo (SP). Details on the isolation source, collection date and sequencing methods for the selected SVA isolates are provided in Table S1 (available in the online Supplementary Material). Viral isolates were inoculated in the H1299 cell (human non-small cell lung carcinoma cell, American Type Culture Collection [ATCC] CRL-5803™) line for virus amplification prior to nucleic acid extraction. The supernatant from cell cultures showing cytopathic effect was collected and stored at −80 °C. Based on the temporal distribution and geographic origin of the viruses that were successfully recovered, 501 out of the 614 SVA isolates were selected for genome sequencing using ABI3130xl Sanger sequencing (n=50) and Oxford Nanopore (n=451) sequencing platforms.

RNA extraction and cDNA synthesis

RNA was extracted from 200 µl of the cell culture supernatant using the BioGene DNA/RNA viral kit (Bioclin Quibasa, Brazil), according to the manufacturer's instructions. cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher, USA), in a 20 µl reaction using random primers. After the reverse transcription reaction was complete, the cDNA was treated with RNase H (1 U) at 37 °C for 20 min to ensure digestion of any residual SVA RNA. After incubation, the RNase H was inactivated by heating at 65 °C for 10 min. cDNA samples were then stored at −80 °C until further use.

RT-PCR and Sanger sequencing genome assembly

A primer walking reverse transcriptase-polymerase chain reaction (RT-PCR) sequencing approach [23] was initially used for sequencing the full genome of 50 SVA isolates. Nine pairs of overlapping primers (Table S3) covering the entire genome

of the SVA were used in the RT-PCR reactions. The RT-PCR reactions were performed by using the GoTaq® Colorless Master Mix (Promega® Corporation, USA) and consisted of 25 µl of the GoTaq® Colorless Master Mix, 2 µl of each primer (20 mM), 19 µl of nuclease-free water and 2 µl of the cDNA sample. The assay conditions were optimized for each set of primers with a known positive control. The RT-PCR products were subjected to electrophoresis on 1% agarose gels to confirm target amplification and then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) per the manufacturer's instructions. Purified amplicons were quantified with the Qubit® 3.0 Fluorometer and the Qubit™ dsDNA BR Assay Kit (Life Technologies, USA), followed by sequencing with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing products were purified using BigDye® XTerminator™ v3.1 (Applied Biosystems, USA) and analysed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA).

The sequence reads were assembled using the phred/phrap/consed package [24]. All consensus bases had a Phred quality score of ≥20. Genome completeness was assessed using BLASTn [25]. The single ORF of SVA was identified using ORFinder (NCBI) and confirmed using BLASTx.

Nanopore sequencing, genome assembly and analyses

For targeted whole-genome next-generation sequencing, cDNA from 451 SVA isolates was prepared for sequencing. An initial consensus sequence was generated by aligning all the sequences derived by Sanger sequences as described above with additional Brazilian sequences available on GenBank. Six pairs of primers (Table S4) were designed using Primer3 (v.2.3.7) [26] implemented in the Geneious Prime Software (v.2023.2.1) (https://www.geneious.com). The library preparation started with two multiplex RT-PCR reactions using two pools of primers (pools 1 and 2), followed by purification of pooled PCR products using AMPure XP beads (Beckman Coulter, USA) at a 1:1 bead-to-DNA volume ratio. DNA concentrations were quantified using Qubit® 3.0 Fluorometer as described above.

Library preparation followed an End Prep reaction, using the NEBNext Ultra™ II end repair/dA-tailing module (New England Biolabs, USA), followed by native barcoding using EXP-NBD196 (Oxford Nanopore Technologies, UK) and NEBNext Ultra II Ligation Module (New England Biolabs, USA). The pooled barcode libraries were purified using AMPure XP beads (Beckman Coulter, USA) and subjected to the AMII Adapter ligation, using Quick T4 DNA ligase (New England Biolabs, USA), as part of the Ligation Sequencing Kit (SQK-LSK109, Oxford Nanopore Technologies, UK). Final libraries were loaded in R9.4 flow cells in a MinION Mk1B (Oxford Nanopore Technologies, UK) for 16 h sequencing runs.

Raw FAST5 reads were basecalled and demultiplexed using Guppy v.6.5.7 in high accuracy mode with parameters --require_barcodes_both_ends --detect_mid_strand_barcodes -min_score_barcode_mid 60 and model dna_r9.4.1_450bps_hac. Consensus sequences were generated using a previously published method [27], with the addition of Cutadapt to remove primer sequences from the reads [28]. SVA nucleotide sequences available in May 2024 were downloaded from GenBank. Sequences with partial coverage of the coding region, missing isolation source information, duplicates from the same historical strain or those associated with patents were excluded from the dataset. The final dataset comprised 805 full-length ORF sequences (~6,546 nt), including 514 sequences of Brazilian SVA (501 generated in this study) and 291 sequences collected globally from Canada (*n*=14), Chile (*n*=1), China (*n*=152), Colombia (*n*=1), India (*n*=1), Mexico (*n*=1), Thailand (*n*=6), USA (*n*=114) and Vietnam (*n*=1). Nucleotide alignments were generated using MAFFT v7.4.9 [29] with default parameters.

Phylogenetic and phylogeographic analysis

The Nextstrain pipeline [30] was used to analyse the final dataset and infer the evolutionary relationships between isolates. Maximum likelihood trees were inferred using IQ-TREE 2 [31] with ModelFinder [32] for best-fit model selection (-m MFP). Branch support was assessed with 1,000 ultrafast bootstrap (UFBoot) replicates [33] (-bb 1000) and 1,000 Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) replicates [34] (-alrt 1000). Nodes with ≥95% UFBoot support and ≥80% SH-aLRT values were considered strongly supported. The initial phylogenetic tree was refined with TreeTime [35] for a time-resolved phylogeny analysis, based on the year of isolation. The phylogenetic tree and map with the predicted dispersal lines were visualized using the Auspice tool kit (available at https://auspice.us).

Protein structure prediction

All structural data on SVA proteins were obtained from the Research Collaboratory for Structural Bioinformatics PDB database, and the corresponding three-dimensional coordinates were downloaded in .pdb format [36]. Using PyMOL 2.3.4 software, the protein structure was analysed and visualized to provide a two-dimensional graphical depiction of protein interactions [37].

Recombination analysis

Potential recombination events were determined using RDP5 software [38] with seven different methods: RDP, GENECONV, Bootscan, MaxChi, Chimaera, SIScan and 3Seq. Analyses were performed on both the sequences generated in this study and those retrieved from public databases. Positive events were selected based on the detection by at least six of the seven methods with a *P*-value equal to or less than 1×10^{-5}. To illustrate the recombinant breakpoint and involved genomic regions, the Simplot++ software [39] was used, with parameters: window 200 bp, step 20 bp and distance model Kimura 2-parameter.

RESULTS

Sequence analysis

The full-length or nearly full-length genomes of 501 SVA isolates were obtained, with sequence lengths ranging from 7,124 to 7,203 nucleotides. No deletions or insertions were detected across these sequences. The SVA genome sequences were deposited in GenBank (NCBI) under accession numbers (OR567038 to OR567087 and PQ117094 to PQ117544).

Pairwise sequence comparisons of the full-ORF coding sequences among Brazilian SVA isolates from this study and those available in GenBank revealed an identity of 97.16 and 94.27% at the amino acid and nucleotide levels, respectively.

Phylogenetic and substitution rate analysis

The topology of the phylogenetic tree generated in this study was largely consistent with previously published SVA phylogenies [5]. All Brazilian SVA sequences clustered in a well-supported (SH-aLRT=99.1%, UFBoot=100%) monophyletic clade distinct from SVA sequences/isolates found in other countries (Fig. 1a). Within the Brazilian clade, two main subclades were identified, one predominantly composed of sequences from states in the Midwest (GO, MT and MS), and the other from the Southern region states (PR, SC and RS) (Fig. 1b).

The Nextstrain pipeline analysis estimated a mean substitution rate of 3.6×10^{-3} substitutions/site/year for the global dataset (n=805 sequences) and 3.71×10^{-3} substitutions/site/year for the Brazilian sequence subset (Fig. S5), suggesting that the evolutionary rate of this virus is relatively stable across geographic regions, with no strong evidence of increased substitution rates in Brazilian viruses.

Global and Brazilian SVA dispersal patterns

The dispersal pathways of SVA were investigated using phylogeographic inference analysis, based on whole-genome sequences, location and date of sample collection. The geographic distribution and predicted dispersal lines are presented in Fig. 2 for the global- and in Fig. 3 for the Brazilian SVA sequences. Based on phylogeographic dispersal analysis and early identification, USA and Canada are presumably the initial source of SVA from where the virus appears to have spread to other regions. Dispersal analysis suggests that from the USA, SVA may have spread to Canada, China, Mexico and several South American countries including Brazil, Colombia and Chile, with Brazil being the first country outside of North America to detect the virus in 2014 (Fig. 2c–f). Interestingly, current phylogeographic analysis suggests potential repeated dispersal of SVA between the USA and Canada (Fig. 2f). Furthermore, dispersal of the virus from China back to the USA was also inferred (Fig. 2a, b). Given the lack of precise collection dates for these key samples in China and in the USA, the inferred directional dispersion of the virus should be interpreted with caution. This analysis also suggests a link between SVA isolates detected in Canada to those detected in India and Thailand (Fig. 2e, f), and between those detected in China and Vietnam (Fig. 2d). No potential spread, based on the available sequences, was identified between South American countries.

We also analysed the dispersal pathways within the Brazilian territory (Fig. 3). After the initial identification of SVA in the SC state, we observed potential dispersal to the states of MG, GO, MT, PR and RS. Secondary spread was predicted to SP and MS. Extensive cross-state viral dispersal was detected between PR and GO, which was supported by the close phylogenetic relationship between SVA sequences detected in these states.

Mutational profiles of SVA circulating in Brazil

The Brazilian SVA sequences were grouped based on the year of isolation to track the evolutionary patterns and to detect amino acid substitutions in the coding sequence (CDS) region of the SVA genome over time. The total number of sequences studied based on the year of sample collection in pigs was as follows: 2015 (n=9), 2016 (n=3), 2018 (n=3), 2019 (n=34), 2020 (n=66), 2021 (n=177) and 2022 (n=222). Substitution were observed at 37 positions across the full-length SVA ORF (Table S2). These specific mutations were distributed throughout the polyprotein, except for VP4 and non-structural protein 2A, which had no mutations.

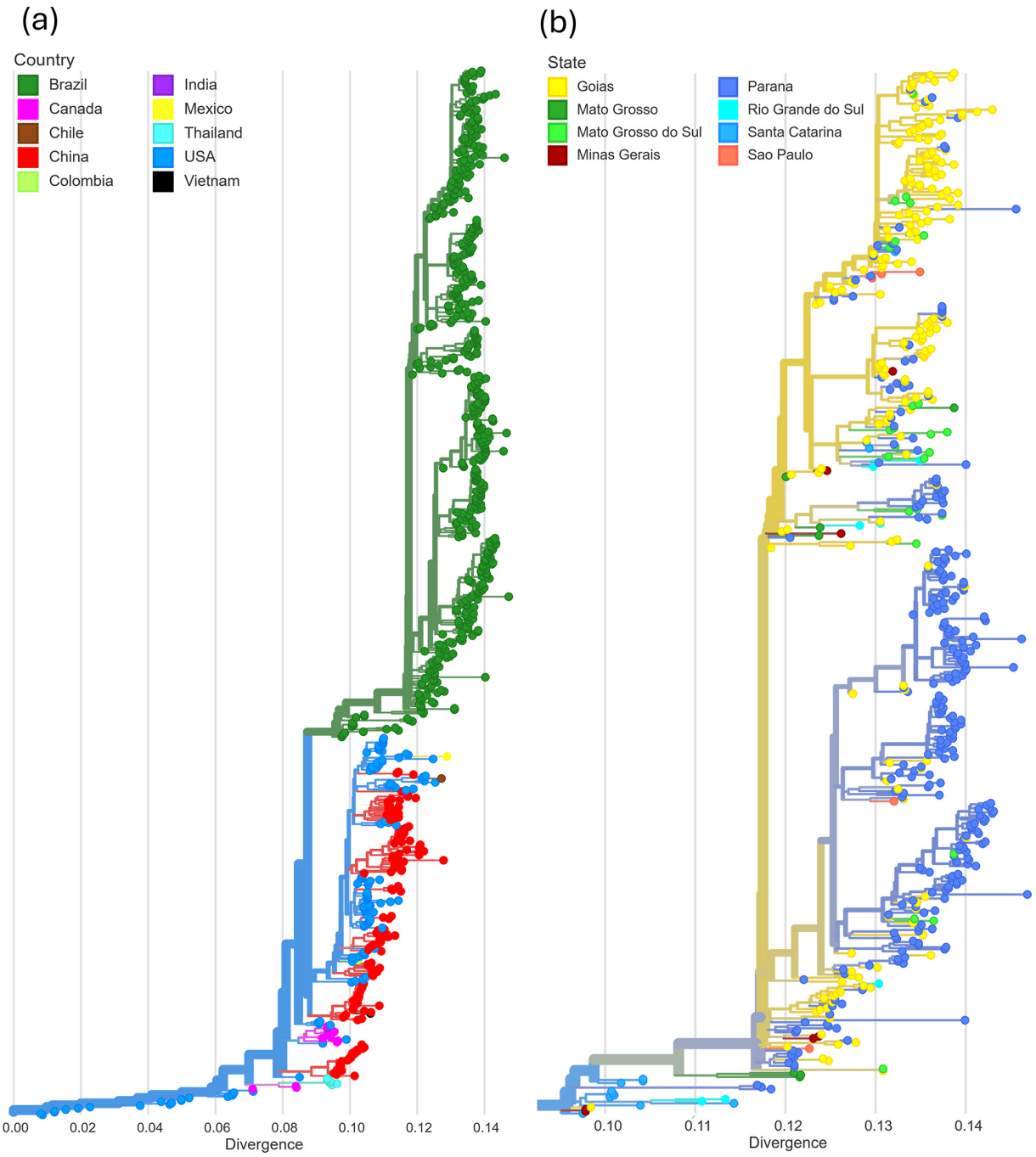

Fig. 1. Global and Brazilian phylogeny of SVA. Maximum-likelihood phylogenetic tree was inferred from full-length ORF nucleotide sequences. The analysis includes 805 sequences: 304 publicly available from GenBank and 501 newly generated in this study. (a) Global phylogeny (*n*=805). (b) Detailed subtree of sequences from Brazil (*n*=514), revealing regional clustering within the country. Tips are coloured by country of origin (a) or by Brazilian state of origin (b).

To further investigate the potential relevance of the identified mutations, the protein structure of the Senecavirus capsid (PDB ID: 6ADR [40]) was retrieved from the PDB database. The structure, which contains the entire viral capsid – comprising VP4, VP2, VP3 and VP1 – was visualized using PyMOL software. Focusing specifically on these structural proteins of SVA, we identified the amino acids that have undergone evolutionary changes over time in Brazil from 2015 to 2022 (Fig. 4). Notably, most of these amino acid substitutions mapped to surface-exposed residues of the viral capsid proteins.

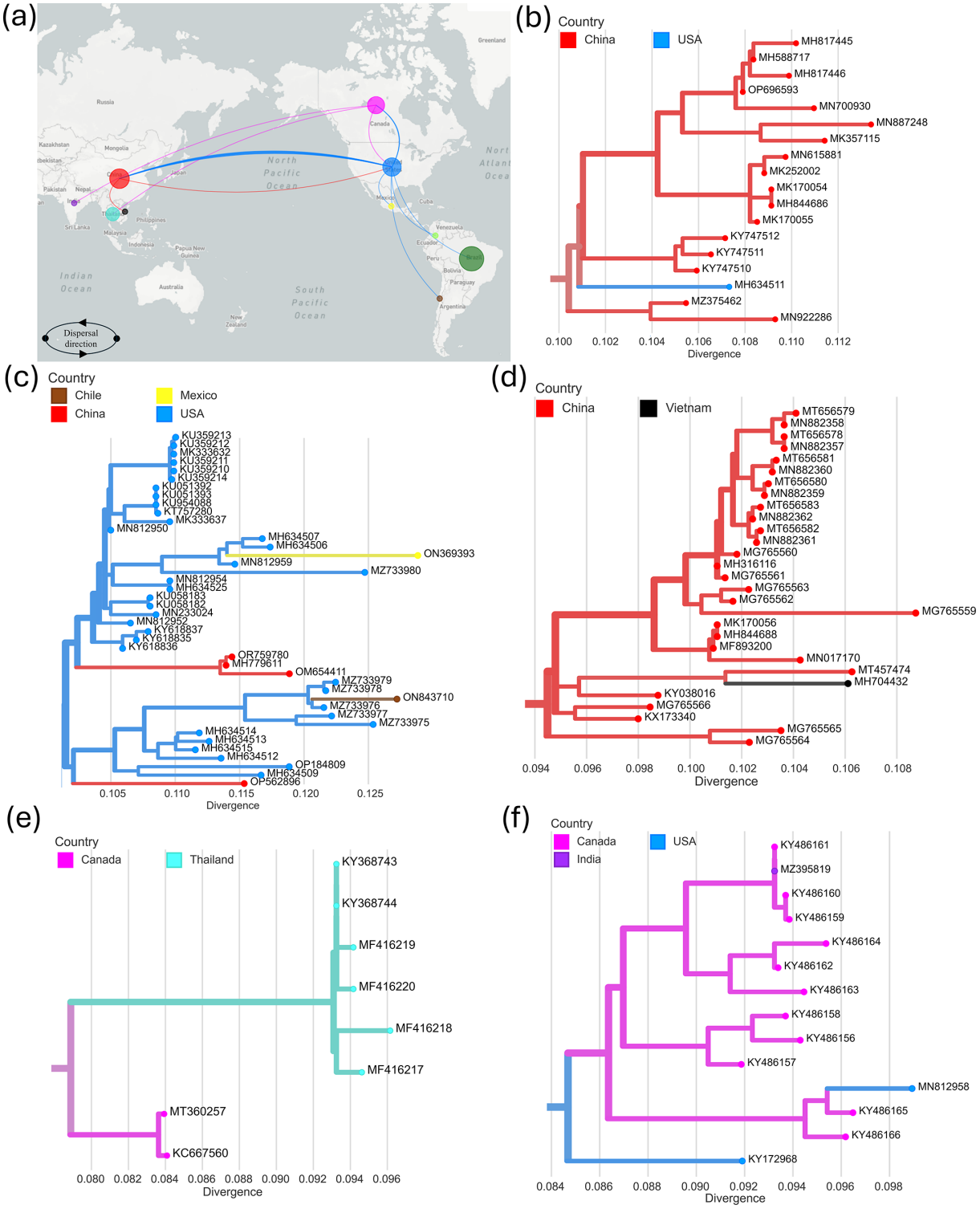

Fig. 2. Global dispersal dynamics of SVA. (a) Dispersal lines between countries inferred using phylogeographic reconstruction in Nextstrain. Curved lines connect inferred dispersal pathways, coloured by the country of origin of the viral ancestor (dispersal lines follow a default counterclockwise direction). Tree tips are coloured by the country of sample collection (b–f). Subtrees highlighting the close phylogenetic clustering of sequences from different countries, providing the underlying genetic relationship between sequences that underlie the inter-country dispersal events illustrated in (a).

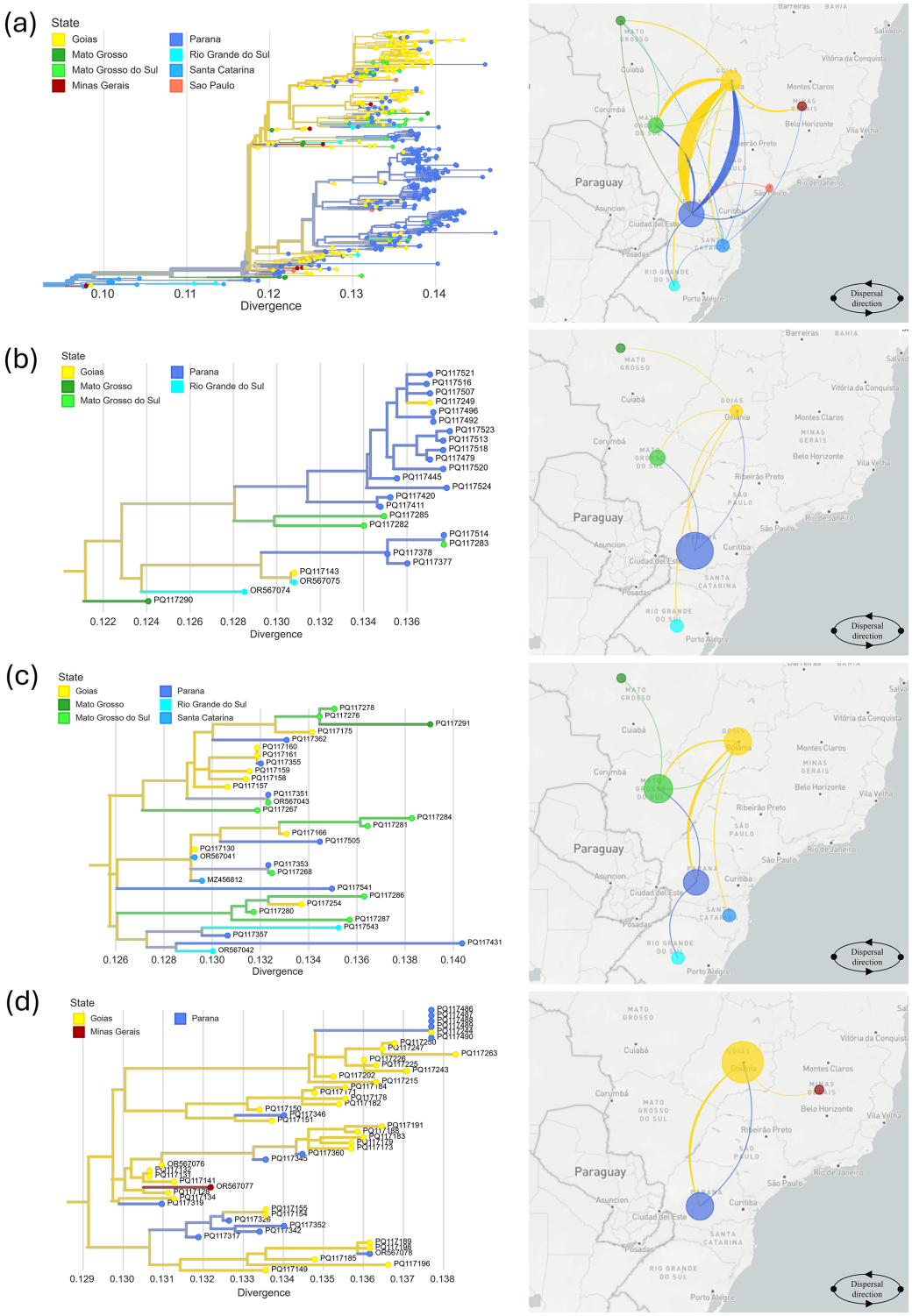

Fig. 3. Phylogeographic dispersal of SVA within Brazil. Partial maps show the distribution of samples by state and predicted inter-state transmission routes. Curved lines connect inferred transmission pathways, coloured by the state of origin of the viral ancestor (dispersal lines follow a default counterclockwise direction). Tree tips are coloured by the state of sample collection. (a) Subtree of all sequences from Brazil (*n*=514) with two main phylogenetic clusters, based on the region of isolation, and the predicted transmission lines. (b–d) Enlarged views of key branches from the main subtree, highlighting the close genetic relationships between viruses from different Brazilian states. These specific phylogenetic clusters provide the underlying evidence for the robust national dissemination routes shown in (a).

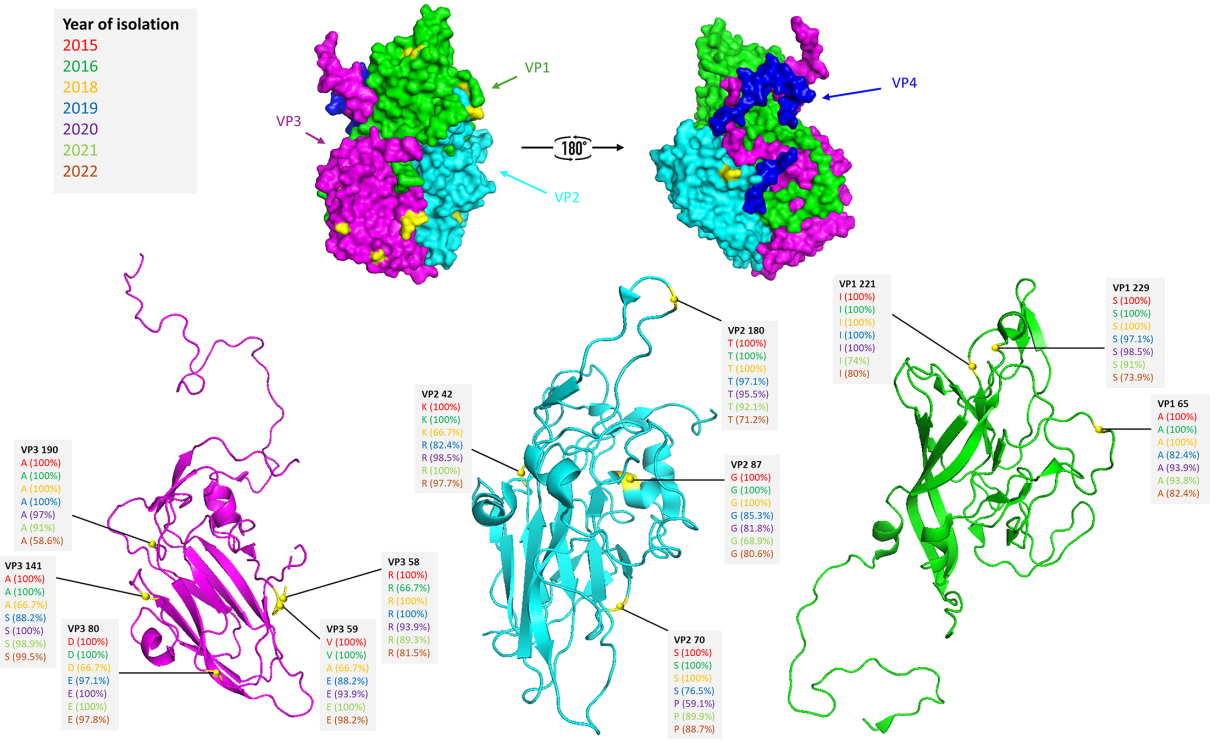

Fig. 4. Three-dimensional scheme of the structural proteins of SVA and evolutionary adaptations/changes in the Brazilian SVA sequences. Amino acids highlighted in yellow in the predicted protein structures are undergoing evolution over the years (2015–2022). VP3 protein is represented in purple, VP2 in cyan, VP1 in green and VP4 in blue. In the legend frames, the most frequent amino acid residues in each position and in the specific year are shown.

To characterize the amino acid substitutions of Brazilian SVA isolates, we aligned 805 SVA sequences (sequences from China, USA, Thailand, etc.). This analysis identified seven exclusive mutations exhibiting high frequency within the Brazilian SVA sequences. These mutations are located in structural protein genes: VP2: K42R and S70P; and non-structural proteins: 2B: K118R; 3A: T73I; 3C: V171A; and 3D: V54T and A276S. These specific mutations were not found in sequences of SVA isolates from other countries. Furthermore, we identified 10 additional mutations exhibiting high frequency within the Brazilian SVA isolates but with a very low frequency in SVA sequences from other geographic locations (Table 1).

Recombination events

To investigate potential recombination events within the Brazilian SVA isolates, the RDP5 software was used for the analysis. Although multiple putative recombination events were initially detected, only five recombination events involving Brazilian isolates met the selection criteria (detected by at least six of the seven methods with a *P*-value equal to or less than 1×10^{-5}) and are illustrated in Fig. 5. Three of these events involved breakpoints within structural protein-coding regions, affecting VP2 (partial), VP3 (full length) and VP1 (partial) genes, while the remaining two events involved breakpoints within non-structural protein-coding regions.

DISCUSSION

In this study, 501 SVA isolates from distinct geographical regions of Brazil were subjected to full genome sequencing. To the best of our knowledge, at the time this study was concluded, only 13 SVA genomes from Brazil were available in GenBank [5, 22]. Our results show that the Brazilian SVA sequences are genetically distinct from those of other countries and form a monophyletic cluster, suggesting a single introduction of the virus into Brazil, followed by rapid dissemination across the country's major swine-producing regions. Interestingly, the Brazilian clade presented two major subclades comprising isolates from the Midwest region states and another containing mainly SVA isolated in the southern region, suggesting that SVA may be evolving independently in the swine population of these two regions. However, phylogeographic and dispersal analysis demonstrates dispersal of the virus across the country, which may explain the detection of viruses/sequences within each subclade in the Midwestern and Southern states. Given that swine production in Brazil is highly integrated, with the

Table 1. Amino acid mutation sites identified in the SVA structural and non-structural proteins. Amino acid residues in bold represent the mutations found exclusively in Brazilian SVA sequences. Multiple amino acids in the same position were ordered according to frequency in the sequences. *N*=number of sequences of determined countries.

Country	***N***	**Amino acid mutation sites**																
		VP2		**VP3**				**2B**		**3A**			**3C**			**3D**		
		42	**70**	**59**	**80**	**141**	**113**	**118**	**28**	**73**	**84**	**81**	**86**	**171**	**54**	**138**	**276**	**400**
Brazil (2015–2016)	12	K	S	V	D	A	T	R	V	T	A	V	V	V	A/V	T/I	A	L
USA	115	K/N	S	V/A/E	D/A/N	A/T	T/S/N	K	V/A/I	T/A/S	A/V/T	L/V/I	M/I	V/I	V	A/T	A	L/S/V
China	154	K/N	S	V/A/M	D/E	A/T/S	T	K	V/A	T/A	A/T	L/V/I	M/V/T/I	V	V/I	A/V/T	A	L/V/S/A
Canada	14	K	S	V	D	T	T	K	V	T/A	V/T/A	V	M	V	V	A	A	L
Thailand	6	K	S	V	D	T	T	K	V	T	A	V	M	V	V	A	A	L
Chile	1	K	S	E	D	A	T	K	V	T	A	L	M	V	V	A	A	L
Colombia	1	K	S	V	D	A	T	K	V	T	A	L	M	V	V	A	A	L
Mexico	1	K	S	V	D	A	T	K	V	A	A	L	M	V	V	T	A	L
India	1	K	S	V	D	T	T	K	V	T	V	V	M	V	V	A	A	L
Vietnam	1	K	S	V	D	T	T	K	V	A	A	I	V	V	V	A	A	S
This study	501	**R**/K/I/S	**P**/S	E/V/A/K/M	E/D/G	S/A/L	S/T/C	**R**/K	A/V/T	**I**/V/T/L	T/A/I/V/N	V/I	V	**A**/V/T	**T**/A	T/I/A	**S**/A	S/L

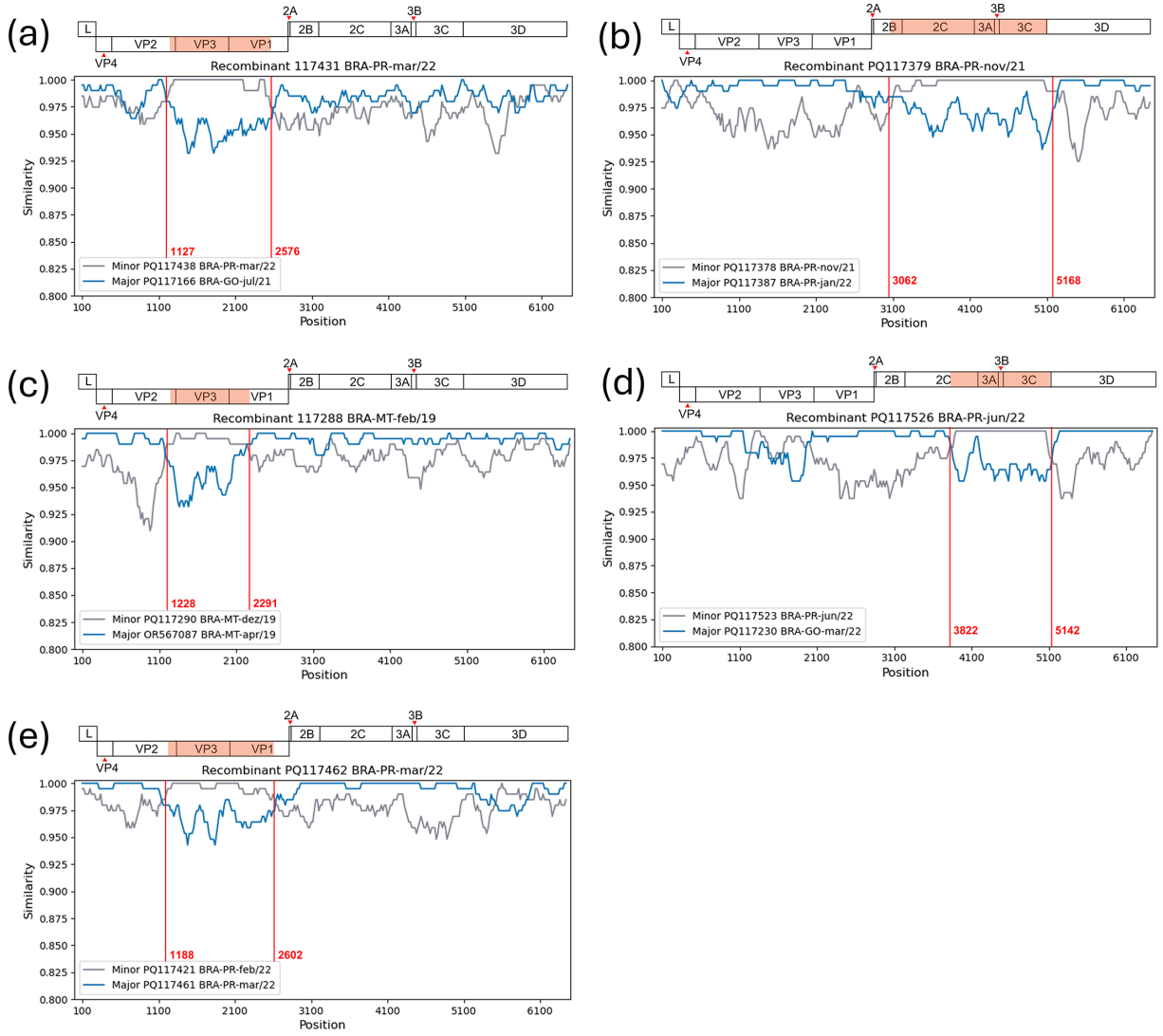

Fig. 5. Recombination events detected among Brazilian SVA isolates using RDP5 software. The graphs show the nucleotide similarity of putative major and minor parents to the respective recombinants. Events involving structural regions (a, c, e) and non-structural regions (b, d) were observed. Breakpoint positions are marked in red. Putative major and minor parent sequences are shown in blue and grey, respectively.

same agro-industrial companies operating across different states, the virus may circulate widely through animal movement within this interconnected system. This is likely facilitated by the ability of the virus to establish persistence and a carrier state in infected animals after they recover from clinical disease [41]. Similarly, the ability of the virus to establish a subclinical carrier state in infected animals [41, 42] may also have contributed to the dispersal of the virus at the global level.

In addition, pairwise sequence comparisons of the ORF of the Brazilian SVA isolates characterized here and those available in GenBank showed an identity of 97.16% at the amino acid level. This result is similar to what was described in other countries, with the SVA isolates from the USA showing the lowest similarity of 96.34%, followed by China with 96.61%, Canada with 98.81% and Thailand with 99.18%. The lowest percent identity observed in the North American sequences can be related to the higher interval between the years of isolation (1988–2020), when compared with other countries, such as China (2015–2023) and Brazil (2015–2022).

The substitution rate estimated in our study was 3.6×10^{-3} substitutions/site/year. These results are similar to the substitution rates (3.35×10^{-3} and 3.72×10^{-3} substitutions/site/year) found in previous studies analysing the global dataset of SVA [43, 44]. When compared to substitution rates reported for other RNA viruses that infect swine, the SVA mutation rate is intermediate. For example, SVA substitution rates are higher than substitution rates estimated for classical swine fever virus at 2.06×10^{-3} substitutions/site/year [45] or lower when compared to porcine reproductive and respiratory syndrome virus (PRRSV) type

2, which were estimated at 8.6×10^{-3} substitutions/site/year [46]. While SVA vaccination has not been widely implemented, the recent licensing of an inactivated vaccine in Brazil (MAPA registration No. 10.443/2021) raises the possibility that positive selection pressure may be at play, which may drive virus evolution and mutation rates in the coming years.

Analysis of the amino acid substitutions within the structural and non-structural proteins revealed unique mutations with high frequency in Brazilian SVA sequences. For example, the 3D polymerase protein displayed predominant mutations at positions V54T and A276S, which became prevalent and fixed in Brazilian SVA isolates in 2018 and 2019. The presence of these genetic signatures in Brazilian SVA isolates and their absence in sequences obtained in other countries indicate that Brazil may not have been the primary source of SVA to other countries, contrary to the inference made by Wu *et al.* [43]. Another mutation identified in our study that supports this hypothesis is the 2B (K118R), observed exclusively in Brazilian SVA sequences isolated since 2015. Consistent with these observations, a recent study suggests that the introduction of SVA into Brazil likely occurred as a single event, giving rise to the entire lineage of Brazilian SVA isolates, with no evidence of subsequent spread from Brazil to other regions [19]. This is also supported by our phylogeographic and dispersal analysis in this study, with our comprehensive dataset that includes samples collected over a 4–5-year period.

Interestingly, our analysis also revealed a VP2-specific mutation T180A that is becoming more frequent in Brazilian SVA isolates over the years (63/222 in 2022). This mutation is located within the DF loop of VP2, a region known to interact with the host cell receptor Antrax Toxin Receptor 1 (ANTXR1) in conjunction with the CD loop of VP1 [40]. Notably, another mutation in the same region, VP2 S177A, has been linked to a 17-fold increase in viral titre compared to the wild-type virus [40]. Single alanine substitutions were synthesized in the linear B-cell epitope ^{177}SLGTYYR^{183} to compare the affinity of a monoclonal antibody targeting this epitope and showed that the T180A substitution decreased the reactivity of the antibody with this epitope. Additionally, the VP1 A65T and VP2 K42R mutations occur within B-cell linear epitopes in a motif that is likely under immune pressure [47, 48]. The actual impact of these mutations in infection and pathogenesis and on immune evasion needs to be investigated in future studies.

Our recombination analysis revealed the occurrence of recombination events in the SVA genome of Brazilian isolates. The analysis showed a similar pattern in breakpoint positions that was described for SVA isolates from China [49, 50], highlighting these regions in the SVA genome as possible hotspots for recombination. The recombination events involving the genes VP2 (partial), VP3 and VP1 (partial) are the most frequent among Brazilian sequences of SVA. A similar pattern of recombination was recently described in SVA genome sequences from Canada, the USA and China [19]. Conserved recombination breakpoint hotspots were described in other single-stranded positive-sense RNA genome viruses, such as members of *Coronaviridae* family [51] and *Arteriviridae* family [52], supporting the hypothesis that the SVA genome may also contain such hotspots. Our analyses involved 514 sequences collected from 2015 to 2022; however, revealed only 5 recombination events with strong support, suggesting that these events may not significantly impact the evolution of SVA in Brazil.

The sequences obtained in our study were from SVA samples collected from commercial swine herds during a passive surveillance effort implemented by LFDA/MAPA and a result of differential diagnosis from FMD virus in VD outbreaks. The eight Brazilian states with SVA-positive samples are the top producers of pork in the country, indicating the virus circulation in the primary pig-producing regions in Brazil. Thus, we also predicted phylogeographic dynamics to understand the patterns of virus dispersal in the country. This analysis revealed intense movement of the virus between PR and GO states in the last years (Fig. 3). This is likely linked to the transportation of animals (subclinically infected carriers) or subproducts between these states but also can be associated with the transportation of feed ingredients potentially contaminated with the virus [53]. Moreover, animals infected with SVA can develop persistent infections and continue shedding the virus even after the resolution of clinical signs, further supporting the hypothesis of sustained viral circulation [41].

Recent research on the evolutionary dynamics of SVA has consistently highlighted two primary factors driving its genetic diversity: the accumulation of point mutations and recombination events [44, 54]. The subsequent global spread of these novel strains creates a complex and shifting epidemiological landscape. Consequently, a key conclusion from this work is that the SVA is an evolving threat, where effective surveillance at a global scale is necessary to track the emergence and spread of new lineages, which can potentially impact virulence, transmission and compromise vaccine development.

In summary, this study provides valuable insights into the molecular evolution and genetic diversity of SVA in swine populations from major pork-producing regions of Brazil. The extensive dataset of complete SVA genomes provided a robust and reliable source of data for analysis of SVA's evolutionary dynamics in the country. Nevertheless, the precise functional implications of the mutations identified within the various viral proteins and their contributions to the infection biology, pathogenicity and virulence of SVA isolates require further investigation.

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Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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